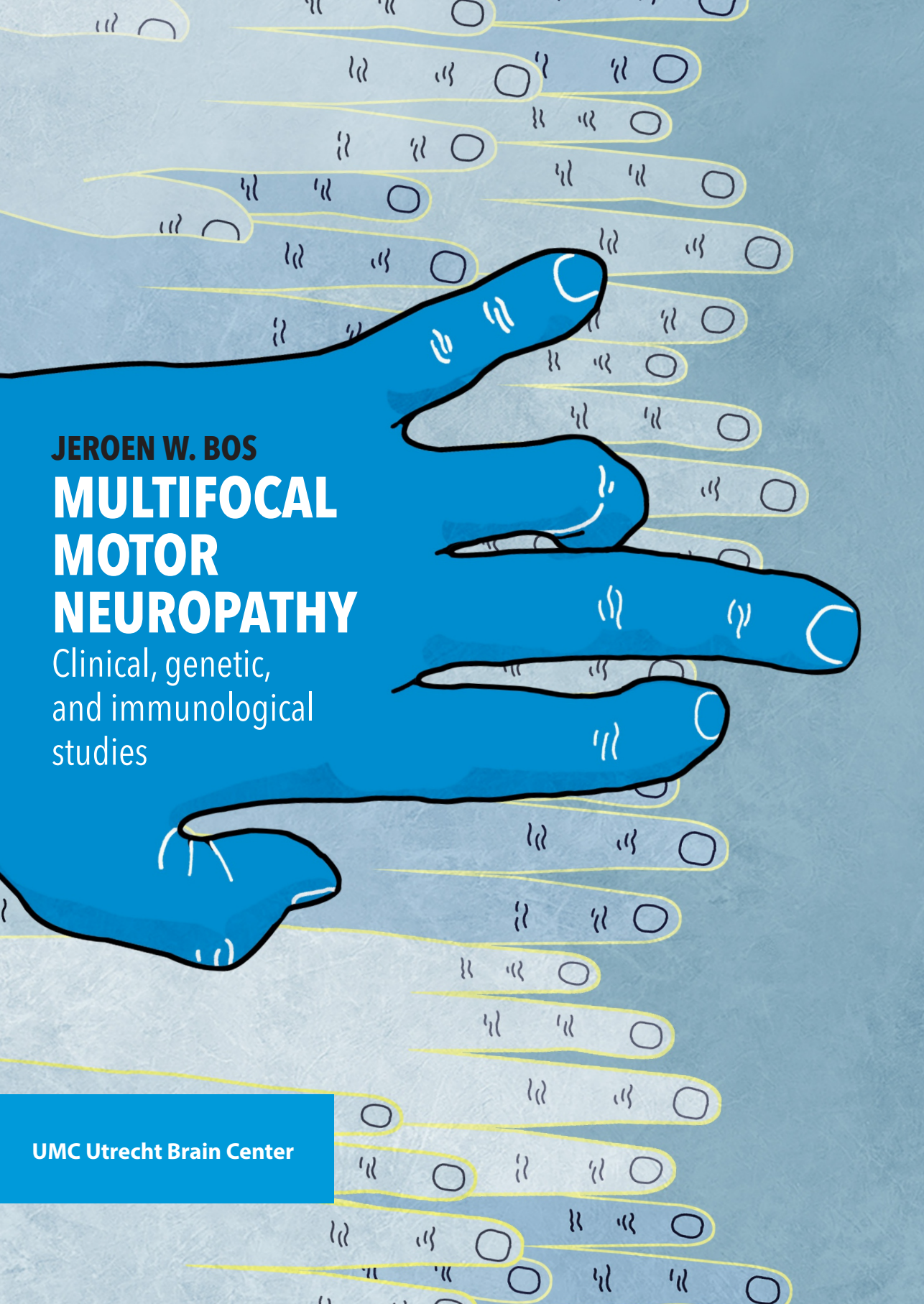

JEROEN W. BOS

MULTIFOCAL MOTOR NEUROPATHY

Clinical, genetic, and immunological studies

UMC Utrecht Brain Center

Multifocal Motor Neuropathy

Clinical, genetic, and immunological studies

Jeroen W. Bos

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Voor mijn ouders

“My right hand has become very weak. […] I cannot lift heavy things. My fingers can grip hold of any suitcase, but I cannot hang a coat on a hook. I find it difficult to brush my teeth. When I write, my hand gets tired. I can only play slowly and pianissimo.”
(1958)

“[My doctors] are both extremely satisfied with the condition of my hands and legs. After all, the fact that I cannot play the piano and that I can walk up steps only with the greatest of difficulty has no importance. One need not play the piano, and one can avoid going up steps and stairs. One can just sit at home.”
(1966)

- Dimitri Shostakovich (1906-1975), famous Russian composer, describing in his letters the symptoms and consequences of his motor neuron disease

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CHAPTER

General introduction and thesis outline

Introduction

Multifocal motor neuropathy (MMN) is a rare disorder that causes asymmetric weakness, more often in arms than legs. It is a mimic of amyotrophic laterals sclerosis (ALS) and the importance of proper diagnosis lies not only in its difference with regard to survival, but also to the possibility of treatment. Postponement of treatment has been found associated with permanent deficits, which can be severe and debilitating.[1] The number of diagnoses in the Netherlands (population size 17.3 million) is an estimated 5-10 times per year and a comparison with the incidence of ALS cases (400-500 patients per year) clearly illustrates the diagnostic challenge.[2] Despite the low prevalence figures that internationally range between 0.3-1.3 per 100.000, costs of MMN to society are high, caused by the often lifelong need for treatment with high doses of immunoglobulins.[1,3-6]
Both the clinical presentation and pathophysiology of MMN are uncommon. First, the clinical picture of markedly asymmetric muscle weakness that predominates in the arms without sensory abnormalities is highly unusual for neuropathies that are normally characterized by symmetrical, predominantly sensory or sensory-motor deficits in the legs more than the arms. Second, although inflammation underlies MMN pathophysiology since patients respond to immunoglobulin treatment, disease onset is usually quite insidious. Unlike the prototypical inflammatory disorders of the nervous system, such as multiple sclerosis (MS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), MMN disease course is not characterized by relapses and remissions, but rather by slow but constant progression. Third, MMN does not have the classic characteristics of an autoimmune disease, such as the epidemiological predominance of women or the response to treatment with corticosteroids. Indeed, prednisone is not effective for treatment of MMN and may even paradoxically worsen muscle weakness. Finally, a currently incompletely unraveled, T-cell independent immunopathology probably underlies MMN, which may be a feature unique tot MMN, but could also be relevant for other immune-mediated neurological disorders, such as (variants of) the Guillain-Barré syndrome and anti-MAG neuropathy.

MMN: a short clinical history

The first known case report of MMN was probably provided by Roth *et al.* in 1986, with a case description of a male patient who developed cramps and muscle weakness of the right hand without sensory abnormalities at the age of 44 with conduction blocks found on motor nerve conduction studies. It was however the seminal publication by the group of Pestronk *et al*. in 1988 that added the presence of antibodies directed at the glycolipid GM1 and the response to immunomodulating treatment to the clinical description.[7,8] Because muscle strength improved after treatment with cyclophosphamide, MMN was recognized as a distinct, immune-mediated, and treatable condition.

Clinical deficits and MMN disease course

MMN mostly affects men (75% of patients with MMN are male) at a relatively young age (the mean age at onset is at 40 years) and the onset is most often in the arms. Its natural history is characterized by slowly progressive muscle weakness, which is strikingly asymmetrical, multifocal and mostly affects distal arm muscles.[1,3,9] MMN mimics the first stages of lower-motor neuron dominant cases of amyotrophic lateral sclerosis (ALS). Its status as a mimic of this invariably fatal disease likely explains why MMN was often (and sometimes still is) regarded as a 'benign' disease. Several studies have shown that this is a misunderstanding, as

MMN is a progressive disorder and 20% of patients with MMN develop severe disability of hand function.[1,10]

Diagnosis of MMN

The European Federation of Neurological Societies (EFNS) published the diagnostic consensus criteria for MMN in 2010, which are summarized in Table 1.[11] For a diagnosis of MMN, the presence of clinical symptoms consistent with MMN, i.e. an asymmetrical, multifocal distribution of muscle weakness in the absence of cranial nerve palsies, marked sensory symptoms, bulbar symptoms or signs consistent with upper motor neuron involvement, needs to be supported by abnormalities of ancillary diagnostic tests. This includes the presence of motor nerve conduction blocks during nerve conduction studies, defined as a loss of CMAP area and amplitude upon more proximal nerve stimulation of >30%, but preferably >50%. Results of nerve conduction study results, and results of other ancillary investigations that serve as supportive criteria, such as the absence of pleocytosis with moderately increased protein concentration (<1 g/L) in cerebrospinal fluid, the presence of IgM antibodies directed against GM1, abnormal magnetic resonance imaging of the brachial plexus, and a response to treatment with immunoglobulins, are used to group patients in diagnostic categories termed definite, probable, and possible MMN. It is important to note that even though the term 'possible MMN' might suggest diagnostic uncertainty to the reader, these patients, by definition, have a typical clinical phenotype consistent with MMN, have normal sensory nerve conduction studies, and respond to treatment with immunoglobulins. Recent studies have shown that ultrasound can be used to reliably document the presence of pathological thickening of peripheral nerves in the upper arm and the brachial plexus in a large majority of patients with MMN. Peripheral nerve ultrasound has very high sensitivity for the detection of MMN and other inflammatory neuropathies, which suggests it is an excellent first-tier tool to identify patients who might have MMN.[12] Moreover, patients with thickened nerves who do not fulfill current MMN diagnostic criteria, may nonetheless benefit from treatment with immunoglobulins.[13] These data suggest that ultrasound investigations deserve a more prominent place in the diagnostic work-up of patients with asymmetric weakness compatible with MMN.

Treatment

The first proposed treatment for MMN was cyclophosphamide, but this approach has been left after the proven efficacy of immunoglobulin treatment. Immunoglobulins can be administered intravenously (IVIg) or subcutaneously (scIg) and consist of IgG and minimal quantities of IgA and IgM antibodies, pooled from hundreds of blood donors.[14-17] IVIg treatment partially improves muscle weakness of patients with MMN and is believed to improve long-term outcome. Although its exact working mechanism is not known, it probably exerts most of its function in patients with MMN through binding to components of the complement cascade, limiting complement activation.[17-19] Subcutaneous immunoglobulin treatment is a more recent alternative to IVIg, which shows similar efficacy and for some patients improves their independence and flexibility.[20]

Table 1. Diagnostic criteria for multifocal motor neuropathy[11]

I. Clinical criteria	
Core criteria	1. Slowly progressive, focal, and asymmetric limb weakness, for longer than one month
	2. No objective sensory abnormalities except for minor vibration sense abnormalities in the lower limbs
Supportive criteria	3. Predominant upper limb involvement
	4. Decreased or absent tendon reflexes in the affected limb
	5. Absence of cranial nerve involvement
	6. Cramps and fasciculations in the affected limb
	7. Response in terms of disability or muscle strength to 15mmune-modulatory treatment
Exclusion criteria	8. Upper motor neuron signs
	9. Marked bulbar involvement
	10. Marked sensory involvement
	11. Diffuse symmetric weakness during the initial weeks
II. Electrophysiological criteria for conduction block	
Definite motor CB	1. Negative peak CMAP area reduction on proximal vs. distal stimulation of at least 50% whatever the nerve segment length. Negative peak CMAP amplitude on stimulation of the distal part of the segment with motor CB must be >20% of the lower limit of normal and >1mV and increase of proximal to distal negative peak CMAP duration must be ≤30%
Probable motor CB	2. Negative peak CMAP area reduction of at least 30% over a long segment of an upper limb with increase of proximal to distal negative peak CMAP duration ≤30% OR Negative peak CMAP area reduction of at least 50% (same as definite) with an increase of proximal to distal negative peak CMAP duration >30%
Sensory NCS	3. Normal sensory nerve conduction in upper limb segments with CB
III. Supportive criteria	1. Elevated anti-GM1 IgM antibodies
	2. Increased CSF protein (< 1 g/l)
	3. Brachial plexus MRI showing increased signal intensity on T2-weighted imaging associated with a diffuse nerve swelling of the brachial plexus
	4. Objective clinical improvement following IVIg treatment

Diagnostic categories	
Definite MMN	Clinical criteria 1, 2 and 8-11 + electrophysiological criteria 1 and 3 in one nerve
Probable MMN	Clinical criteria 1, 2 and 8-11 + electrophysiological criteria 2 and 3 in two nerves Clinical criteria 1, 2 and 8-11 + electrophysiological criteria 2 and 3 in one nerve + at least two supportive criteria 1-4
Possible MMN	Clinical criteria 1, 2 and 8-11 + normal sensory nerve conduction studies + supportive criteria 4 Clinical criteria 1 with clinical signs present in only one nerve, + clinical criteria 2 and 8-11 + electrophysiological criteria 1 or 2 and 3 in one nerve

CB = conduction block, CMAP = compound muscle action potential, CSF = cerebrospinal fluid, MMN = multifocal motor neuropathy, MRI = magnetic resonance imaging, IVIg = intravenous immunoglobulins

Unfortunately, the efficacy of immunoglobulin treatment is partial, and most patients will experience a slowly progressive disease course, leading to increasing muscle weakness and disability.[1,10,15,16] Treatment with other immunosuppressant drugs like prednisone, azathioprine, methotrexate, interferon-beta and rituximab have not been proven effective and prednisone and plasmapheresis may even worsen symptoms.[15] Interestingly, although activation of the complement cascade is central in the pathophysiology underlying MMN, treatment with eculizumab, an anti-C5 monoclonal antibody, is probably ineffective.[15,16,21] Therefore, identifying new and more effective treatment strategies for patients with MMN remains important. To achieve this, we need to improve our detailed understanding of the immunopathology underlying MMN.

Immunopathogenesis of MMN

From the first description by the group of Pestronk in 1988, MMN has been associated with IgM antibodies directed at the ganglioside GM1, a glycolipid that is expressed by motor nerves more than by sensory nerves, in particular at and close to the nodes of Ranvier.[8,9] Because of this association, and because patients with MMN benefit from immunomodulatory treatment, MMN is considered an immune-mediated neuropathy. Yet, strikingly, nerve biopsies performed in patients with MMN show a marked absence of true demyelination or infiltration by cells of the immune system, in contrast to the histopathology seen in patients with CIDP.[22] Therefore, major questions regarding the pathogenesis of MMN are still to be answered, including the identification of factors associated with MMN susceptibility, the origin of anti-

GM1 IgM antibodies and the immunological environment determining their pathogenicity. Their answers may form the rationale for new treatment strategies, and these questions have therefore formed the basis of this thesis.

Immunogenetics

Our knowledge on genetic susceptibility underlying MMN is very limited. Previous studies have shown that patients with MMN, more often than controls, have first-degree relatives with other autoimmune diseases, such as type 1 diabetes, Hashimoto's thyroid disease and celiac disease.[23] This may be explained by a more general susceptibility for autoimmune diseases that could be genetic. Although genetic associations with altered B-cell function have not been found, one study reported that patients with MMN more often carry an MHC class II variant called HLA-DRB1*15.[24,25] MHC class II genes form part of a family of genes primarily involved in the presentation of peptides by antigen-presenting cells, such as dendritic cells and B cells, to $CD4^+$ T helper cells.[26] From an immunological perspective, it is quite difficult to directly correlate this genetic association with the production of IgM antibodies to the glycolipid (i.e. non-peptide) GM1, which are presumably produced in a T cell-independent manner. The fact that this MHC class II allele has been found to be associated with increased autoantibodies in rheumatoid arthritis and CIDP offers an alternative explanation that needs confirmation.[27,28]

Anti-GM1 IgM antibodies engage the complement system

GM1 is a ganglioside that is part of a group of molecules known as glycosphingolipids. Gangliosides all share a common molecular structure, consisting of a ceramide core, to which one or multiple sugars (hexoses) are attached. The hydrophobic stalk is incorporated in the cell's lipid bilayer, exposing sugars as potential epitopes for anti-ganglioside antibodies.[29] IgG antibodies that target GM1 have been shown to bind to the axolemma of motor neurons, thereby disrupting clusters of voltage-gated sodium channels at the nodes of Ranvier, and, in the paranodal region, interfering with normal axon-Schwann cell interactions.[30]

There is a striking variation in the prevalence of IgM antibodies to GM1 between different cohorts of patients with MMN, ranging from 20-80%. More recent studies that employed well-described and standardized ELISA and glycoarray techniques converge to an antibody prevalence figures of at least 40% of patients with MMN.[1,31-34] Much of the variation of anti-GM1 IgM prevalence numbers is likely caused by differences in the techniques used.[35,36] The presence of anti-GM1 IgM antibodies in MMN is probably underestimated when solid-phase assays are used, as shown by recent studies using induced-pluripotent stem cell (iPSC) techniques.[37] The use of iPSC-derived motor neurons in combination with serum samples from supposedly anti-GM1 IgM *seronegative* patients actually showed the presence of such antibodies at immunologically relevant levels, but below ELISA detection limits. The iPSC approach has also helped to show the pathogenic potential of anti-GM1 IgM antibodies, through the damaging effects of complement activation. These *in vitro* findings are compatible with the finding that patients with MMN and high anti-GM1 IgM antibody titers have more severe muscle weakness.[31] The association of antibody titers with clinical characteristics is accompanied by the finding that high innate activity of the classical pathway (i.e. the pathway activated by antibodies) of complement is associated with more severe muscle weakness.[19,38]

The current working model of MMN pathophysiology consists of the tandem activity of antibody and complement. Experiments with iPSC-derived motor neurons have demonstrated that, upon binding of IgM antibodies, classical pathway activation leads to deposition of

complement, including the membrane-attack complex (MAC), on motor neuron cells, leading to structural motor neuron damage.[3,9,37]

The origin of anti-GM1 antibodies

If anti-GM1 IgM antibodies are instrumental in the activation of the classical complement cascade and thereby play a central role in the immunopathogenesis of MMN, the most important question is how and where they are elicited. Nores *et al*. showed in 2004 that anti-GM1 IgM antibodies are absent in umbilical cord blood but appear in serum during the first months of life. The appearance of anti-GM1 IgM antibodies showed a perfect concordance with anti-bacterial anti-glycan IgM antibodies, suggesting that they most likely formed as a normal innate immune response to the formation of the microbiome by bacteria colonizing the respiratory tract or gut.[39] Changes in the microbiome can cause changes in titers and isotypes of anti-GM1 antibodies, as has been shown in variants of the Guillain-Barre syndrome (GBS).[40] GBS, in its classical form, is a postinfectious, monophasic inflammatory neuropathy that leads to flaccid paralysis, usually with marked sensory abnormalities, that develops in days to weeks. Though most patients respond to immunotherapy, some patients with GBS are left with severe neurological deficits, and in some, GBS is lethal.[41] Serum of patients with GBS who suffer from the pure-motor axonal variant (i.e. AMAN) often contains anti-GM1 antibodies of the IgG isotype. AMAN and the presence of these antibodies is strongly associated with preceding gastrointestinal infections with *Campylobacter (C.) jejuni*, a gram-negative bacterium that can express structures on its outer membrane that resemble GM1.[42,43] The presumed pathological mechanism of AMAN is one in which these GM1-like epitopes on the outer cell membrane of *C. Jejuni* elicit the humoral immune response in the host, that allows the production of IgG antibodies that cause the collateral damage to nerve cells. This mechanism is known as *molecular mimicry*.[44] Although molecular mimicry is relevant for AMAN, studies have failed to show a clear association between MMN and preceding *C. jejuni* infection, in accordance with MMN not being known as a typical post-infectious inflammatory neuropathy.[45] Moreover, in contrast to GBS, anti-GM1 IgM antibodies have an oligoclonal origin in MMN, and infection-driven molecular mimicry driving anti-GM1 production fails to explain the absence of immunoglobulin class switching as seen in patients with MMN.[46]

Outline of this thesis

The aims of this thesis are to improve our understanding of the clinical outcome and pathophysiology of MMN, more specifically:

1. To study the natural history of MMN;
   - In **chapter 2**, we describe the results of a Dutch combined cross-sectional and follow-up study on patients with MMN, and investigated clinical and immunological parameters associated with MMN disease course.
2. To identify genetic susceptibility factors of MMN;
   - In **chapter 3**, we report the outcome of a genetic study on genetic variation in the MHC class II DRB1, DQA1 and DQB1 loci.
   - In **chapter 4**, we investigated whether altered copy numbers of the *SMN1* and *SMN2* genes, encoding the survival motor neuron (SMN) protein, associated with various

motor neuron diseases, including MMN, and lower- and upper motor neuron-dominant forms of ALS (PMA and PLS).

- In **chapter 5**, we took a closer look on the associations between common polymorphisms in the promotor regions of genes encoding the membrane-bound complement regulators CD46, CD55 and CD59 and MMN, and correlated these polymorphisms to MMN disease course.

3. To study immunological processes that underlie MMN, in particular the presence and origin of antibodies.

   - In **chapter 6**, we studied innate immune responses to LPS in patients with MMN and controls by stimulating whole blood with LPS and measuring immune-regulating proteins before and after stimulation.

   - In **chapter 7**, we investigated the potential role of Schwann cells in MMN by performing IgM binding experiments on Schwann cells and determining the role of anti-GM2 IgM antibodies in MMN susceptibility and disease course.

   - In **chapter 8**, we performed a study on the microbiota that make up the gut microbiome in patients with MMN and controls, and correlated bacterial diversity and their relative abundance to the presence of anti-GM1 IgM antibodies.

References

1. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology 2010a;75(9):818-825

2. De Jongh AD, Van Eijk RPA, Peters SM, et al. Incidence, prevalence and geographic clustering of motor neuron disease in the Netherlands. Neurology 2021;96(8):e1227-e1236

3. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

4. Löscher WN, Oberreiter E-M, Erdler M, et al. Multifocal motor neuropathy in Austria: a nationwide survey of clinical features and response to treatment. J Neurol 2018;265(12):2834-2840

5. Miyashiro A, Matsui N, Shimanati Y, et al. Are multifocal motor neuropathy patients underdiagnosed? An epidemiological survey in Japan. Muscle Nerve 2014;49(3):357-361

6. Mahdi-Rogers M, Hughes RAC. Epidemiology of chronic inflammatory neuropathies in southeast England. Eur J Neurol 2014;21(1):28-33

7. Roth G, Rohr J, Magistris MR et al. Motor neuropathy with proximal multifocal persistent conduction block, fasciculations and myokymia, evolution to tetraplegia. Euro Neurol 1986;25:416-423

8. Pestronk A, Cornblath DR, Ilyas AA, et al. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. Ann Neurol 1988;24(1):73-78

9. Harschnitz O, Jongbloed BA, Franssen H, et al. MMN: from immunological cross-talk to conduction block. J Clin Immunol 2014;34(Suppl 1):S112-119

10. Lange DJ, Weimer LH, Trojaborg W, et al. Multifocal motor neuropathy with conduction blok: slow but not benign. Arch Neurol 2006;63(12):1778-1781

11. Van Schaik IN, Leger JM, Nobile-Orazio E, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripheral Nerv Syst 2010;15(4):295-301

12. Goedee HS, Van der Pol WL, Van Asseldonk JH, et al. Diagnostic value of sonography in treatment-naive chronic inflammatory neuropathies. Neurology 2017;88(2):143-151

13. Herraets IJT, Goedee HS, Telleman JA, et al. Nerve ultrasound improves detection of treatment-responsive chronic inflammatory neuropathies. Neurology 2020;94(14):e1470-e1479

14. Van den Berg LH, Kerkhoff H, Oey PL, et al. Treatment of multifocal motor neuropathy with high-dose intravenous immunoglobulins: a double blind, placebo controlled study. J Neurol Neurosurg Psychiatry 1995;59(3):248-252

15. Briani C, Cocito D, Campagnolo M, et al. Update on therapy of chronic immune-mediated neuropathies. Neurol Sci 2021

16. Umapathi T, Hughes RAC, Nobile-Orazio E, et al. Immunosuppressant and immunomodulatory treatments for multifocal motor neuropathy. Cochrane Database Syst Rev 2015;2015(3):CD003217

17. Liu X, Cao W, Li T. High-dose intravenous immunoglobulins in the treatment of severe acute viral pneumonia: the known mechanisms and clinical effects. Front Immunol 2020;11:1660

18. Lutz HA, Stammler P, Bianchi V, et al. Intravenously applied IgG stimulates complement attenuation in a complement-dependent autoimmune disease at the amplifying C3 convertase level. Blood 2004;103(2):465-472

19. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol 2010;229(1-2):256-262

20. Herraets IJT, Bakers JNE, Van Eijk RPA, et al. Human immune globulin 10% with recombinant human hyaluronidase in multifocal motor neuropathy. J Neurol 2019;266(11):2734-2742

21. Fitzpatrick AM, Mann CA, Barry S, et al. An open label clinical trial of complement inhibition in multifocal motor neuropathy. J Peripher Nerv Syst 2011;16(2):84-91

22. Tracy JA, Taylor BV, Kiernan M, et al. Nerve pathology distinguishes focal motor chronic inflammatory demyelinating polyradiculoneuropathy from multifocal motor neuropathy. J Clin Neuromuscul Dis 2020;22(1):1-10

23. Cats EA, Bertens ASB, Veldink JH, et al. Associated autoimmune diseases in patients with multifocal motor neuropathy and their family members. J Neurol 2012;259(6):1137-1141

24. Vlam L, Cats EA, Seelen M, et al. Multifocal motor neuropathy is not associated with genetic variation in PTPN22, BANK1, Blk, FCGR2B, CD1A/E, and TAG-1 genes. J Peripher Nerv Syst 2011;16(3):175-179

25. Sutedja NA, Otten HG, Cats EA, et al. Increased frequency of HLA-DRB1*15 in patients with multifocal motor neuropathy. Neurology 2010;74(10):828-83

26. Dendrou CA, Petersen J, Rossjohn J. HLA variation and disease. Nat Rev Immunol 2018;18(5):325-339

27. De Vries RRP, Huizinga TW, Toes REM. Redefining the HLA and RA association: to be or not to be anti-CCP positive. J Autoimm 2005;25;21-25

28. Martín-Aguilar, Lleixà C, Pascual-Goñi E, et al. Clinical and laboratory features in anti-NF155 autoimmune neuropathy. Neurol Neuroimmunol Neuroinflamm 2021;9(1):e1098

29. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002;125:2591-2625

30. Susuki K, Rasband MN, Tohyama K, et al. Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. J Neurosci 2007;27(15):3956-3967

31. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010;75(22):1961-1967

32. Galban-Horcajo F, Vlam L, Delmont E, et al. The diagnostic utility of determining anti-GM1:GalC complex antibodies in multifocal motor neuropathy: a validation study. J Neuromuscul Dis 2015;2(2):157-165

33. Nobile-Orazio E, Gallia F, Terenghi F, et al. How useful are anti-neural IgM antibodies in the diagnosis of chronic immune-mediated neuropathies? J Neurol Sci 2008;266(1-2):156-163

34. Nobile-Orazio E, Giannotta C, Musset L, et al. Sensitivity and predictive value of anti-GM1/galactocerebroside IgM antibodies in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry 2014;85(7):754-758

35. Kuijf ML, Van Doorn PA, Tio-Gilles AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci 2005;239(1):37-44

36. Van Schaik IN, Bossuyt PM, Brand A, et al. Diagnostic value of GM1 antibodies in motor neuron disorders and neuropathies: a meta-analysis. Neurology 1995;45(8):1570-1577

37. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol 2016;80(1):71-88

38. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2015;2(4):e119

39. Alaniz ME, Lardone RD, Yudowski SL, et al. Normally occurring human anti-GM1 immunoglobulin M antibodies and the immune response to bacteria. Infect Immun 2004;72(4):2148-2151

40. Brooks PT, Bell JA, Bejcek CE, et al. An antibiotic depleted microbiome drives severe Campylobacter jejuni-mediated type 1/17 colitis, type 2 autoimmunity and neurologic sequelae in a mouse model. J Neuroimmunol 2019;337:577048

41. Shahrizaila N, Lehmann HC, Kuwabara S. Guillain-Barré syndrome. Lancet 2021;397(10280):1214-1228

42. Yuki N, Yoshino H, Sato S, et al. Acute axonal polyneuropathy associated with anti-GM1 antibodies following Campylobacter enteritis. Neurology 1990;40(12):1900-1902

43. Jacobs BC, Van Doorn PA, Schmitz PI, et al. Campylobacter jejuni infections and anti-GM1 antibodies in Guillain-Barré syndrome. Ann Neurol 1996;40(2):181-187

44. Van den Berg B, Walgaard C, Drenthen J, et al. Guillain-Barré syndrome: pathogenesis, diagnosis, treatment and prognosis. Nat Reve Neurol 2014;10(8):469-482

45. Terenghi F, Allaria S, Scarlato G, et al. Multifocal motor neuropathy and Campylobacter Jejuni reactivity. Neurology 2002;59(2):282-284

46. Cats EA, Van der Pol WL, Tio-Gillen AP, et al. Clonality of anti-GM1 IgM antibodies in multifocal motor neuropathy and the Guillain-Barré syndrome. J Neurol Neurosurg Psychiatry 2015;86(5):502-504

CHAPTER

2

Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study

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Neurology, 2020

Abstract

Objective To assess the clinical course of multifocal motor neuropathy (MMN) in a large cohort of patients and to identify predictive factors of a progressive disease course.

Methods Between May 2015 and February 2016, we collected clinical data from 100 patients with MMN of whom 60 had participated in a nationwide cross-sectional cohort study in 2007. We documented clinical characteristics using standardized questionnaires and performed a standardized neurological examination. We used multiple linear regression analysis to identify factors that correlated with worse outcome.

Results We found that age of diagnosis (45.2 vs. 48.6 years, $p<0.02$) significantly increased between 2007 and 2015-2016, whereas diagnostic delay decreased with 15 months. Seven out of ten outcome measures deteriorated over time (all $p<0.01$). Patients who had a lower MRC sum score and absence of one or more reflexes at the baseline visit showed a greater functional loss at follow up ($p=0.007$ and $p=0.016$).

Conclusions Our study shows that MMN is a progressive disease. Although 87% of patients received maintenance treatment, muscle strength, reflexes, vibration sense, and the Self-Evaluation Scale significantly deteriorated over time. Lower MRC sum score and absence of reflexes predicted a more progressive disease course.

Classification of evidence This study provides Class II evidence that lower MRC sum score and the absence of reflexes predict a more progressive disease course in patients with MMN.

Introduction

Multifocal motor neuropathy (MMN) is a pure motor disorder characterized by slowly progressive asymmetric distal weakness mainly in the hands, the absence of upper motor neuron signs and presence of one or more abnormal ancillary investigations, i.e. abnormal nerve conduction or conduction block (CB), thickening or T2 hyperintensity on MRI of the brachial plexus, sonographic nerve thickening, increased protein content in the CSF or the presence of anti-GM1 IgM antibodies in serum.[1-7] Administration of intravenous or subcutaneous immunoglobulins transiently improves muscle strength and maintenance treatment is therefore needed.[8-12]

Consensus criteria have facilitated diagnosis of MMN and shortened diagnostic delays, but we know less of the disease course and outcome.[4,7] Early case reports suggested that its course is not benign in individual patients, but few studies have longitudinally addressed natural history in larger patients cohorts.[13-15] Early treatment may improve long-term outcome, but accumulating axonal damage nevertheless results in significant disability in up to one fifth of patients.[4,16] More detailed insight in MMN's clinical course would help to identify correlates of worse outcome and thereby patients at higher risk for developing severe deficits, and eventually to investigate efficacy of other treatment approaches.

We have previously reported the characteristics of a relatively large cross-sectional cohort of patients with MMN in The Netherlands.[4] In order to gain more insight in the clinical course of MMN, we performed a combined cross-sectional and follow-up study in a cohort of 100 patients with the aim to identify factors that predict a progressive disease course of MMN.

Methods

Study design and patients

This cross-sectional cohort study was performed between May 2015 and February 2016 in the UMC Utrecht, a large tertiary referral center for neuromuscular disorders in The Netherlands. We invited all patients listed in the MMN database of the UMC Utrecht who met the following inclusion criteria: 1) a diagnosis of definite, probable or possible MMN according to the EFNS/PNS criteria and 2) age ≥18 years.[7] A subgroup of our patients previously participated in a similar cross-sectional cohort study in 2007.[4]

Neurological examination and questionnaires

We documented clinical characteristics of patients with MMN (including but not limited to site of onset and age at symptom onset) using a standardized questionnaire and collected the Overall Disability Sum Score (ODSS), the Self-Evaluation Scale (SES), the Rasch-built Overall Disability Score for MMN (MMN-RODS) and the Fatigue Severity Scale (FSS).[17-22]

All patients underwent a standardized neurological examination (Table e-1).[4] This consisted of bilateral grading of motor function of 18 muscle groups using the Medical Research Council (MRC) scale to calculate the MRC sum score with a maximum of 180 points. Sensory function was tested using a Rydell-Seiffer tuning fork to assess vibration sense in arms and legs bilaterally. Vibration sense was graded from normal (grade 0) to abnormal at the acromioclavicular joint or anterior superior iliac spine (grade 4).[4,23] Tendon reflexes of biceps, triceps, knee and ankle were performed on both sides and scored as normal, brisk or absent. We used data obtained during a previous study in 2007 as baseline data.[4] To minimize inter-

observer variability, one of the authors (EAC) who collected clinical data during the 2007 study trained the author (BAJ) who performed the clinical examination in 2015-2016, with special emphasis on the interpretation of MRC and Rydell-Seiffer scales.[4]

Nerve conduction studies and other ancillary investigations

One of the authors (HSG), who has extensive experience in clinical neurophysiology, evaluated available nerve conduction study results using the EFNS/PNS criteria for CB and other abnormalities.[7] All patients underwent a standardized NCS protocol and stimulation was up to Erb's point.[24] CB was defined as definite CB (compound muscle action potential (CMAP) area reduction of at least 50%) or probable CB (CMAP area reduction of 30-50%)[7], and axonal loss as a decreased distal CMAP (distal CMAP amplitude below the lower limit of normal) in ≥1 nerves, including the median, ulnar, radial, musculocutaneous, peroneal, and tibial nerves.[4,25,26] We also collected all available results of laboratory studies (in particular the presence of anti-GM1 IgM antibodies in serum and analysis of cerebrospinal fluid) and of MRI of the brachial plexus.[7]

Statistical analyses

MMN cohort data

We stratified the MMN patients into two groups: 1) patients diagnosed before our previous study in 2007, and 2) patients diagnosed after 2007, to explore differences in clinical characteristics.[4] Depending on the distribution of the variable, we compared groups using the Mann-Whitney U test (for continuous data) and the χ^2 test (for categorical data). To account for right skew in time-related covariates, we log-transformed (natural) duration of treatment, months untreated and time to diagnosis. Univariate linear regression analyses were performed to identify changes in clinical characteristics over calendar time. Dependent variables were age at diagnosis, time to diagnosis (log-transformed) and age at onset of symptoms. The independent variable was the year of diagnosis. Subsequently, we calculated the mean MRC score per muscle group for patients with longer and shorter disease duration (defined as equal to or larger than the median disease duration). We corrected the obtained p-values for multiple testing using the Benjamini Hochberg method. Multiple linear regression analysis was used with backward elimination based on p-value selection to predict the MRC sum score 2015-2016 based on sex, symptom onset in a leg, presence of anti-GM1 IgM antibodies, FSS (0-63), duration of treatment in months (log-transformed), months untreated (log-transformed) and age at onset of symptoms in years.

Longitudinal follow-up data

The mean yearly rate of decline of each outcome measure was estimated between visit 1 (2007) and visit 2 (2015-2016) and tested using a one-sample t-test (i.e. assessing whether the yearly rate of decline is other than zero). Multiple linear regression analysis was performed with backward elimination based on p-value selection to predict the yearly rate of decline in MRC sum score based on sex, presence of anti-GM1 IgM antibodies, symptom onset in leg, months untreated (log-transformed), age at onset of symptoms in years, ODSS (0-8), MRC sum score (0-180) and sum score of reflexes (0-8). The last three variables were analyzed with data of the first visit (2007). Patients were asked to describe their disease course as stable, gradually but slowly progressive, gradually progressive, stepwise progressive or gradually improving.

Standard protocol approvals, registrations, and patient consent
The local medical ethics committee of the UMC Utrecht approved the research protocol (NL50354.041.14). All included patients gave written informed consent.

Data availability statement
The data that support the findings of this study will be available on request from the corresponding author.

Results

We identified a total of 142 patients with MMN. Hundred patients (70.4%) agreed to participate of whom 60 patients previously participated in a nationwide cross sectional cohort study in 2007.[4] Reasons for not participating are shown in Figure 1.

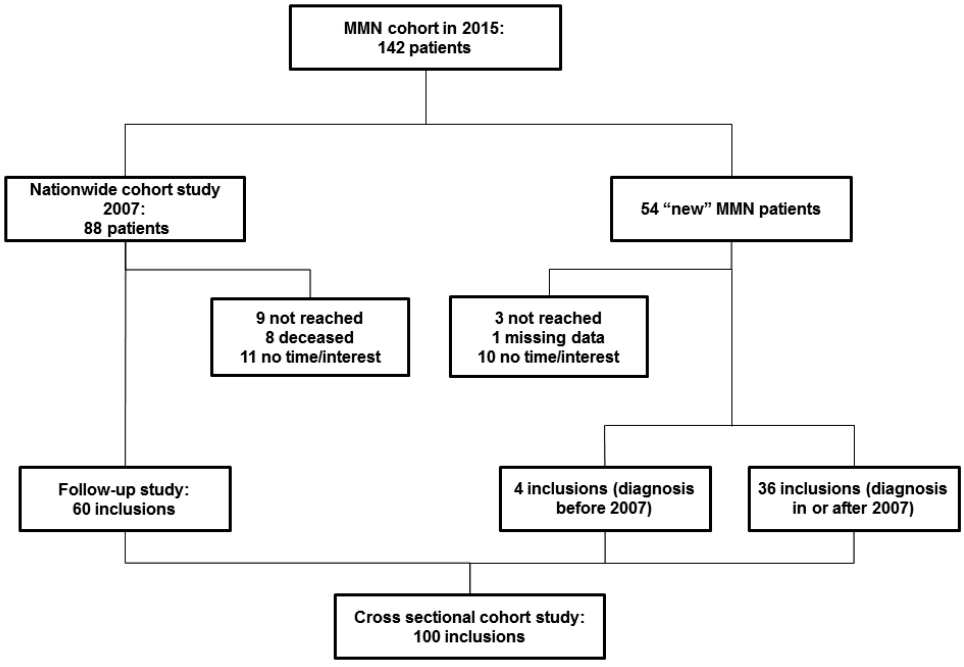

Figure 1 Flowchart of study
MMN = multifocal motor neuropathy

Clinical characteristics

Patient characteristics (sex, age at onset of symptoms, MMN diagnosis according to EFNS/PNS criteria and additional investigations i.e. NCS, MRI brachial plexus, CSF protein and presence of anti-GM1 IgM antibodies) between participants (n=100) and non-participants (n=42), were not significantly different, except for the onset of muscle weakness (p=0.04). Median age at onset of symptoms and age of diagnosis were significantly higher in patients diagnosed after 2007 (p<0.01 and p=0.02; Table 1).

We performed univariate linear regression analysis with year of diagnosis as independent variable. Both median age at onset of symptoms and median age of diagnosis significantly increased over time (both p<0.01) (Figure 2). Median time from symptom onset to diagnosis (i.e. diagnostic delay) decreased over time (6.4 years (range 1-27) in period 1996 to 2000; 1,8 years (range 1-29) in period 2011-2015) but was significantly longer for patients with onset of symptoms in a leg and for patients with higher age at diagnosis (p=0.01, p<0.01). We use a starting dose of 0.4 g/kg immunoglobulins per 3-4 weeks and then tailor the dose (if needed up to 1g/kg) until patients remain stable during the treatment interval.[2] The starting dose was significantly higher for patients diagnosed before 2007 (p<0.01), probably due to a different treatment regime with repeated loading doses of immunoglobulins in the period before 1995 rather than lower-dosed weekly to monthly maintenance therapy. We found no significant differences in clinical characteristics between males and females.

Weakness, sensory function, and tendon reflexes

The distribution of muscle weakness was distal more than proximal and more pronounced in hand than in foot or lower leg muscles (Table e-2). Finger flexion and plantar foot flexion were relatively spared compared to hand and finger extension and dorsal foot flexion. Patients with longer disease duration had significantly more weakness in hand and lower leg/foot muscles compared to patients with shorter disease duration (all p<0.05) (Figure 3, Table e-2). We found abnormal vibration sense on the toes in 57 patients (57.6%). Median disease duration was longer in these patients compared to those without sensory findings (median 16.1 years, range (1.3-46.5) versus 11.5 range (1.9-30.5); p=0.03). We found at least one absent reflex in 79 patients (79.8%). Sixteen of these patients (20.2%) had generalized areflexia (Table e-3). We did not find a relation between the presence of conduction block (definite and/or probable) and the absence of reflexes (p>0.10).

Table 1 Clinical characteristics

	Diagnosis before 2007 (n=64)	Diagnosis in or after 2007 (n=36)	*p*-value
Male	46 (72%)	29 (81%)	0.34
Age at onset of symptoms	40.3 (21.4-53.8)	45.2 (30.1-67.2)	<0.01
Age of diagnosis	45.2 (25.2-71.1)	48.6 (30.9-73.5)	0.02
Time to diagnosis (months)[a]	42.0 (3.0-433.0)	27.0 (6.0-345.0)	0.10
Time from disease onset until treatment (months)	42.0 (3.0-435.9)	27.5 (3.9-346.0)	0.09
Maintenance treatment immunoglobulins	55 (86%)	32 (89%)	0.67
Starting maintenance therapy IVIg per week (gram)	10.0 (5.0-33.0)	8.0 (4.0-12.0)	<0.01
Onset of muscle weakness			
Distal arm	41 (64%)	25 (70%)	0.08
Proximal arm	3 (4%)	3 (8%)	
Distal leg	18 (28%)	4 (11%)	
Proximal leg	1 (2%)	-	
Distal symmetrical	1 (2%)	4 (11%)	
Number of affected limbs at inclusion			
0	2 (3%)	1 (3%)	0.15
1	7 (11%)	8 (22%)	
2	12 (19%)	12 (33%)	
3	18 (28%)	7 (20%)	
4	25 (39%)	8 (22%)	

Table 1 (continued)

	Diagnosis before 2007 (n=64)	Diagnosis in or after 2007 (n=36)	*p*-value
Electrophysiological criteria according to EFNS/PNS criteria			
Definite	45 (70%)	29 (81%)	0.32
Probable	15 (23%)	4 (11%)	
Negative	4 (6%)	3 (8%)	
NCS with axonal degeneration	31 (48%)	13 (36%)	0.23
MRI abnormalities brachial plexus	22/43 (51%)	8/17 (47%)	0.77
Laboratory: increased CSF protein	12/16 (75%)	8/10 (80%)	0.77
Anti-GM1 IgM antibodies	38/61 (62%)	17/29 (59%)	0.74
MMN diagnosis according to EFNS/PNS criteria			
Definite	45 (70%)	29 (81%)	0.32
Probable	15 (24%)	4 (11%)	
Possible	4 (6%)	3 (8%)	

Data are shown in median (range) or number of patients (%), unless stated otherwise.
[a] log transformed variable
Anti-GM1 IgM antibodies = presence of anti-GM1 IgM antibodies, IVIg = intravenous immunoglobulins

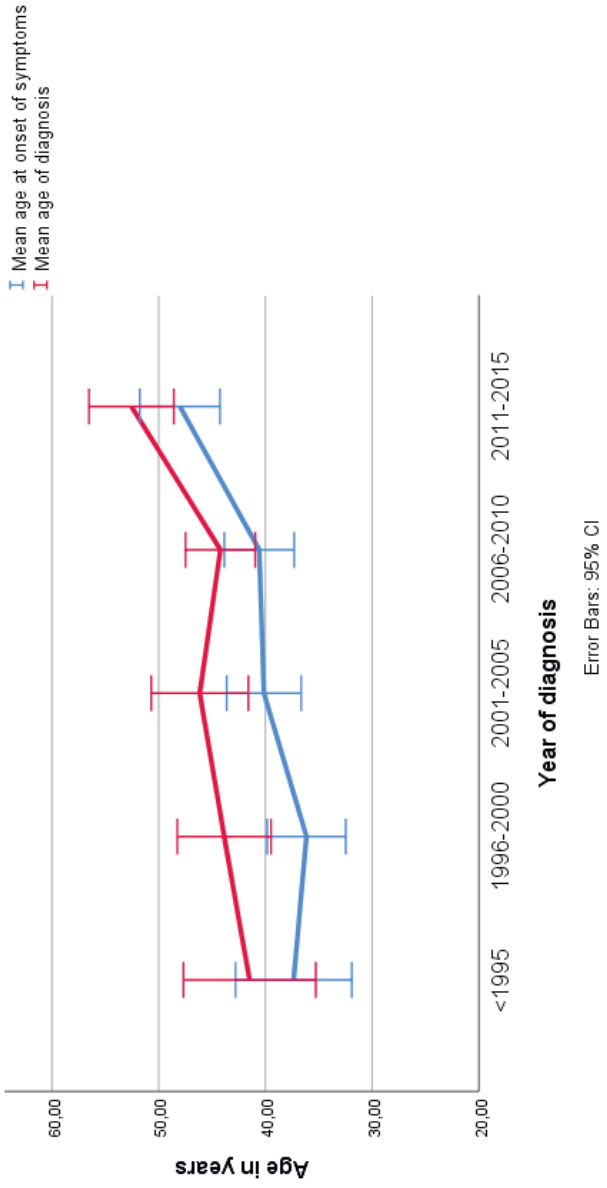

Figure 2 Clinical characteristics over time
Mean age at onset of symptoms and mean age of diagnosis over time. 95% CI = 95% confidence interval

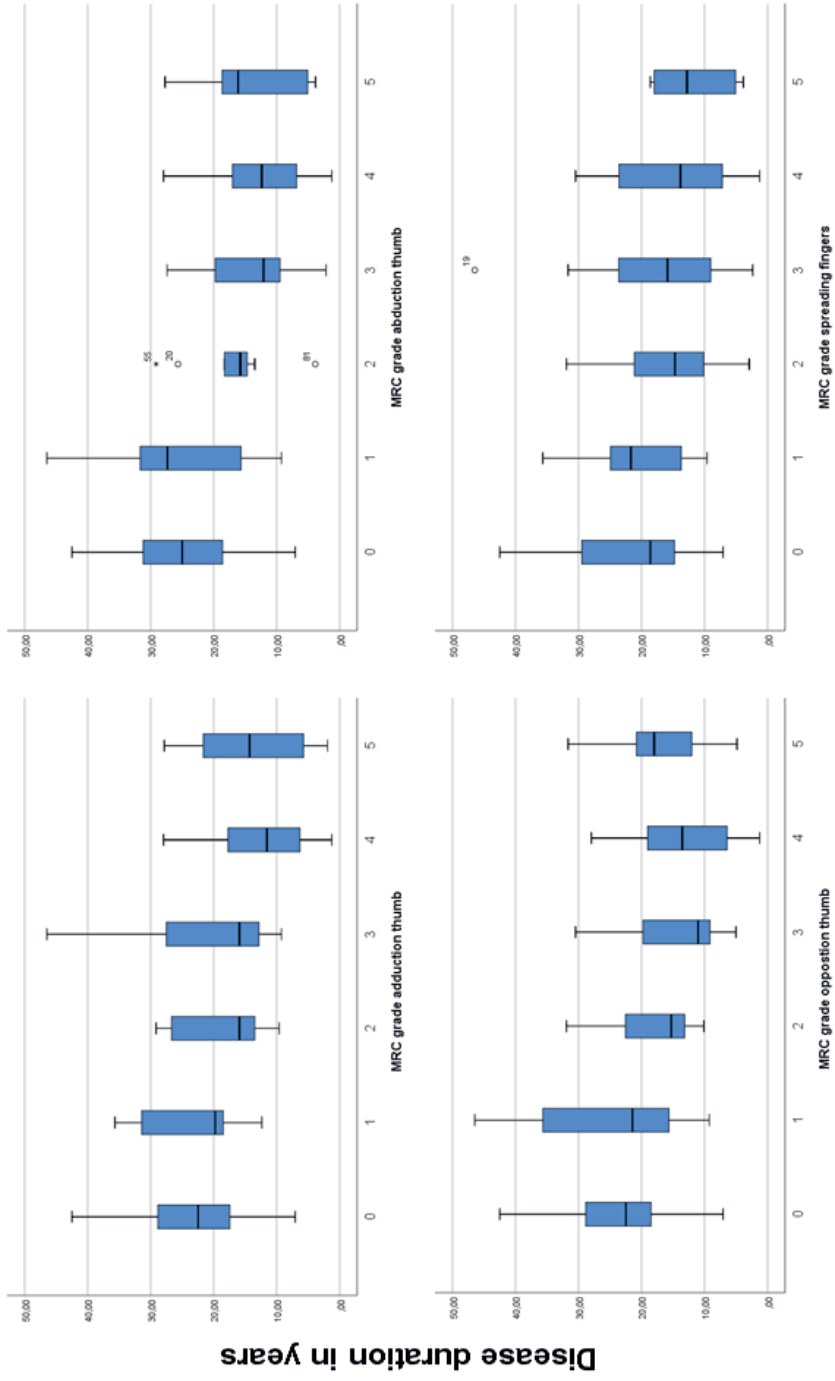

Figure 3 Correlation of MRC grade and disease duration per muscle group
The boxplots provide the variability in disease duration per MRC grade (0-5). MRC = Medical Research Council of weakest side

Nerve conduction studies and laboratory investigations
One or more definite CBs were present in 74 patients (74.0%), only probable CB in 19 patients (19.0%) and no CB in 7 patients (7.0%). The diagnosis of MMN in these 7 patients without CB was based on the presence of anti-GM1 IgM antibody titers (4 patients; 57.1%), abnormal CSF protein concentrations (protein level >0.4 gram/liter (g/L)) (2 patients; 28.6%), an abnormal MRI of the brachial plexus (3 patients; 42.9%), and response to immunoglobulin therapy in all patients. We found evidence of axonal damage during NCS in 71 patients (71.0%), the presence of anti-GM1 IgM antibodies in 55/90 patients (61.1%) and abnormal CSF protein concentrations (>0.4g/L) in 20/26 (76.9%) patients.

Disability questionnaires
Results of the disability questionnaires are shown in Table e-3. Median ODSS of the arms was 2 (range 0-4), of the legs 1 (range 0-5), and of arms and legs combined 3 (range 0-8). Twelve patients (12.1%) reported no disability of the arms, and 34 patients (34.7%) did not experience disability of the legs.

Correlates of outcome
Results from multiple linear regression analysis are summarized in Table e-4. Lower MRC sum score correlated with longer disease duration without treatment, presence of anti-GM1 IgM antibodies and lower age at onset of symptoms (p=0.024, p=0.046 and p=0.006).

Outcome measures over time
Mean differences between visit 1 (2007) and visit 2 (2015-2016) of different outcome measures are shown in Table 2. Except for ODSS, FSS and vigorimetry of the left hand, all outcome measures deteriorated over time (all p<0.01). The difference in MRC sum score between 2015 and 2007 was significantly larger in patients with axonal damage compared to patients without axonal damage (5.2 points versus 13.8 points; p =0.014). Most patients indicated that their disease course was stable (25.0%) or mildly progressive (61.7%). The dose of immunoglobulin treatment significantly increased over time (p <0.001).

Predictors of progression
Multiple linear regression showed that faster progression, i.e. a larger difference of the MRC sum score of visit 1 (2007) and visit 2 (2015-2016) per year correlated with the reflexes sum score (i.e. absent reflexes) and a lower MRC sum score in 2007 (p=0.016 and p=0.007) (Table e-5).

Table 2 Outcome measures over time

	Mean difference/year	**95% CI**	***p***
ODSS (0-12 points)	-0.004	[0.03, -0.04]	0.81
MRC sum score (0-180 points)	-1.361	[-0.97, -1.75]	<0.01
SES (0-25 points)	0.352	[0.54, 0.16]	<0.01
FSS (0-63 points)	-0.94	[-0.25, -1.63]	<0.01
Vibration sense (abnormal in 0-4 limbs)	0.121	[0.15, 0.09]	<0.01
Reflexes arm (absence in 0-4 reflexes)	0.055	[-0.02,-0.09]	<0.01
Reflexes leg (absence in 0-4 reflexes)	0.072	[-0.03, -0.11]	<0.01
Reflexes sum score (absence in 0-8 reflexes)	0.121	[-0.06, -0.18]	<0.01
Grip strength right (kPa)	-1.127	[-0.39, -1.87]	<0.01
Grip strength left (kPa)	-0.770	[0.04, -1.58]	0.06
Number of affected muscle groups	0.465	[0.36-0.58]	<0.01

Mean difference per year was calculated as the difference between visit 1 (2007) and visit 2 (2015-2016) divided by the follow-up duration.
Absence of reflexes arm: biceps and triceps reflexes (0-4); and leg: knee and ankle reflexes (0-4), kPa = Kilopascal, ODSS = Overall Disability Sum Score, MRC = Medical Research Council, SES = Self-Evaluation Scale, FSS = Fatigue Severity Scale, 95% CI = 95% confidence interval

Discussion

This study aimed to document clinical outcomes of patients with MMN and identify predictors of disease progression. We combined cross-sectional data with longitudinal data with a mean duration between visits of eight years. Our clinical observations confirmed that MMN is a progressive disorder in the large majority of patients even when they receive immunoglobulin maintenance treatment. Virtually all selected outcome measures significantly deteriorated over time. Factors with prognostic value of a progressive disease course were absence of reflexes and a lower MRC sum score at baseline.

A previous study described the natural history of 38 treatment-naive patients with MMN retrospectively. Patients with longer disease duration (n=10) had significantly lower MRC sum scores and a higher number of affected regions. None of the patients experienced spontaneous improvement or a relapsing remitting course.[14] Taylor et al. longitudinally assessed 18 patients with MMN and found a slowly worsening of muscle weakness, i.e. a change in neurological impairment score (NIS) of 1.3 points/per year.[15] We performed multiple linear regression analysis to determine predictors of a progressive disease course and found that absence of at least one reflex and a lower MRC sum score at baseline were associated with a larger decrease of the MRC sum score over time. This amounted to a difference of 1.36 MRC point decrease of the MRC sum score per year in patients with generalized areflexia compared to those with normal reflexes. These findings can help to identify patients with a more progressive disease course. Until the development of more effective treatment strategies for MMN, the identification of patients at greater risk may ultimately help to tailor the dosing or frequency of immunoglobulin treatment in the future.

We used two approaches to analyze cross-sectional data. First, we compared patients with a diagnosis before and after 2007, and thereby with longer and shorter disease duration. The distribution pattern of muscle weakness in patients with shorter and longer disease duration was similar but the severity of weakness of hand and lower leg/foot muscles was significantly increased in the latter. This finding supports the longitudinal data and also shows that proximal muscle groups are relatively spared. The second approach consisted of multiple linear regression analysis to determine factors that were associated with more severe weakness. Previous studies showed that axonal damage is highly associated with muscle weakness and therefore we performed the analysis without axonal damage as an independent factor.[4,16] We found that presence of anti-GM1 IgM antibodies and 'years untreated' were associated with more severe weakness, which is similar to findings of smaller previous studies.[27-29] These data imply that to prevent permanent weakness, reducing time to diagnosis and providing earlier treatment are crucial. Increased awareness of MMN and possibly the extension of reliable diagnostic tools, such as nerve ultrasound might serve this goal. We think that MMN should also be actively excluded in older patients or those with asymmetric weakness in a leg.

The follow-up data showed that almost all outcome measures significantly deteriorated over time. However, there were some exceptions, most notably vigorimetry of the left hand. Although we cannot explain this finding, we previously observed that weakness is more common in the dominant hand.[4] This has also been reported for other inflammatory asymmetric syndromes such as neuralgic amyotrophy.[30] Moreover, fatigue seemed to improve over time. Fatigue is a common symptom of chronic immune-mediated disorders but without

intervention, at best remains stable but often deteriorates over time.[4,31,32] A possible explanation for the improvement of fatigue in MMN could be that patients get used to the feeling of fatigue or adapted by changing frequency or intensity of their daily activities (e.g. change or quit their jobs, improve their lifestyles). We do not think that immunoglobulin therapy provides an explanation for the reduction in reported fatigue, since both in 2007 and in 2015 approximately 85% of the patients received maintenance therapy.

Median age at onset of symptoms and age of diagnosis significantly increased over time. The higher median age at diagnosis could be explained by an already increased awareness of MMN, resulting in more frequent clinical suspicion in older patients presenting with asymmetric weakness. Moreover, the addition of novel diagnostic techniques other than nerve conduction studies such as nerve ultrasound or the more frequent use of immunoglobulin trials to assess response to treatment could have led to the higher median age at diagnosis.[1,6,7,33] The cause of the increase of age at onset is unknown although it is not unique for MMN. Similar trends have been observed in amyotrophic lateral sclerosis (ALS) (unpublished data of ALS cohort of 2900 patients in UMC Utrecht The Netherlands).[34,35] We can therefore not exclude the possibility that this trend is caused by changes in an altered referral pattern of patients with motor neuron disorders in our center.

Despite the fact that MMN is considered a pure motor neuropathy, we found vibration sense abnormalities in 57% of the patients. These deficits were confined to the feet in 97% of the patients and in general occurred in patients with longer disease duration. Vibration sense also significantly deteriorated over time, which is similar to previous studies that showed reduced sensory nerve action potentials years after MMN onset.[36,37]

Our study has some limitations. Neurological examination at both study visits was performed by different investigators. However, the authors who performed neurological examination were trained prior to the second tier of the study to minimize differences in performance, evaluation and interpretation of the MRC and Rydell-Seiffer scales. The large majority of patients received immunoglobulin maintenance treatment, which will have attenuated the true progression of MMN. Moreover, the relation of disease course with immunoglobulin therapy was not a primary aim of our study. We usually tailor treatment dose and frequency to maintain stable function between gifts.[2] Although we found a significant increase in dose of immunoglobulin over time, possible relations between the therapy and progression should be a topic for future studies.

Our study shows that MMN is a progressive disorder in the large majority of patients despite immunoglobulin maintenance treatment. Diagnostic delays are more common in older patients or with onset of weakness in one of the legs. Absence of reflexes and lower MRC sum score at baseline predict a more progressive disease course. Whether these patients would benefit from more aggressive treatment approaches with immunoglobulins needs to be established.

References

1. Goedee HS, Jongbloed BA, van Asseldonk JH, et al. A comparative study of brachial plexus sonography and magnetic resonance imaging in chronic inflammatory demyelinating neuropathy and multifocal motor neuropathy. Eur J Neurol. 2017;24:1307-1313.

2. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol. 2011;8:48-58.

3. Van Asseldonk JT, Franssen H, Van den Berg-Vos RM, et al. Multifocal motor neuropathy. Lancet Neurol. 2005;4:309-319.

4. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology. 2010;75:818-825.

5. Loscher WN, Oberreiter EM, Erdler M, et al. Multifocal motor neuropathy in Austria: a nationwide survey of clinical features and response to treatment. J Neurol. 2018;265:28342840.

6. Goedee HS, van der Pol WL, van Asseldonk JH, et al. Diagnostic value of sonography in treatment-naive chronic inflammatory neuropathies. Neurology. 2017;88:143-151.

7. Joint Task Force of the E, the PNS. European Federation of Neurological

Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripher Nerv Syst. 2010;15:295-301.

8. Van Schaik IN, van den Berg LH, de Haan R, et al. Intravenous immunoglobulin for multifocal motor neuropathy. Cochrane Database Syst Rev. 2005;18:CD004429.

9. Leger JM, Chassande B, Musset L, et al. Intravenous immunoglobulin therapy in multifocal motor neuropathy: a double-blind, placebo-controlled study. Brain. 2001;124:145-153.

10. Eftimov F, Vermeulen M, de Haan RJ, et al. Subcutaneous immunoglobulin therapy for multifocal motor neuropathy. J Peripher Nerv Syst. 2009;14:93-100.

11. Harbo T, Andersen H, Hess A, et al. Subcutaneous versus intravenous immunoglobulin in multifocal motor neuropathy: a randomized, single-blinded cross-over trial. Eur J Neurol. 2009;16:631-638.

12. Markvardsen LH, Harbo T. Subcutaneous immunoglobulin treatment in CIDP and MMN. Efficacy, treatment satisfaction and costs. J Neurol Sci. 2017;378:19-25.

13. Lange DJ, Weimer LH, Trojaborg W, et al. Multifocal motor neuropathy with conduction block: slow but not benign. Arch Neurol. 2006;63:1778-1781.

14. Van den Berg-Vos RM, Franssen H, Visser J, et al. Disease severity in multifocal motor neuropathy and its association with the response to immunoglobulin treatment. J Neurol. 2002;249:330-336.

15. Taylor BV, Wright RA, Harper CM, et al. Natural history of 46 patients with multifocal motor neuropathy with conduction block. Muscle Nerve. 2000;23:900-908.

16. Van Asseldonk JT, Van den Berg LH, Kalmijn S, et al. Axon loss is an important determinant of weakness in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry. 2006;77:743-747.

17. Vanhoutte EK, Faber CG, van Nes SI, et al. Rasch-built Overall Disability Scale for Multifocal motor neuropathy (MMN-RODS(©). J Peripher Nerv Syst. 2015;20:296-305.

18. Merkies IS, Schmitz PI. Getting closer to patients: the INCAT Overall Disability Sum Score relates better to patients' own clinical judgement in immune-mediated polyneuropathies. J Neurol Neurosurg Psychiatry. 2006;77:970-972.

19. Van Nes SI, Vanhoutte EK, van Doorn PA, et al. Rasch-built Overall Disability Scale (R-ODS) for immune-mediated peripheral neuropathies. Neurology. 2011;76:337-345.

20. Rietberg MB, Van Wegen EE, Kwakkel G. Measuring fatigue in patients with multiple sclerosis: reproducibility, responsiveness and concurrent validity of three Dutch selfreport questionnaires. Disabil Rehabil. 2010;32:1870-1876.

21. Cats EA, van der Pol WL, Piepers S, et al. New liquid intravenous immunoglobulin (10 % IVIg) for treatment of multifocal motor neuropathy: a prospective study of efficacy, safety and tolerability. J Neurol. 2008;255:1598-1599.

22. Krupp LB, LaRocca NG, Muir-Nash J, et al. The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus. Arch Neurol. 1989;46:1121-1123.

23. Martina IS, van Koningsveld R, Schmitz PI, et al. Measuring vibration threshold with a graduated tuning fork in normal aging and in patients with polyneuropathy. European Inflammatory Neuropathy Cause and Treatment (INCAT) group. J Neurol Neurosurg Psychiatry. 1998;65:743-747.

24. Van den Berg-Vos RM, Franssen H, Wokke JH, et al. Multifocal motor neuropathy: long-term clinical and electrophysiological assessment of intravenous immunoglobulin maintenance treatment. Brain. 2002 Aug;125:1875-1886.

25. Van Asseldonk JT, Van den Berg LH, Van den Berg-Vos RM, et al. Demyelination and axonal loss in multifocal motor neuropathy: distribution and relation to weakness. Brain. 2003;126:186-198.

26. Hattori N, Yamamoto M, Yoshihara T, et al. Demyelinating and axonal features of Charcot-Marie-Tooth disease with mutations of myelin-related proteins (PMP22, MPZ and Cx32): a clinicopathological study of 205 Japanese patients. Brain. 2003;126:134-151.

27. Al-Zuhairy A, Sindrup SH, Andersen H, et al. A population-based and cross-sectional study of the long-term prognosis in multifocal motor neuropathy. J Peripher Nerv Syst. 2019;24:64-71.

28. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of antiGM1 IgM antibodies with clinical features. Neurology. 2010;75:1961-1967.

29. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm. 2015;2:e119.

30. Van Alfen N, van Engelen BG. The clinical spectrum of neuralgic amyotrophy in 246 cases. Brain. 2006;129:438-450.

31. Merkies IS, Faber CG. Fatigue in immune-mediated neuropathies. Neuromuscul Disord. 2012;22:S203-207.

32. Merkies IS, Schmitz PI, Samijn JP, et al. Fatigue in immune-mediated polyneuropathies. European Inflammatory Neuropathy Cause and Treatment (INCAT) Group. Neurology. 1999;53:1648-1654.

33. Goedee HS, Herraets I, Visser LH, et al. Nerve ultrasound can identify treatmentresponsive chronic neuropathies without electrodiagnostic features of demyelination. Muscle Nerve. 2019;60:415-419

34. Kobayashi C, Kinoshita T, Miyazaki D, Hineno A, Nakamura A. Increasing incidence and age at onset of amyotrophic lateral sclerosis in Nagano Prefecture, Japan. Shinshu MedJ. 2016;64:239-246.

35. Koch-Henriksen N, Thygesen LC, Stenager E, et al. Incidence of MS has increased markedly over six decades in Denmark particularly with late onset and in women. Neurology. 2018;90:e1954-e1963.

36. Delmont E, Benaim C, Launay M, set al. Do patients having a decrease in SNAP amplitude during the course of MMN present with a different condition? J Neurol. 2009;256:1876-1880.

37. Lambrecq V, Krim E, Rouanet-Larriviere M, et al. Sensory loss in multifocal motor neuropathy: a clinical and electrophysiological study. Muscle Nerve. 2009;39:131-136.

Supplementary tables

Table e-1 Specification of neurological examination and questionnaires	
Modality	**Description**
MRC score	Bilateral measurement of motor function of:
	Abduction of the arm
	Flexion and extension of the forearm wrist, and fingers
	Spreading of the fingers
	Abduction, adduction and opposition of the thumb
	Flexion of the hip
	Flexion and extension of the knee and foot
	Extension and flexion of the toes
	MRC sum score: 0-180 points
Vibration sense	Bilateral assessment of sensory function using Rydell-Seiffer tuning fork:
	Normal (grade 0)
	Abnormal hallux valgus (grade 1)
	Abnormal ankle (grade 2)
	Abnormal knee (grade 3)
	Abnormal at the acromioclavicular joint or anterior superior iliac spine (grade 4)
Vigorimetry	Bilateral measurement of grip strength in Kilopascals (kPa) with the Martin Vigorimeter (Martin Medizintechnik, Tuttlingen, Germany)

Table e-2 Mean MRC grade per muscle group

	Total cohort (n=100)	**Disease duration < 180.6 months (n=50)**	**Disease duration ≥ 180.6 months (n=50)**	***p*-value**
Proximal arm				
Elbow extension	4.7	4.7	4.6	0.33
Elbow flexion	4.4	4.5	4.3	0.63
Shoulder abduction	4.5	4.5	4.5	0.38
Wrist flexion	4.4	4.5	4.3	0.63
Wrist extension	3.9	4.1	3.6	0.20
Hand				
Flexion fingers	4.5	4.7	4.3	0.01
Extension fingers	3.3	3.7	3.0	0.03
Adduction thumb	3.4	3.9	2.9	0.01
Opposition thumb	3.2	3.7	2.7	0.03
Spreading fingers	3.1	3.5	2.7	0.03
Abduction thumb	3.0	3.6	2.4	0.01
Upper Leg				
Hip flexion	4.9	4.8	4.9	0.33
Knee flexion	4.9	4.9	4.9	0.73
Knee extension	4.9	5.0	4.9	0.38
Lower leg/foot				
Foot plantar flexion	4.3	4.8	3.9	0.03
Flexion toes	4.2	4.7	3.7	0.03
Foot dorsal flexion	3.4	4.2	2.6	0.01
Extension toes	3.5	4.1	2.9	0.04

Table e-3 Outcome measures	
	All inclusions (n=100)
MRC sum score	165 (69-180)
Vibration sense	
Abnormal in at least one limb	57 (58)
Normal	42 (42)
Reflexes	
At least one reflex abnormal	79 (80)
Generalized areflexia	16 (16)
Normal	20 (20)
ODSS	
Arms	2 (0-4)
Legs	1 (0-5)
Total	3 (0-8)
SES	10 (1-25)
FSS	37 (9-61)

Data are shown in median (range) or in number of patients (%).
MRC = Medical Research Council, ODSS = Overall Disability Sum Scale, SES = Self-Evaluation Scale, FSS = Fatigue Severity Scale

Table e-4 Multiple linear regression analysis for determinants of weakness

	Model 1		Model 2		Model 3		Model 4		Model 5		
Variable	RC	SE	RC	SE	RC	SE	RC	SE	RC	SE	95% CI
Anti-GM1 IgM antibodies	-10.87	5.27[b]	-10.68	5.17[b]	-10.81	5.18[b]	-11.10	5.20[b]	-10.65	5.24[b]	[-21.2,-0.21]
Sex	7.29	6.19	7.12	6.10							
Symptom onset leg	-8.52	6.32	-8.51	6.28	-8.98	6.28	-9.75	6.28			
Age at onset of symptoms	0.44	0.34	0.45	0.34	0.51	0.33	0.67	0.31[b]	0.832	0.30[c]	[0.24,1.42]
Months untreated[a]	-4.65	2.53	-4.55	2.47	-4.85	2.46	-5.09	2.47[b]	-5.69	2.46[b]	[-10.6,-0.79]
Duration of treatment[a]	-4.55	2.98	-4.49	2.94	-3.68	2.87					
FSS	-0.04	0.17									
Adjusted R^2	0.21		0.22		0.22		0.21		0.19		

Multiple linear regression analyses to predict the MRC sum score 2015-2016 based on anti-GM1 IgM antibodies (presence =1, absence =0), sex (male =0, female = 1), symptom onset in a leg (arm =0, leg =1), age at onset of symptoms in years, months untreated (log-transformed), duration of treatment in months (log-transformed) and FSS (0-63). The models show the different steps of the backward selection. In model 1 all independent factors were included and in model 5 only the independent factors that were significantly associated with a lower MRC sum score.

[a] log transformed variable, [b] $p<0.05$, [c] $p<0.01$, FSS = Fatigue Severity Scale, RC = regression coefficient, 95% CI = 95% confidence interval, SE = standard error

Table e-5 Predictors of progression

	Model 1		Model 2		Model 3		Model 4		Model 5		Model 6		
Variable	RC	SE	RC	SE	RC	SE	RC	SE	RC	SE	RC	SE	95% CI
Anti-GM1 IgM antibodies	0.18	0.34											
Sex	-0.52	0.37	-0.51	0.36	-0.47	0.36	-0.52	0.35	-0.50	0.35			
Symptom onset leg	-0.33	0.38	-0.33	0.37	-0.36	0.37	-0.32	0.36					
Months untreated[a]	-0.11	0.17	-0.11	0.17									
Reflexes sum score 2007	-0.17	0.08[b]	-0.17	0.07[b]	-0.16	0.07[b]	-0.16	0.07[b]	-0.17	0.07[b]	-0.18	0.07[b]	[-0.32,-0.03]
MRC sum score 2007	-0.03	0.02	-0.03	0.02	-0.03	0.02	-0.04	0.01[b]	-0.03	0.01[b]	-0.03	0.01[c]	[-0.06,-0.01]
ODSS 2007	0.15	0.16	0.16	0.16	0.15	0.16							
Age at onset of symptoms	-0.03		-0.03	0.02	-0.04	0.02	-0.04	0.02	-0.03	0.02	-0.03	0.02	
Adjusted R^2	0.42		0.43		0.43		0.43		0.44		0.43		

Multiple linear regression analysis to predict the difference of the MRC sum score between visit 1 (2007) and visit 2 (2015-2016) per year based on anti-GM1 IgM antibodies (presence =1, absence =0), sex (male =0, female =1), symptom onset in leg (arm=0, leg =1), months untreated (log-transformed), age at onset of symptoms in years, sum score of reflexes (0-8), MRC sum score (0-180) and ODSS (0-8). The last three variables were analysed with data of the first visit (2007). The models show the different steps of the backward selection. In model 1 all independent factors were included and in model 6 only the independent factors that were significantly associated with the difference of the MRC sum score between visit 1 (2007) and visit 2 (2015-2016) per year.

[a] log transformed variable, [b] $p<0.05$, [c] $p<0.01$, MRC = Medical Research Council, ODSS = Overall Disability Sum Score, RC = regression coefficient

CHAPTER

High-resolution mapping identifies HLA class II associations with multifocal motor neuropathy

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Abstract

Objective To gain further insight in the immunopathology underlying multifocal motor neuropathy (MMN) by exploring the association between MMN and the human leukocyte antigen (HLA) class II DRB1, DQB1 and DQA loci in depth and by correlating associated haplotypes to detailed clinical and anti-ganglioside antibody data.

Methods We performed high-resolution HLA-class II typing for the DRB1, DQB1 and DQA1 loci in 126 well-characterized MMN patients and assessed disease associations with haplotypes. We used a cohort of 1305 random individuals as a reference for haplotype distribution in the Dutch population.

Results The DRB1*15:01-DQB1*06:02 haplotype (OR 1.6 (95% CI 1.1-2.2), p <.05) and the DRB1*12:01-DQB1*03:01 haplotype (OR 2.7 (95% CI 1.2-5.5), p<.05) were more frequent in patients with MMN than in controls. These haplotypes were not associated with disease course, response to treatment or anti-ganglioside antibodies.

Conclusions Multifocal motor neuropathy is associated with the DRB1*15:01-DQB1*06:02 and DRB1*12:01-DQB1*03:01 haplotypes. These HLA molecules or gene variants in their immediate vicinity may promote the specific inflammatory processes underlying MMN.

Introduction

Multifocal motor neuropathy (MMN) is a rare neuropathy with a distinct clinical phenotype. It is characterized by distal, asymmetric, primarily upper limb weakness without significant sensory abnormalities. MMN affects mostly young to middle-aged men and although treatment with intravenous or subcutaneous immunoglobulins (IVIg of scIg) often leads to improvement in muscle strength, remission is rare. In the majority of patients disease course is slowly progressive, leading to severe disability of hand and arm function in a significant subgroup of patients.[1-3]

MMN is probably an immune-mediated disorder. From its first description, the presence of IgM antibodies directed at GM1, a glycolipid constituent of motor and sensory nerves, has been noted. These antibodies are probably mono- or oligoclonal and have the capacity to activate the classical complement pathway.[4-6] The interaction of antibodies and complement was recently shown to interfere with motor neuron function and to induce cell damage and death in an induced pluripotent stem cell (iPSC) derived model for MMN.[7,8]

It is as yet not known which genetic and environmental factors may contribute to MMN susceptibility, including the propensity for increased anti-GM1 IgM titers. Common genetic factors for autoimmunity may play a role as suggested by slightly increased risk for autoimmune diseases and the increased frequency of the HLA-DRB1*15 allele in Dutch patients.[9,10] In order to gain further insight in the pathogenesis of MMN, we performed a high-resolution HLA-DRB1, -DQA1 and -DQB1 typing in MMN patients and assessed their association with MMN susceptibility and MMN disease course.

Materials and methods

Study population

Patients were diagnosed and enrolled at the outpatient clinic of the University Medical Center of Utrecht (UMCU), a tertiary referral center and national expertise center for MMN. All patients fulfilled diagnostic criteria for definite, probable or possible MMN as specified in both the initial criteria described by our group and the follow-up EFNS diagnostic criteria.[11,12] These criteria primarily rely on the presence of the specific finding of conduction block, or – in its absence - a combination of abnormal ancillary investigations or a response to treatment with intravenously administered immunoglobulins that suggests MMN.

Clinical data were obtained from the MMN database, when necessary supplemented with the most recent data from UMCU patient files.[1] Data included sex, age at onset, age at diagnosis, delay until diagnosis, presence of anti-GM1 IgM antibodies, response to IVIg treatment, last recorded IVIg dosage per month, abnormalities on MR imaging of the brachial plexus, muscle strength and number of affected limb regions at the first visit and last follow-up visit. Onset of disease was defined as first complaints of muscle weakness. Diagnostic delay was defined as the time that lapsed between first symptom onset and MMN diagnosis. Muscle strength was graded on a 6-point Medical Research Council (MRC) scale, ranging from 0 (no contraction) to 5 (normal muscle strength against resistance) on the patients' first and last visit to the outpatient clinic. We documented MRC scores of shoulder abduction, elbow flexion, elbow extension, wrist flexion, wrist extension, finger flexion, finger extension, finger spreading, hip flexion, knee flexion, knee extension, foot dorsal flexion and foot plantar flexion. MRC sum scores (maximum score 130) were obtained by summation of the MRC values of all tested

muscle groups. In addition, the number of affected limb regions, i.e. bilateral proximal and distal limb regions (maximum score 8) on patients' first and last follow-up visit were recorded. Nerve conduction studies were performed as described previously.[1,10] In short, motor and sensory function were assessed bilaterally in median, ulnar, radial, musculocutaneous, peroneal and tibial nerves. The number of definite conduction blocks, defined as CMAP area reduction of at least 50% (definite conduction block), or of 30%-50% (probable conduction block) over 2.5 cm inching, was scored in nerves with a distal CMAP > 1 mV. IgM antibodies against GM1 were assessed using a standardized ELISA in most patients.[13] The presence and titers of antibodies against the gangliosides GM1, GD1b, asialo-GM1 and GM2 were tested using a standardized ELISA as described earlier.[14] Anti-ganglioside cross-reactivity patterns were assessed in anti-GM1 IgM positive and negative patients.[15] A neuroradiologist reported on hyperintensity or thickening of the brachial plexus on MR imaging.
All patients with MMN were Dutch. The control group for HLA typing consisted of a random sample of 1305 Dutch individuals living in or near the city of Leiden, The Netherlands.[16]

HLA typing
We used the commercially available NGSgo-AmpX kit (GenDx), containing HLA-DRB1, HLA-DQB1 and HLA-DQA1 specific primers. Regions of interest were amplified by polymerase chain reaction according to the manufacturer's instructions.
A library preparation using NGSgo-LibrX and NGSgo-IndX kits was performed on the PCR products prior to next-generation sequencing (NGS). We used the MiSeq reagent kit v2 (500 cycles) MS-102-2003 for forward and reverse MiSeq sequencing. We used the freely available NGSengine software (GenDx) for data analysis and HLA typing. We used the standard World Health Organization nomenclature.

Statistical analysis
We carried out statistical analyses using R statistics version 3.4.1 (2017) and used the Genetics package to calculate allele and haplotype frequencies. When alleles or haplotypes were found in at least 5% of the MMN population, we used a Chi-squared or Fisher's Exact test, as appropriate, to compare these frequencies to the control cohort. We used odds ratios with a 95% confidence interval as a measure of association. When correlating haplotypes to clinical parameters, we performed pairwise comparisons between patients that either did or did not carry an associated haplotype. Continuous clinical variables, i.e. age at onset, age at diagnosis, number of definite conduction blocks, the anti-GM1 IgM antibody titer, IVIg dosage, first and last visit MRC sum scores and first and last visit number of affected regions,were compared using a Student's t test or a Mann-Whitney U test, as appropriate. We compared dichotomous variables, i.e. the presence of anti-GM1 IgM antibodies,with a Chi-squared or Fisher's Exact test. We corrected continuous variables for sex, diagnostic delay, age at diagnosis, disease duration and treatment with immunoglobulins using a multivariate linear model. We used the common rule of thumb of at least 10 observations per correcting variable.
Given the aim of this study, the small patient population and the independence of variables, we deemed a p-value correction method using the Bonferroni method too conservative for comparing the HLA data. Therefore, we used a false discovery rate (fdr) method instead. Since the clinical variables are not independent, we used a Bonferroni correction method to correct the p-values of comparisons of clinical data. In both cases, a corrected p-value < .05 was considered statistically significant.

Standard protocol approvals, registrations, and patient consents
The locally appointed ethics committee of the University Medical Center Utrecht gave approval for this study (protocol NL-50354.041.14). All included patients gave written informed consent prior to inclusion in this study.

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

Results

Study population
We obtained full high-resolution HLA typing from 126 out of 130 (97%) initially enrolled patients with MMN. Baseline characteristics are shown in table 1. Nine patients (7.1%) were lost to follow-up after their first visit to the UMCU. Nerve conduction data from our hospital were available of all patients with the exception of 9 (7.1%) who underwent NCS elsewhere prior to referral to our hospital. These NCS were revised by experts for a previous study and presence and site of conduction block were recorded in our files.[1] Brachial plexus MRI results were available of 73 (58%) of patients. Anti-GM1 IgM antibody testing results (positive or negative) were available for 88 (70%) patients. Follow-up data were present in 117 MMN patients, with a median follow-up time of over 9 years.

HLA class II typing
High-resolution HLA typing yielded 26 DRB1 and 16 DQB1 alleles in 126 MMN patients. Results are shown in table 2.
Allele frequencies of DRB1*15:01 and DRB1*12:01 were significantly higher in MMN patients. The DRB1*15:01-DQB1*06:02 and DRB1*12:01-DQB1*03:01 haplotype frequencies were significantly higher in MMN patients (OR 1.6 (1.1-2.2) and 2.7 (1.2-5.5)), as shown in table 3. Comparisons of HLA-DQA1 allele frequencies were not possible since we lacked information in the control cohort. All patients carrying the HLA-DRB1*15:01-DQB1*06:02 haplotype also carried the HLA-DQA1*01:02 allele. Eleven out of 12 patients (92%) carrying the HLA-DRB1*12:01-DQB1*03:01 haplotype carried the HLA-DQA1*05:05 allele. Therefore, HLA-DQA1*01:02 and HLA-DQA1*05:05 seem to form part of the extended haplotypes of HLA-DRB1*15:01-DQB1*06:02 and HLA-DRB1*12:01-DQB1*03:01, respectively.

Table 1 Baseline characteristics of MMN patients (N=126)		
		N (%)
Male, n (%)	96 (76)	126 (100)
Age at onset, years (SD) [$]	41 (10.5)	126 (100)
Age at diagnosis, years (SD) [$]	48 (10.9)	126 (100)
Delay until diagnosis, months [§]	49 (3-585)	126 (100)
Definite MMN, n (%)	94 (75)	126 (100)
Probable MMN, n (%)	22 (17)	126 (100)
Possible MMN, n (%)	10 (8)	126 (100)
Follow-up, months [§]	109 (4-345)	119 (94)
Positive anti-GM1 IgM antibody, n (%)	74 (67)	111 (88)
Anti-GM1 IgM antibody titer [§]	1:200 (0 – 1:51200)	88 (70)
Abnormal brachial plexus MRI, n (%)	33 (26)	73 (58)
Response to IVIg therapy, n (%)	94 (93)	101 (80)
IVIg dosage per 4 weeks (range) [§]	53 (0-275)	101 (80)
Definite CB on diagnostic EMG, n (range) [§]	1 (0-8)	117 (93)
MRC sum score on first visit (range) [§]	122 (85-130)	126 (100)
MRC sum score on last visit (range) [§]	120 (51-130)	117 (93)
Affected limb regions on first visit, n (range) [§]	3 (1-8)	126 (100)
Affected limb regions on last visit, n (range) [§]	4 (0-8)	117 (93)

[$] Values displayed as mean (SD)

[§] Values displayed as median (range)

Abbreviations: CB = Conduction block, IVIg = Intravenous immunoglobulins, MMN = Multifocal motor neuropathy, MRC = Medical resource council, MRI = Magnetic resonance imaging

.

Table 2 HLA class II allele frequencies in MMN patients and controls

DRB1 locus	MMN	MMN (%$)	Controls	p-value	DQB1 locus	MMN	MMN (%$)	Controls	p-value
DRB1*01:01	0.103	18.3	0.107	0.840	DQB1*02:01	0.111	21.4	0.139	0.222
DRB1*03:01	0.111	21.4	0.136	0.268	DQB1*02:02	0.083	16.7	0.072	0.526
DRB1*04:01	0.067	12.7	0.097	0.126	DQB1*03:01	0.154	27.8	0.157	0.923
DRB1*04:02	0.004	0.8	0.004	-	DQB1*03:02	0.087	16.7	0.111	0.234
DRB1*04:03	0.016	3.2	0.008	-	DQB1*03:03	0.032	6.3	0.040	0.509
DRB1*04:04	0.028	5.6	0.038	0.400	DQB1*03:05	0.004	0.8	0.000	-
DRB1*04:07	0.008	1.6	0.009	-	DQB1*04:02	0.012	2.4	0.026	-
DRB1*07:01	0.111	22.2	0.107	0.851	DQB1*05:01	0.139	25.4	0.136	0.899
DRB1*08:01	0.012	2.4	0.026	-	DQB1*05:02	0.008	1.6	0.013	-
DRB1*08:03	0.008	1.6	0.002	-	DQB1*05:03	0.016	3.2	0.034	-
DRB1*09:01	0.008	1.6	0.009	-	DQB1*06:01	0.008	1.6	0.005	-
DRB1*10:01	0.020	4.0	0.016	-	DQB1*06:02	0.206	36.5	0.145	0.009
DRB1*11:01	0.048	9.5	0.058	0.503	DQB1*06:03	0.073	13.5	0.065	0.515
DRB1*11:03	0.000	0.8	0.003	-	DQB1*06:04	0.056	11.1	0.042	0.318
DRB1*11:04	0.012	2.4	0.018	-	DQB1*06:09	0.004	0.8	0.007	-
DRB1*12:01	0.044	8.7	0.016	0.0061*	DQB1*06:49	0.004	0.8	0.000	-
DRB1*12:02	0.008	1.6	0.002	-					
DRB1*13:01	0.071	12.7	0.062	0.559					
DRB1*13:02	0.063	12.7	0.048	0.288					
DRB1*13:03	0.004	0.8	0.007	-					
DRB1*14:01	0.008	0.8	0.001	-					
DRB1*14:54	0.012	2.4	0.033	-					
DRB1*15:01	0.210	37.3	0.149	0.0096*					
DRB1*15:02	0.012	2.4	0.005	-					
DRB1*16:01	0.004	0.8	0.011	-					
DRB1*16:02	0.004	0.8	0.000	-					

Allele frequency = (N alleles)/(2*N). The allele frequencies of alleles present in at least 5% of MMN patients were compared with the control cohort.

* Statistically significant after post-hoc p-value adjustment

$ Percentage of MMN population (n=126) carrying at least one allele

Table 3 Haplotype frequencies of MMN associated DRB1 alleles in MMN patients and controls

HLA Class II Haplotype	MMN	MMN (%)[$]	Controls	p-value	OR (95% CI)
DRB1*12:01-DQB1*03:01	0.044	8.7	0.016	0.006[*]	2.7 (1.2-5.5)
DRB1*15:01-DQB1*05:01	0.000	0.0	0.000	-	-
DRB1*15:01-DQB1*05:02	0.000	0.0	0.001	-	-
DRB1*15:01-DQB1*06:01	0.000	0.0	0.001	-	-
DRB1*15:01-DQB1*06:02	0.206	36.5	0.142	0.009[*]	1.6 (1.1-2.2)
DRB1*15:01-DQB1*06:03	0.000	0.0	0.003	-	-
DRB1*15:01-DQB1*06:04	0.004	0.8	0.004	-	-
DRB1*15:01-DQB1*06:39	0.000	0.0	0.000	-	-

Haplotypes frequencies of haplotypes found in at least 5% of MMN patients were compared between groups. Haplotype frequency = (N haplotype copies)/(2*N).
* Statistically significant after p-value adjustment
[$] Percentage of MMN patients (n=126) carrying at least on haplotype copy

Clinical correlation

MMN patients were stratified as carriers of the HLA-DRB1*15:01-DQB1*06:02, HLA-DRB1*12:01-DQB1*03:01 or neither of these haplotypes. One subject was excluded from the analysis since this patient carried both. Given the small sample size of patients carrying the HLA-DRB1*12:01-DQB1*03:01 haplotype, correction for continuous variables was carried out for the HLA-DRB1*15:01-DQB1*06:02 group only. Results are shown in table 4.

There was no correlation between HLA-DRB1*12:01-DQB1*03:01 haplotype carriership and clinical parameters. Patients carrying the HLA-DRB1*15:01-DQB1*06:02 haplotype had a trend towards a higher median MRC sum scores at the first visit and last follow-up visit and a lower number of affected regions on the last follow-up visit.

Table 4 Clinical parameters of MMN patients grouped by carriership of the associated haplotypes

				p-values	
	Negative (n=70)	**DR12 (n=10)**	**DR15 (n=45)**	DR12	DR15
Age at onset[$]	42 (10.9)	37 (11.3)	41 (9.7)	0.122	0.745
Age at diagnosis[$]	48 (10.9)	40 (11.7)	48 (10.1)	0.017	0.908
Definite CBs, n (range)[§]	1 (0-8)	1 (1-3)	1 (0-4)	0.750	0.199
Anti-Gm1 IgM positive, %	67	40	73	0.158	0.691
Anti-GM1 IgM antibody titer[§]	1:400 (0 – 1:51200)	1:100 (0 – 1:51200)	1:300 (0-1:51200)	0.890	0.210
IVIg dosage§	53 (0-275)	58 (0-200)	60 (0-225)	0.842	0.850
First visit MRC sum score[§]	120 (85-130)	122 (101-128)	123 (96-130)	0.482	0.150
First visit affected regions, n[§]	3 (1-8)	3 (1-8)	2 (1-7)	0.966	0.140
Last follow-up visit MRC sum score§	119 (51-130)	121 (109-130)	121 (75-130)	0.196	0.014
Last follow-up visit affected regions, n[§]	4 (0-8)	4 (0-8)	3 (0-6)	0.704	0.001[*]

Comparisons were made between the negative group and the DR12 and DR15 group separately
DR12 = DRB1*12:01-DQB1*03:01 haplotype carriers; DR15 = DRB1*15:01-DQB1*06:02 haplotype carriers
* Statistically significant at p <.05 after correction for multiple testing
[$] Values displayed as mean (SD)
[§] Values displayed as median (range)

As shown in table 4, the presence of anti-GM1 IgM antibodies did not differ between patients grouped by haplotype carriership. To further assess the possible pathogenic role of the associated haplotypes, we determined their correlation with anti-GM1 IgM antibody cross-reactivity patterns. As described previously, anti-GM1 IgM antibodies can show cross-reactivity with other gangliosides, notably asialo-GM1 (aGM1), GD1b and GM2, indicative of fine epitope specificity of anti-GM1 IgM antibodies. Three main specificity patterns have been described. First, some anti-GM1 IgM antibodies are GM1 specific and do no cross-react with either aGM1, GD1b or GM2. Second, anti-GM1 IgM antibodies targeting the terminal Gal (β1-3) GalNac structure show cross-reactivity with asialo-GM1 and GD1b. Third, antibodies targeting a shared internal sialylated sugar cross-react with GM2.[15] In patients without anti-GM1 IgM antibodies, usually no IgM antibodies against aGM1, GD1b or GM2 are found.
Data on the presence of anti-GM1, -GM2, -GD1b and –asialo-GM1 IgM antibodies were available for 81 patients (64%). Associations with anti-GM1 IgM cross-reactivity patterns could not be formally tested for the HLA-DRB1*12:01-DQB1*03:01 haplotype due to very low numbers (n=7). The presence of the HLA-DRB1*15:01-DQB1*06:02 haplotype did not differ from the other 45 patients (Chi-squared test, χ^2 = 0.30, p-value .58). HLA-DRB1*15:01-DQB1*06:02 was not associated with a specific anti-GM1 IgM cross-reactivity pattern, both in anti-GM1 IgM positive and negative patients (table 5).

Table 5. HLA-DRB1*15:01-DQB1*06:02 status in cross-reactivity patterns of anti-GM1, -GD1b, -asialo-GM1 (aGM1) and -GM2 IgM antibodies

		HLA-DRB1*15:01-DQB1*06:02	
Anti-GM1 IgM	**Cross-reactivity pattern**	Positive (n (%))	Negative (n (%))
Anti-GM1 IgM$^+$ (n=44)	Anti-GM1 IgM$^+$ only	3 (33%)	6 (67%)
	Anti-GM1/GD1b/aGM1 IgM$^+$	2 (18%)	9 (82%)
	Anti-GM1/GM2$^+$	2 (67%)	1 (33%)
	Other	7 (33%)	14 (67%)
Anti-GM1 IgM$^-$ (n=37)	Anti-GM1/GD1b/aGM1/GM2 IgM$^-$	11 (48%)	12 (52%)
	Other	6 (43%)	8 (57%)

Percentages shown are within cross-reactivity patterns. A Fisher's Exact test was performed in the anti-GM1 IgM positive and negative groups separately (p = .44 and 1.00, respectively).

Discussion

This study corroborates our previous finding of an association of HLA-DRB1*15 with MMN but not with its clinical characteristics, and extends the association to an extended haplotype, likely HLA-DRB1*15:01-DQB1*06:02-HLA-DQA1*01:02.[10] Moreover, we identified HLA-DRB1*12:01 as a second risk factor, independent of HLA-DRB1*15:01-DQB1*06:02, as part of a haplotype with DQB1*03:01 and possibly HLA-DQA1*05:05. Almost half of MMN patients carried one of these haplotypes (9% and 37%, respectively). These associations may improve our understanding of the immunopathology in MMN.

In a previous smaller study of 74 patients with MMN we found an association with the HLA class II DRB1*15 allele group.[10] A major drawback of the previous study was its lack of statistical power to detect associated alleles with a prevalence below 15%. The larger sample size and refined methodology in this study allowed us to perform more detailed analysis of the HLA-DRB1 locus in MMN. Our data show that the association of MMN with DRB1*15 is explained by an increased frequency of the DRB1*15:01-DQB1*06:02 haplotype. We found a second association with the DRB1*12:01 allele and more specifically, the DRB1*12:01-DQB1*03:01 haplotype. This new association is not explained by ethnic imbalance between patient and control groups. None of the patients had an ethnic background in which allele frequencies of DRB1*12:01 are reportedly higher, i.e. Asian and African American populations.[17]

The finding of an HLA class II association with MMN, when interpreted as a role for $CD4^+$ cells in the pathophysiological processes underlying an immune response against the glycolipid GM1, is puzzling.[8] Both HLA-DRB1*15:01 and HLA-DRB1*12:01 have been found associated with autoimmune disorders, including those of the central and peripheral nervous system. DRB1*15:01 has been found associated with the anti-neurofascin-155 positive chronic inflammatory demyelinating polyneuropathy (CIDP) and both alleles may converge in their reported associations with the multiple sclerosis – NMO spectrum.[18-23] Although specific characteristics of HLA molecules, such as the affinity of DRB1*15:01 to accommodate constituents of the myelin sheath including myelin-bound protein (MBP) and possibly ceramide, an important constituent of gangliosides, may suggest a direct pathogenic role, the initiation of the anti-GM1 IgM immune response is probably T-cell independent.[21] Indeed, we have shown that HLA-DRB1*15:01-DQB1*06:02 is not associated with the presence of anti-GM1 IgM antibodies, nor with its titer or anti-ganglioside cross-reactivity patterns. Also, we have previously shown that only a small minority of patients with MMN show signs of anti-GM1 IgM isotype switching to IgG (1%) or IgA (5%), all with a titer of 1:400 or less and all having an anti-GM1 IgM titer that far exceeds the isotype titer.[14] We therefore think it is unlikely that the HLA class II associations are based on directly propagating antigen presentation or germinal center CD4+ T-cell interaction with activated B cells. However, CD4+ cells, including T-helper cells and regulatory T cells, could play another role in MMN pathogenesis, for example in the formation and maintenance of anti-GM1 IgM immunological memory by interacting with long-lived IgM-secreting plasma cells.[24]

Dissecting the pathophysiological role of HLA associations is often difficult. DRB1*15:01 and DQB1*06:02, and DRB1*12:01 and DQB1*03:01 were in strong linkage disequilibrium (LD) and this may complicate the direct identification of functional effects of the alleles constituting a

haplotype, not in the least because alleles within a haplotype may contribute to different pathogenic pathways.[25,26] Moreover, HLA alleles could be in LD with unidentified disease-associated genes in their vicinity, including the HLA class III region that encompasses major components of the complement system. This is exemplified by the association of DRB1*15:01 with MS, which is better explained by its strong LD with an 11 SNP string (DRB1*15:01-*a1* haplotype).[19] Future studies are needed to explore the possibility of LD as explanation of the associations of HLA haplotypes with MMN in more detail.

We did not find associations of HLA haplotypes with MMN disease course or response to treatment. Although patients carrying the DRB1*15:01-DQB1*06:02 had a trend towards higher MRC sum scores and a lower number of affected limb regions on their first visit, the clinical relevance of this finding is questionable. The lack of clear associations is in accordance with our previous study.[10]

To the best of our knowledge, this is the only study on high-resolution HLA-class II genetics in MMN and given the rarity of this disease, the study population can be considered large. We believe that the robustness of our data is exemplified by the fact that we have corroborated the previous HLA-DRB1*15 association, in an expanded patient cohort and with a new and larger Dutch control cohort. Since the UMCU is a national tertiary referral center for MMN, we had the unique opportunity to correlate HLA genetics to clinical parameters and the presence of anti-ganglioside antibodies. Detailed clinical follow-up information was present in 93% of our patients, with a median follow-up time of over 9 years. Potential weaknesses are the fact that approximately 20% of our patients when first seen at our hospital already used IVIg, although this was statistically corrected for. The assessments of muscle strength, both at patients' first visit and last follow-up visit, were performed by more than one physician, all neurologists specialized in neuromuscular disorders. Ethnicity of MMN patients was not formally recorded, but all were Dutch, matching the ethnic background of the control population.

In conclusion, this study shows that the DRB1*12:01-DQB1*03:01 and DRB1*15:01-DQB1*06:02 haplotypes are associated with multifocal motor neuropathy. Future studies should assess possible population differences and determine the underlying pathogenic mechanisms in MMN.

References

1. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology 2010a;75(9):818-825

2. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

3. Yeh WZ, Dyck PJ, Van den Berg LH, et al. Multifocal motor neuropathy: controversies and priorities. J Neurol Neurosurg Psychiatry 2020;91:140-148

4. Cats EA, Van der Pol WL, Tio-Gillen AP, et al. Clonality of anti-GM1 IgM antibodies in multifocal motor neuropathy and the Guillain-Barré syndrome. J Neurol Neurosurg Psychiatry 2015;86:502-504

5. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol 2010;229(1-2):256-262

6. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2015;2(4):e119

7. Harschnitz O, Jongbloed BA, Franssen H, et al. MMN: from immunological cross-talk to conduction block. J Clin Immunol 2014;34 Suppl 1:S112-119

8. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol 2016;80(1):71-88

9. Cats EA, Bertens AS, Veldink JH, et al. Associated autoimmune diseases in patients with multifocal motor neuropathy and their family members. J Neurol 2012;259(6):1137-1141

10. Sutedja NA, Otten HG, Cats EA, et al. Increased frequency of HLA-DRB1*15 in patients with multifocal motor neuropathy. Neurology 2010;74(10):828-83

11. Van den Berg LH, Kerkhoff H, Oey PL, et al. Treatment of multifocal motor neuropathy with high dose intravenous immunoglobulins: a double blind, placebo controlled study. J Neurol Neurosurg Psychiatry 1995;59(3):248-252

12. Van Schaik IN, Leger JM, Nobile-Orazio E, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripheral Nerv Syst 2010;15(4):295-301

13. Kuijf ML, van Doorn PA, Tio-Gillen AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci 2005;239(1):37-44

14. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010b;75(22):1961-1967

15. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002;125:2591-2625

16. www.allelefrequencies.net. Accessed November 2018

17. Maiers M, Gragert L, Klitz W. High-resolution HLA alleles and haplotypes in the United States population. Hum Immunol 2007;68(9):779-788

18. Gianfrancesco MA, Stridh P, Shao X, et al. Genetic risk factors for pediatric-onset multiple sclerosis. Mult Scler 2017 Published online first: 1 October 2017

19. Goodin DS, Khankhanian P, Gourraud PA, et al. Highly conserved extended haplotypes of the major histocompatibility complex and their relationship to multiple sclerosis susceptibility. PLoS One 2018;13(2):e0190043

20. Martinez-Martinez L, Lleixa MC, Boera-Carnicero G, et al. Anti-NF155 chronic inflammatory demyelinating polyradiculoneuropathy strongly associates to HLA-DRB15. J Neuroinflammation 2017;14(1):224-229

21. Misra MK, Damotte V, Hollenbach JA. Structure-based selection of human metabolite binding P4 pocket of DRB1*15:01 and DRB1*15:03, with implications for multiple sclerosis. Genes Immun 2019;20(1):46-55

22. Moutsianas L, Jostins L, Beecham AH, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. Nat Genet 2015;47(10):1107-1113

23. Soelberg K, Nilsson AC, Nielsen C, et al. Autoimmune and immunogenetic profile of patients with optic neuritis in a population-based cohort. Multi Scler Relat Disord 2018;21:97-102

24. Lightman SM, Utley A, Lee KP. Survival of long-lived plasma cells (LLPC): piecing together the puzzle. Frontiers in Immunology 2019;10(965).

25. Kaushansky N, Altmann DM, David CS, et al. DQB1*0602 rather than DRB1*1501 confers susceptibility to multiple sclerosis-like disease induced by proteolipid protein (PLP). J Neuroinflammation 2012;9:29

26. Kaushansky N, Ben-Nun A. DQB1*06:02-associated pathogenic anti-myelin autoimmunity in multiple sclerosis-like disease: potential functioning of DQB1*06:02 as a disease-predisposing allele. Front Oncol 2014;4:280

CHAPTER

SMN1 duplications are associated with progressive muscular atrophy, but not with multifocal motor neuropathy and primary lateral sclerosis

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Abstract

Objective To assess the association between copy number variation in the *SMN* locus and multifocal motor neuropathy (MMN), progressive muscular atrophy (PMA) and primary lateral sclerosis (PLS) susceptibility and to determine the association of *SMN1* and *SMN2* copy number with MMN, PMA and PLS disease course.

Methods In this monocenter study, we used multiplex ligation-dependent probe amplification (MLPA) to determine *SMN1* and *SMN2* copy number in Dutch patients with MMN, PMA and PLS and controls. We stratified clinical parameters for *SMN1* and *SMN2* copy number. We analyzed *SMN1* and *SMN2* exons 1-6, intron 6 and exon 8 copy number to study the genetic architecture of *SMN1* duplications.

Results *SMN1* and *SMN2* copy number were determined in 132 patients with MMN, 150 patients with PMA, 104 patients with PLS and 956 control subjects. MMN and PLS were not associated with copy number variation in *SMN1* or *SMN2*. In contrast, patients with PMA more often than controls carried *SMN1* duplications (≥ 3 SMN1 copies, 12.0% vs. 5.0%, OR 2.69 (1.43 – 4.91), p .0020). *SMN1* and *SMN2* copy number status was not associated with either MMN, PLS or PMA disease course. In case of *SMN1* exon 7 duplications, exon 1-6, exon 8 and introns 6 and 7 were also duplicated, suggesting full *SMN1* duplications.

Conclusions *SMN1* duplications are associated with PMA, but not with PLS and MMN. *SMN1* duplications in PMA are balanced duplications. The results of this study highlight the primary effect of altered *SMN* copy number on lower motor neurons.

Introduction

The *survival motor neuron* (*SMN*) locus, including *SMN1* and the nearly identical *SMN2,* has gone through extensive duplication, deletion and gene conversion events, causing variation in *SMN1* and *SMN2* copy number.[1] Heterogeneity at this locus has been associated with motor neuron disorders. Homozygous *SMN1* deletions lead to lower motor neuron (LMN) degeneration in hereditary proximal spinal muscular atrophy (SMA), the severity of which is inversely correlated to *SMN2* copy number (CN).[2,3] *SMN1* duplications, in contrast, have been associated with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by progressive loss of both upper and lower motor neurons.[4,5] No study has systematically investigated the association of other motor neuron disorders with *SMN* CN variation (CNV).

Multifocal motor neuropathy (MMN) is a rare, slowly progressive inflammatory neuropathy, characterized by asymmetrical distal muscle weakness, which responds to immunoglobulin treatment .[6,7,8] MMN is associated with specific HLA-haplotypes, suggesting genetic susceptibility underlying disease pathogenesis.[9] The selective motor neuron vulnerability seen in MMN is not fully explained. Progressive muscular atrophy (PMA) is characterized by progressive LMN degeneration, leading to muscular atrophy, muscle weakness and in some cases premature death. Disease progression can be fast and reminiscent of ALS, but is sometimes slow with patients surviving for decades.[5,10] In contrast with the LMN vulnerability in PMA and MMN, primary lateral sclerosis (PLS) is characterized by progressive degeneration of upper motor neurons (UMN) only.[5]

Here, we hypothesized that the *SMN* locus modifies susceptibility for a range of motor neuron disorders and determined *SMN* CNV in three large cohorts of Dutch MMN, PMA and PLS patients.

Methods

Study population

MMN patients, PMA patients, PLS patients and controls

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 of expertise for MMN, ALS and SMA. All patients fulfilled the most recent diagnostic criteria for definite, probable or possible MMN.[11] These criteria mostly 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 the basis of abnormal ancillary investigations and/or a response to treatment with immunoglobulins. All patients with MMN were Dutch.

Patients with PMA and PLS were enrolled through The Prospective ALS study The Netherlands (PAN), a Dutch population-based prospective case-control study.[12] All patients with PMA suffered from a progressive LMN loss without signs or symptoms consistent with UMN involvement, i.e. no pseudobulbar affect, increased jaw jerk, muscle hypertonia, pathological deep tendon reflexes or pathological signs including extensor plantar (Babinski) reflex or ankle clonus. Patients with PLS showed selective UMN loss, i.e. pseudobulbar dysarthria, pseudobulbar affect, hypertonia, pathological deep tendon reflexes or pathological signs including extensor plantar (Babinski) reflexes or clonus. None of the patients with PLS showed signs or symptoms of LMN degeneration, i.e. no atrophy, fasciculations or diminished or absent deep tendon reflexes.

Dutch population-based controls were also enrolled through the PAN study.[12] Control subjects enrolled between January 2012 and August 2018 were included in this study. None of the controls was diagnosed with MND after inclusion in this study.

Clinical data

For patients with MMN, PMA and PLS, clinical data were drawn from the MMN or PAN database.[12,13] When needed, these data were supplemented with data from UMCU patient files.

For all patients, recorded data included sex, age at onset, diagnostic delay, site of onset, response to IVIg treatment, presence of anti-GM1 IgM antibodies and muscle strength testing on the first and last visit to the outpatient clinic. Results of nerve conduction studies were used to assess the presence of conduction block in patients with MMN. Survival data from PMA and PLS patients were obtained by checking the last date the patient was known to be alive in the municipal population register (updated at quarterly intervals).

Onset of disease was defined as the first complaint of muscle weakness. Diagnostic delay was defined as the time between onset of muscle weakness and diagnosis. Anti-GM1 IgM antibody testing was performed using standardized ELISA.[14] Results of muscle strength testing on the first and last visit to the outpatient clinic were quantified on the 6-point Medical Research Council (MRC) scale. This scale ranges from 0 (no muscle contraction) to 5 (normal muscle strength against resistance). We documented the MRC scores 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. An MRC sum score was calculated by summation of the MRC scores of all tested muscle groups (range 0-130). As an outcome measure in MMN patients who were untreated at their first visit, a ΔMRC sum score/month was calculated by subtracting the MRC sum score at the first from that at the last visit, divided by the follow-up time.

In patients with MMN, nerve conduction studies were performed as described previously.[9,13] In short, motor and sensory function were tested bilaterally in the median, ulnar, radial, musculocutaneous, peroneal and tibial nerves. In nerves with a distal CMAP of >1 mV, the presence of conduction blocks was assessed, where a definite block was defined as a CMAP reduction of at least 50%, and a probable block as a reduction of 30-50%.

Survival was defined as time between onset of muscle weakness and death or last date known alive.

DNA samples

Genomic DNA was extracted from whole blood using standard DNA isolation methods. Samples from controls, PMA and PLS patients were obtained upon participation in the PAN study. Samples from MMN patients were obtained during the Dutch national cross-sectional studies on MMN performed in 2007 and 2015.

***SMN* CNV analysis**

We used multiplex ligation-dependent probe amplification (MLPA) to assess CNV in the *SMN* locus. We used the SALSA MLPA P021-B1 SMA probe mix (MRC Holland, Amsterdam, The Netherlands),. All analyses were performed as described previously.[2]

SMN1 and *SMN2* exon 7 specific probes were used to determine the *SMN1* and *SMN2* copy number. The *SMN1/2*Δ7-8 gene variant CN was determined by subtracting the median copy number of the seven probes targeting *SMN1* and *SMN2* intron 6, exon 7 and 8 from the median

copy number of the ten probes targeting *SMN1* and *SMN2* exons 1-6. For example, when the median copy number of the intron 6 – exon 8 region of *SMN1* and *SMN2* was three and the median copy number of *SMN1* and *SMN2* exons 1-6 was four, this indicated the presence of one copy of the *SMN1/2*Δ7-8 gene variant.[15]

Statistical analysis

We used R (version 3.5.1) to perform statistical analyses. *SMN1*, *SMN2* and *SMN1/2*Δ7-8 CN in patients with MMN, PMA and PLS were separately compared to controls using a χ^2 or Fisher's Exact test, as appropriate. Odds ratio's and 95% confidence intervals were calculated.

Patients were stratified by *SMN1* CN (1, 2 or ≥3 copies). To assess possible *SMN2* disease modification, clinical parameters within each *SMN1* group were stratified by *SMN2* CN. We compared continuous variables using ANOVA or Kruskal-Wallis testing, as appropriate. In case of a p-value < .05, a pairwise t-test or Wilcoxon signed-rank test was performed. We used a prerequisite of at least five observations per group for pairwise comparisons.

Survival analyses within the PMA and PLS groups were performed using the 'Survminer' package. Kaplan Meier curves were drawn, stratified by *SMN1* CN. At least five observations per stratum were required for survival analyses. The assumption of proportional hazard was tested and hazard ratio's with 95% confidence intervals were calculated. To assess possible *SMN2* disease modification, each *SMN1* group was stratified by *SMN2* CN and the same survival analyses were performed.

P-value adjustment using the Bonferroni method was applied in case of multiple testing. A corrected p-value < .05 was considered statistically significant.

Standard protocol approvals, registrations, and patient consents

The locally appointed ethics committee of the University Medical Center Utrecht gave approval for this study (METC Utrecht, METC.NL.041). All included patients gave written informed consent prior to inclusion in this study.

Data availability statement

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

Results

Study population

We included 137 patients with MMN, 159 patients with PMA, 105 patients with PLS and 981 control subjects. We obtained results from 132 patients with MMN (96.4%), 150 patients with PMA (94.3%), 104 patients with PLS (99%) and 956 control subjects (97.4%). Baseline characteristics are shown in table 1.

Table 1. Baseline characteristics of patients with MMN, PMA and PLS

	MMN (n = 132)	PMA (n = 150)	PLS (n = 104)	Controls (n = 956)
Male sex (n, %)	102 (77)	119 (79)	57 (55)	592 (62)
Age[$]	42 (15)	62 (15)	58 (16)	66 (9)
Delay in months[$]	42 (62)	17 (18)	34 (43)	
Site of onset (n, %)				
Bulbar	0 (0)	1 (1)	25 (24)	
Spinal	132 (100)	146 (97)	79 (76)	
Thoracic/respiratory	0 (0)	3 (2)	0 (0)	
EFNS diagnosis (n, %)				
Definite MMN	94 (73)	NA	NA	
Probable MMN	24 (19)	NA	NA	
Possible MMN	10 (8)	NA	NA	
Anti-GM1 IgM antibodies (n/N, %)	75/116 (65)	2/18 (11)	0/0 (0)	
IVIg response (n/N, %)	118/126 (94)	0/2 (0)	0/0 (0)	
IVIg treatment at first visit (n/N, %)	27/129 (21)	0/150 (0)	0/0 (0)	
Disease duration at first visit in months[§]	31 (3 - 433)	13 (2 - 485)	28 (1 - 325)	
MRC sum score at first visit[§]	122 (85 - 130)	122 (80 - 129)	130 (105 - 130)	
Limbs affected at first visit				
1 (n, %)	35 (34)	32 (21)	13/98 (13)	
4 (n, %)	24 (24)	48 (32)	10/98 (10)	
Riluzole treatment (n, %)	0 (0)	130 (87)	89 (86)	
Follow-up data (n, %)	120 (91)	50 (33)	59 (57)	
Follow-up duration in months[$]	101 (134)	22 (28)	31 (31)	
C9ORF72 repeat expansions (n, %)	NA	2/148 (1.4)	1/104 (1)	
Overall mortality	NA	83 (55)	37 (36)	
Death within 4 years of onset (n, %)	NA	39 (47)	5 (14)	

[$] Values displayed as median (IQR) [§] Values displayed as median (range)

Clinical parameters at first visit are shown for treatment naïve MMN patients only (n=102)

MMN = multifocal motor neuropathy, PLS = primary lateral sclerosis, PMA = progressive muscular atrophy, IVIg = intravenous immunoglobulins, MRC = medical research council, NA = not applicable

Sex, age at onset, diagnostic delay and site of onset were recorded in all patient subjects. Clinical parameters of MMN patients, i.e. the first visit MRC sum score and the number of affected limbs at the first visit are shown for patients who were treatment naïve at their first visit (n=102). Clinical parameters in the PMA group were recorded for all included patients. At the end of this study, 55% and 36% of PMA and PLS patients had deceased, of whom 47% and 13%, respectively, had died within four years after disease onset.

CNV analysis

SMN1, *SMN2* and *SMN1/2*Δ7-8 CN are show in table 2. In controls, we observed 48 *SMN1* duplications, of which one person had 4 and one person had 5 copies. All 11 MMN patients with *SMN1* duplications had 3 copies. Eighteen patients with PMA had *SMN1* duplications, of which three had 4 copies. Seven patients with PLS had a single *SMN1* duplication.

Compared to controls, *SMN1* duplications were found more frequently in patients with PMA (12.0% vs. 5.0%, OR 2.69 (1.43 – 4.91), p .0020), but not in patients with MMN and PLS. This association is further exemplified by the fact that also double *SMN1* duplications (i.e. at least four *SMN1* copies) were found more frequently in PMA patients (2% vs. 0.2% (OR 11.0 (1.25 – 133.56), p .015). No associations with *SMN2* CNV were found, nor with the presence of the *SMN1/2*Δ7-8 gene variant. Average total *SMN* CN was comparable between the patient groups and controls, possibly indicating an *SMN2* deletion event accompanying *SMN1* duplications.

A detailed overview of the *SMN*1:*SMN*2 configuration in control subjects and PMA patients is included in supplementary table e-1.

Clinical correlation

In all patient groups, age at onset and MRC sum score at the first visit were compared by *SMN1* group (1, 2 and ≥3 copies). Due to low numbers in groups with an *SMN1* deletion and duplication, the modifying potential of the *SMN2* CN was analyzed in the group with two *SMN1* copies only. Results are shown in Figure 1.

Table 2. *SMN1*, *SMN2* and *SMN1/2*Δ7-8 copy numbers in controls (n = 956) and patients with MMN (n = 132), PMA (n = 150) and PLS (n = 104)

	Controls	MMN	p	OR (95% CI)	PMA	p	OR (95% CI)	PLS	p	OR (95% CI)
SMN1										
1	20 (2.1)	4 (3.0)	.52	1.46 (0.36-4.46)	4 (2.7)	.56	1.28 (0.31-3.90)	1 (1.0)	.71	0.45 (0.01-2.90)
2	888 (92.9)	117 (88.6)	.12	0.59 (0.32-1.16)	128 (85.3)	.0028*	0.45 (0.26-0.79)	96 (92.3)	.99	0.92 (0.42-2.28)
≥3	48 (5.0)	11 (8.3)	.17	1.72 (0.78-3.47)	18 (12.0)	.0020*	2.69 (1.43-4.91)	7 (6.7)	.48	1.36 (0.50-3.14)
SMN2										
0	78 (8.2)	11 (8.3)	1.00	1.02 (0.48-2.00)	11 (7.3)	.85	0.89 (0.42-1.74)	9 (8.7)	.85	1.07 (0.46-2.22)
1	401 (41.9)	56 (42.4)	.99	1.02 (0.69-1.50)	74 (49.3)	.11	1.34 (0.94-1.93)	35 (33.7)	.13	0.70 (0.44-1.09)
2	451 (47.2)	59 (44.7)	.66	0.91 (0.62-1.33)	60 (40.0)	.12	0.75 (0.52-1.07)	54 (51.9)	.41	1.21 (0.79-1.85)
3	24 (2.5)	6 (4.5)	.29	185 (0.61-4.76)	5 (3.3)	.58	1.34 (0.29-3.66)	4 (3.8)	.35	1.55 (0.38-4.65)
4	2 (0.2)	-	-	-	-	-	-	2 (1.9)	.05	9.31 (0.67-130)
*SMN1/2*Δ7-8										
0	718 (75.1)	102 (77.3)	.66	1.13 (0.72-1.80)	104 (69.3)	.16	0.74 (0.51-1.12)	83 (79.8)	.34	1.31 (0.78-2.27)
1	230 (24.1)	27 (20.5)	.42	0.81 (0.50-1.29)	45 (30.0)	.14	0.35 (0.90-2.00)	20 (19.2)	.33	0.75 (0.43-1.27)
2	8 (0.8)	3 (2.3)	.14	2.75 (0.50-11.6)	1 (0.7)	1.00	0.80 (0.02-6.00)	1 (1.0)	.61	1.15 (0.03-8.72)
Mean CN[$]										
SMN1/SMN2	3.48 (0.72)	3.51 (0.78)	.68	-	3.50 (0.76)	.67	-	3.62 (0.89)	.11	-
*SMN1/SMN2/ SMN1/2*Δ7-8	3.74 (0.64)	3.76 (0.67)	.73	-	3.82 (0.75)	.15	-	3.84 (0.75)	.19	-

[§] Values displayed as n (%) [$] Values displayed as mean (SD)

[*] Statistically significant after Bonferroni p-value adjustment. Uncorrected p-values are shown.

CN = copy number, MMN = multifocal motor neuropathy, OR = odds ratio, PLS = primary lateral sclerosis, PMA = progressive muscular atrophy, SMN = survival motor neuron

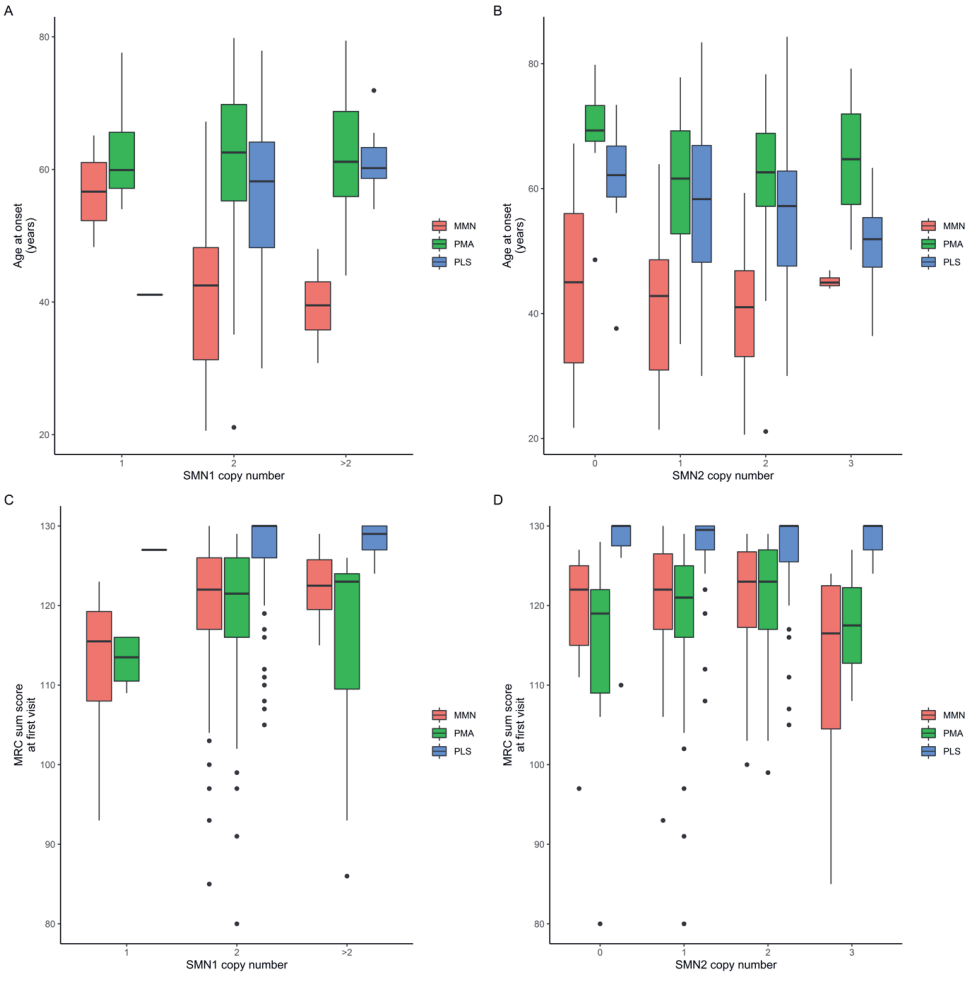

A
Age at onset (years)
SMN1 copy number
B
Age at onset (years)
SMN2 copy number
C
MRC sum score at first visit
SMN1 copy number
D
MRC sum score at first visit
SMN2 copy number
MMN
PMA
PLS

Figure 1. Clinical parameters stratified by *SMN1* and *SMN2* copy number in MMN and PMA patients (previous page)

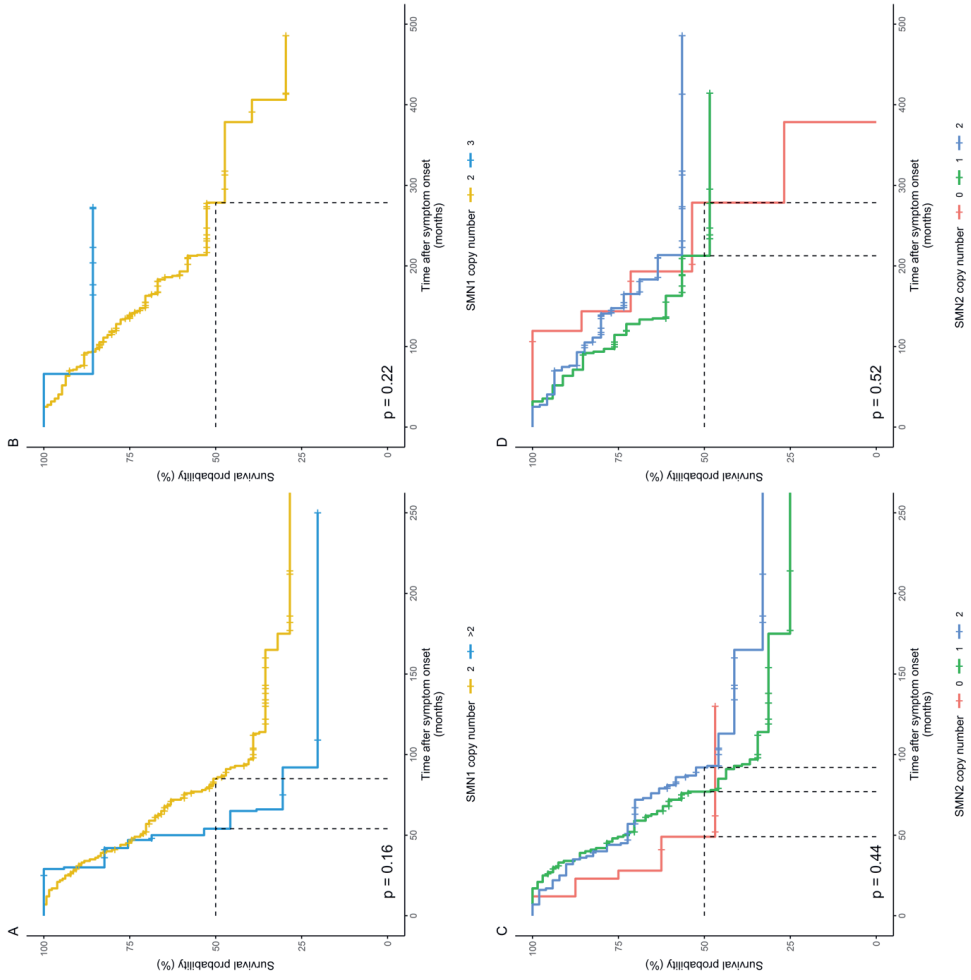
Boxplots showing median age at onset in years and median MRC sum score at the first visit in patients with MMN, PMA and PLS. A) Age at onset in years by SMN1 copy number. B) Age at onset by SMN2 copy number in patients carrying 2 SMN1 copies. C) MRC sum score at first visit by SMN1 copy number. D) MRC sum score at first visit by SMN2 copy number in patients carrying 2 SMN1 copies. No association between the clinical parameters and SMN1 or SMN2 copy number status was found in either disease group (all p-values > .05). MMN = multifocal motor neuropathy, MRC = medical research council, PLS = primary lateral sclerosis, PMA = progressive muscular atrophy, SMN = survival motor neuronWe did not find a positive association between SMN1 and SMN2 CN and median age at onset or median MRC sum scores at the first visit in patients with MMN, PMA or PLS. In the MMN group, SMN1 CN and SMN2 CN within patients carrying two SMN1 copies did not alter the rate of progression as expressed by ΔMRC sum score/month (p-values .36 and .52, respectively, data not shown). SMN1 duplications were not found more frequently in patients with PMA and a survival longer than 48 months (Fisher's exact test p .30, data not shown). Kaplan-Meier curves were drawn for all PMA and PLS patients carrying two SMN1 copies and SMN1 duplications. Due to the low number of patients with an SMN1 deletion, this group was excluded from survival analysis. Kaplan-Meier curves are shown in Figure 2.

Figure 2. Survival by *SMN* copy number in patients with PMA (see right)

Kaplan-Meier curves showing the probability of survival according to disease duration in patients with PMA (panels A and C) and PLS (panels B and D). Panels A and C show overall survival stratified by SMN1 copy number. Panels B and D show overall survival stratified by SMN2 copy number in patients carrying two SMN1 copies. Due to low numbers, survival curves for patients with SMN1 deletions and SMN2 duplications are not shown.

Survival did not differ between patients with PMA carrying two or three SMN1 copies (log-rank test p 0.16, HR 1.6 (0.83 – 3.0), p .16), nor did SMN2 copy number have an effect on survival in patients carrying two SMN1 copies (log-rank test p .44; HR 0 vs. 2 copies: 1.7 (0.57 – 4.8), p .35; HR 1 vs. 2 copies: 1.3 (0.76 – 2.1), p .38). In patients with PLS, SMN1 CN was not associated with survival (log-rank test p .22, HR 0.31 (0.04 - 2.2), p .25), nor did SMN2 CN affect survival in patients carrying two SMN1 copies (log-rank test p .52; HR 0 vs. 2 copies: 1.4 (0.51 - 4.1), p .49; HR 1 vs. 2 copies: 1.4 (0.69 - 3.0), p .33).

HR = hazard ratio, PLS = primary lateral sclerosis, PMA = progressive muscular atrophy, SMN = survival motor neuron


A
B
C
D
p = 0.16
p = 0.22
p = 0.44
p = 0.52
Survival probability (%)
Time after symptom onset (months)
SMN1 copy number
SMN2 copy number

Cox proportional hazard assumption was met in all analyses (p > .05). Although, compared to PMA patients carrying two *SMN1* copies, survival in PMA patients with *SMN1* duplications was lower, this was not statistically significant. *SMN2* CN did not affect survival in patients with PMA or PLS carrying two *SMN1* copies.

***SMN1* duplication analysis**

To gain further insight in the genetic architecture of the *SMN* locus in case of *SMN1* duplications, we further analyzed patients with PMA and controls with three *SMN1* copies (n=15 and 46, respectively). Results are shown in table 3.

In all subjects carrying one *SMN1* duplication as expressed by the *SMN1* exon 7 CN, *SMN1* exon 8 CN matched the *SMN1* exon 7 CN. The same accounts for *SMN2* exon 7 and 8 CN. In 36 controls (78%) and 14 patients with PMA (93%), the *SMN1* and *SMN2* exons 1-6 and intron 6 CN matched the combined *SMN1* and *SMN2* exon 7 copy number, suggesting a balanced and full duplication of *SMN1*. In 10 controls (22%) and 1 patient with PMA (7%), one copy of the *SMN1/2*Δ7-8 gene variant was found. Besides carrying one extra copy of *SMN1* and *SMN2* exons 1-6 and intron 6, these subjects all have a probable full *SMN1* gene duplication.

Regarding the *SMN1*:*SMN2* genetic architecture, there was a trend towards patients with PMA having a higher *SMN2* and thus lower *SMN1/2*Δ7-8 copy number when compared to controls, though this was not statistically significant (Fisher's Exact test p .64 and .26, respectively). Telomeric NAIP exon 5 duplications were found in 45/61 *SMN1* duplications (73%), which did not differ between PMA patients and controls (Fisher's Exact test p .51).

Table 3. Genetic architecture of *SMN1* duplications in controls and patients with PMA

	Controls (n = 46)	**PMA (n = 15)**	**p**
[*SMN1*:*SMN2*] (n, %)			
[3:0]	6 (13)	2 (13)	.64
[3:1]	20 (43)	4 (27)	
[3:2]	16 (35)	8 (53)	
[3:3]	4 (9)	1 (7)	
*SMN1/2*Δ7-8 (n, %)			
0	36 (78)	14 (93)	.26
1	10 (22)	1 (7)	
NAIP-5 (n, %)			
2	11 (24)	5 (33)	.51
3	31 (67)	10 (67)	
4	4 (9)	0 (0)	

SMN = survival motor neuron, PMA = progressive muscular atrophy, NAIP-5 = NLR family apoptosis inhibitory protein exon 5

Discussion

In this study, we investigated the possible association between CNV in the *SMN* locus and the motor neuron disorders MMN, PMA and PLS, and the effect of *SMN1* and *SMN2* CN on MMN, PMA and PLS disease course. We found that, compared to controls, *SMN1* and *SMN2* CN were not associated with MMN and PLS, nor their disease characteristics. PMA, but not its disease course, was associated with *SMN1* duplications (OR 2.69 (1.43 - 4.91), p .0020). These results indicate that CNV in the *SMN* locus is a novel genetic risk factor for PMA.

Previous studies have shown an association between ALS and *SMN1* with a combined OR of 1.76 (95% CI 1.33 – 2.32).[4,17,18] Interestingly, a recent study including >6000 patients with ALS identified no association between ALS and *SMN* CNV.[19] To better understand these conflicting results and detail the relevance of CNV in the *SMN* locus for motor neuron disorders, we have taken a closer look at the extremes of the motor neuron disorder spectrum by analyzing large cohorts of MNDs characterized by inflammation (MMN), predominant LMN (PMA) and UMN degeneration (PLS). We found an association between *SMN1* duplications and PMA, but not PLS and MMN. In PMA, we found a similar effect size identified in a previous study.[20] We hypothesize that our findings highlight the primary effect of altered SMN copy number on lower motor neurons.

PMA and PLS are considered part of a spectrum of MNDs that also includes ALS. In contrast to ALS and PLS, PMA is limited to clinical signs of lower motor neurons.[5] However, up to 50% of patients with PMA may show signs of corticospinal tract degeneration at autopsy.[21] Whether PMA represents a distinct disease remains a topic of ongoing debate and further research is warranted to further detail the clinical heterogeneity associated with PMA We nevertheless think that our PMA cohort differs from a typical ALS cohort on various grounds. Based on detailed clinical and electrophysiological examination, the patients included in this study showed exclusive progressive loss of lower motor neurons, and almost none of the patients had a bulbar onset. Moreover, in 104/159 (65%) survival was longer than four years, and 37/159 (23%) patients had a very long survival of at least eight years (range 96 – 531 months). Analysis of available follow up data of 54/159 (34%) patients showed a conversion to ALS in only three cases (i.e. upper motor neuron signs after the start of this study). Of these patients, none carried an *SMN*1 duplication.

A central question in this study and in the aforementioned studies is what effect *SMN1* duplications have on LMN SMN protein levels. In our analysis, we showed that the genetic architecture of *SMN1* duplications in PMA does not differ from that of controls, and that about three quarters of *SMN1* duplications are large, including the telomeric NAIP gene. Most importantly, we showed that *SMN1* duplications are probably balanced and could theoretically lead to a full-length SMN transcript. Though the transcriptional, translational and post-translational effects in individuals carrying three *SMN1* genes remain unknown, we cannot exclude the possibility that the association between *SMN1* duplications and PMA is caused by an excess of lower motor neuron SMN protein levels. Further supporting this hypothesis may be the fact that previous studies have not shown an association with *SMN1* deletions.[4,17,18] Alternative explanations may be found in the disruption of regulatory sequences by the large duplications in the *SMN* locus. Future research on SMN protein levels in, for example, induced

pluripotent stem cell (iPSC) derived motor neurons obtained from patients with different *SMN1* genotypes could further address this issue.

To the best of our knowledge, this is the first study reporting on CNV in the *SMN* locus in MMN and PLS. The size of the cohorts we included in our analyses is substantial considering the low prevalence of these diseases. Clinically, MMN patients were characterized in detail and we had data from longer periods of follow-up for analysis. Our data virtually exclude the *SMN* locus as a susceptibility locus for inflammatory MNDs. Although our cohort of patients with PMA is also relatively large, it is not possible to draw definite conclusions about the association of *SMN* CNV and disease course due to a relatively small number of patients with *SMN1* duplications. *SMN* copy number varies considerably among different ethnicities.[15] The results of our study therefore cannot readily be extrapolated to ethnicities other than that of the patients in this study, that is, Dutch.

In summary, our study shows that *SMN* CNV underlies motor neuron vulnerability in PMA, but not in MMN and PLS. This extends the role of *SMN* CNV in lower motor neuron diseases beyond SMA and supports the notion that the *SMN* locus is a general modifier for lower motor neuron disease susceptibility. The intriguing finding that, rather than loss of SMN as in SMA, gene duplications are a main genetic risk factor for PMA, warrants further fundamental research to improve our understanding of the cellular mechanisms that underlie this susceptibility.

Acknowledgement

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References

1. Butchbach, MER. Copy number variations in the Survival Motor Neuron genes: implications for spinal muscular atrophy and other neurodegenerative diseases. Front Mol Biosc. 2016;3-7

2. Wadman, RI, Jansen, MD, Stam, M, et al. Intragenic and structural variation in the *SMN* locus and clinical variability in spinal muscular atrophy. Brain Commun 2020;2(2)

3. Wirth, B, Karakaya, M, Kye, MJ, Mendoza-Ferreira, N. Twenty-five years of spinal muscular atrophy research: from phenotype to genotype to therapy, and what comes next. Annu Rev Genomics Hum Genet 2020;21(231-261)

4. Wang, X, Cui, N, Gao, J, Qiu, X., Zheng, F. SMN1 duplications contribute to sporadic amyotrophic lateral sclerosis susceptibility: evidence from a meta-analysis. J Neurol Sci 2014;340(1-2):63-68

5. Van Es, MA, Hardiman, O, Chio, A, et al. Amyotrophic lateral sclerosis. Lancet 2017;390:2084-2098

6. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

7. Harschnitz O, Jongbloed BA, Franssen H, Straver DC, van der Pol WL, van den Berg LH. MMN: from immunological cross-talk to conduction block. J Clin Immunol 2014;34 Suppl 1:S112-119

8. Herraets I, Van Rosmalen M, Bos J, et al. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. Neurology 2020;95(14):1979-1987

9. Sutedja NA, Otten HG, Cats EA, et al. Increased frequency of HLA-DRB1*15 in patients with multifocal motor neuropathy. Neurology 2010;74(10):828-832

10. Liewluck, T, Saperstein, DS. Progressive muscular atrophy. Neurol Clin. 2015;33(4):761-773

11. van Schaik IN, Leger JM, Nobile-Orazio E, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripheral Nerv Syst 2010;15(4):295-301

12. Huisman, MHB, Seelen, M, De Jong, SW, et al. Lifetime physical activity and the risk of amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry 2013; 84(9):976-981s

13. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology 2010;75(9):818-825

14. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010;75(22):1961-1967

15. Vijzelaar, R, Snetselaar, R, Clausen, M, et al. The frequency of SMN gene variants lacking exon 7 and 8 is highly population dependent. PLoS ONE 2019;14-7

16. Vorster, E, Essop, FB, Rodda, JL, Krause, A. Spinal muscular atrophy in the black South African population: a matter of rearrangement? Front Genet 2020;11(54)

17. Blauw, HM, Barnes, CP, Van Vught, PWJ, et al. SMN1 gene duplications are associated with sporadic ALS. Neurology 2012;78(11):776-780

18. Veldink, JH, Kalmijn, A, Van der Hout, AH, et al. SMN genotypes producing less SMN protein increase susceptibility to and severity of sporadic ALS. Neurology 2005;65(6):820-825

19. Moisse, M, Zwamborn, R.A.J., Van Vugt, J., et al. The effect of SMN gene dosage on ALS risk and disease severity. Ann Neurol 2021

20. Kuźma-Kozakiewicz, M, Jędrzejowska, M, Kaźmierczak, B. SMN1 gene duplications are more frequent in patients with progressive muscular atrophy. Amyotroph Lateral Scler Frontotemporal Degener 2013;14(5-6):457-462

21. Ince, PG, Evans, J, Knopp, M, et al. Corticospinal tract degeneration in the progressive muscular atrophy variant of ALS. Neurology 2003;60(8):1252-1258

Supplementary material

Table e-1. Detailed *SMN1*:*SMN2* copy number configuration in controls and patients with PMA.

SMN1	***SMN2***	**Controls (n=956)**	**PMA (n=150)**	**p**	**OR (95% CI)**
1	1	8 (0.8%)	1 (0.7%)	1.00	-
	2	10 (1.0%)	2 (1.3%)	0.67	-
	3	1 (0.1%)	1 (0.7%)	.25	-
	4	2 (0.2%)	0 (0.0%)	1.00	-
2	0	71 (7.4%)	8 (5.3%)	.49	-
	1	372 (38.9%)	67 (44.7%)	.21	-
	2	424 (44.4%)	51 (34.0%)	.022	0.65 (0.44 – 0.94)
	3	20 (2.0%)	2 (1.3%)	.76	-
3	0	6 (0.6%)	2 (1.3%)	.30	-
	1	20 (2.0%)	4 (2.7%)	.55	-
	2	16 (1.7%)	8 (5.3%)	.010	3.30 (1.20 -8.36)
	3	4 (0.4%)	1 (1.3%)	.52	-
4	0	0 (0.0%)	1 (0.7%)	0.14	-
	1	1 (0.1%)	2 (1.3%)	0.05	-
5	0	1 (0.1%)	0 (0.0%)	1.00	-

CI = confidence interval, OR = odds ratio, PMA = progressive muscular atrophy, SMN = survival motor neuron

CHAPTER

5

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

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Abstract

Background and objectives To further substantiate the role of antibody-mediated complement activation in 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 0.009; Del/Del genotype 16.8% vs. 7.7%, p 0.005, OR 2.43 (1.27–4.58)), and patients carrying this deletion had a more favorable disease course (mean difference 0.26 MRC points/year (95% CI 0.040–0.490), p 0.019)). 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 0.032)).

Conclusions 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. Together, these results point out the relevance of the pre-C5 level of the complement cascade in the inflammatory processes underlying MMN.

Introduction

Multifocal motor neuropathy (MMN) is a rare, asymmetric, immune-mediated and 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 (iPSC) 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, amongst 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 tresholds 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.

Methods

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.

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 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 at our hospital.

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.[1-4] We used control DNA samples that were obtained upon participation in the PAN study.

Genotyping

We used two techniques to determine the presence of five *CD46*, *CD55* and *CD59* promotor region variants, that 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 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.[26] 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 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.

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 <0.05 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 <0.017 was considered statistically significant. Odds ratio's and 95% confidence intervals (CI) 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, and disease duration at the first visit and polymorphisms 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 modelling 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 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 <0.05 were considered statistically significant.

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.

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

Results

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.

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[$]	43 (16)	67 (11)
Diagnostic delay in months[$]	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[$]	122 (9)	
IVIg treatment after first visit (n/N, %)	96/103 (93)	
IVIg response	90/96 (94)	
Follow-up data (n/N, %)	102/103 (99)	
Follow-up duration[$]	101 (154)	
Last visit MRC sum score[$]	121.5 (11.5)	

[$] Values displayed as median (IQR)
IVIg = intravenous immunoglobulins, MMN = multifocal motor neuropathy, MRC = Medical research council

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, minor allele frequencies, 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 minor allele frequencies was found for CD55 rs28371582 (controls vs. MMN 0.30 vs 0.36, respectively), though this was not statistically significant (X2 = 2.50, p = 0.11). To compare genotype frequencies, an overall X2 test was performed, which showed a statistically significant different genotype distribution between patient and controls for CD55 rs28371582 (X2 = 9.38, p = 0.009). In a post-hoc analysis, we found that this effect was driven by patients with MMN more often carrying the Del/Del genotype (X2 = 8.05, OR 2.43 (1.27 - 4.58), p = 0.005).

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 0.019)).

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 0.019)), suggesting a more favorable long-term disease course after initiation of IVIg treatment. All other polymorphisms were not associated with 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 >0.05 for all analyses).

Table 2. Minor allele frequencies and genotype frequencies of rs2796267, rs2796268, rs28371582, rs28371583 and rs141385724 in patients with MMN (n=133) and controls (n=380). Chi-squared tests were performed and uncorrected p-values are shown.

Gene	Polymorphism	Alleles	MAF			Genotype frequencies (n, %)					
			Controls	MMN	p		Controls	MMN	p	OR (95% CI)	p
CD46	rs2796267	A vs. 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 vs. 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 vs. Del	0.30	0.36	.11	Ins/Ins	178 (47.0%)	59 (45.0%)	0.009*	0.93 (0.61 – 1.41)	0.70
						Ins/Del	172 (45.4%)	50 (38.2%)		0.74 (0.48 – 1.14)	0.15
						Del/Del	29 (7.7%)	22 (16.8%)		2.43 (1.27 – 4.58)	0.005*
						ND	1 (0.3%)	2 (1.5%)			
	rs28371583	A vs. 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	- vs. 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%)			

* Statistically significant after p-value adjustment

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

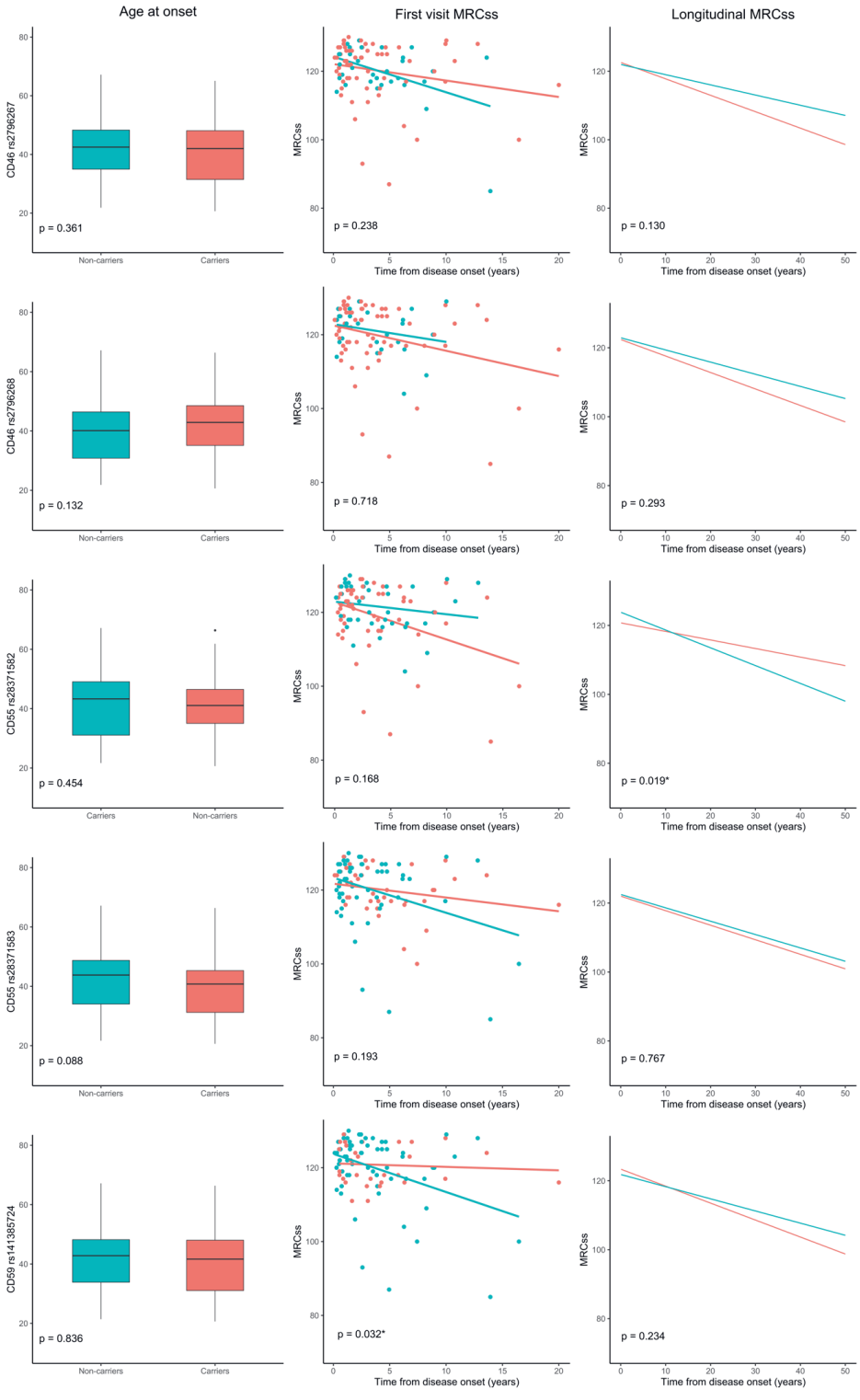

Age at onset
First visit MRCss
Longitudinal MRCss
CD46 rs2796267
p = 0.361
p = 0.238
p = 0.130
CD46 rs2796268
p = 0.132
p = 0.718
p = 0.293
CD55 rs28371582
p = 0.454
p = 0.168
p = 0.019*
CD55 rs28371583
p = 0.088
p = 0.193
p = 0.767
CD59 rs1418572
p = 0.836
p = 0.032*
p = 0.234
Non-carriers
Carriers
MRCss
Time from disease onset (years)

Figure 1 (previous page). *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 = 0.032). 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 = 0.019).*

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 = 0.009; Del/Del genotype 16.8 vs. 7.7%, OR 2.43 (1.27 - 4.58), p = 0.005), whilst *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 Fig. 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.[30-32] 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.[33,34] 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.

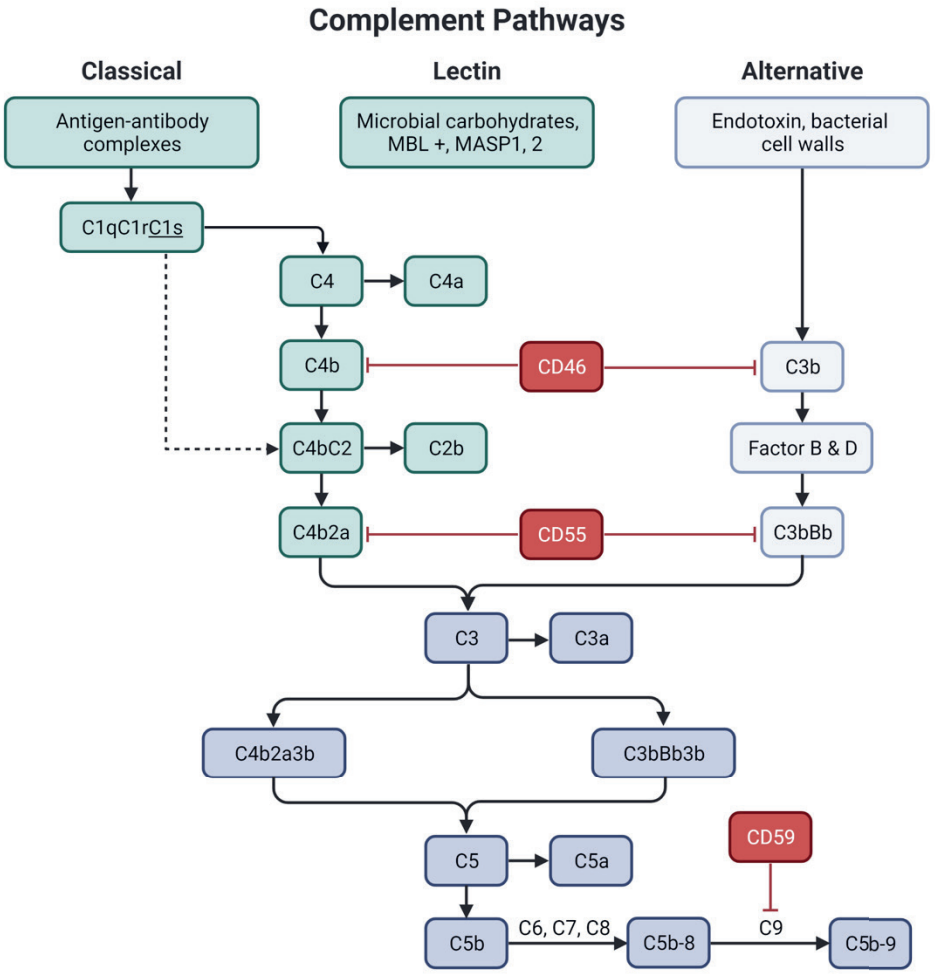


Figure 2. *The complement pathways and cascades that follow upon activation. Anti-GM1 IgM antibodies activate the classical pathway, ultimately leading to formation of the membrane attack complex (MAC). The membrane-bound complement regulatory proteins CD46, CD55 and CD59 each inhibit the complement cascade at different levels. CD46 (membrance cofactor protein (MCP)) acts as a cofactor for the cleavage-mediated inactivation of C3b and C4b. CD55 (decay accelerating factor (DAF)) 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 (MAC-IP)), that inhibits the polymerization of C9 and thus MAC formation.*

We found two associations of genetic variability of CRPs and MMN disease course. First, treatment-naïve patients carrying *CD59* rs141385724 showed a trend to 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, C3), rather than the terminal pathway (C5-C9, i.e., MAC).[36] We hypothesize that CD59 expression on peripheral nerves is sufficiently 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.

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.

References

1. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. *Neurology.* 2010;75(9):818-825

2. Briani C, Cocito D, Campagnolo M, et al. Update on therapy of chronic immune-mediated neuropathies. *Neurol Sci.* 2022;43(Suppl 2):605-614

3. Beadon K, Guimarães-Costa R, Léger J-M. Multifocal motor neuropathy. *Curr Opin Neurol.* 2018;31(5):559-564

4. Herraets I, Van Rosmalen M, Bos J, et al. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. *Neurology.* 2020;95(14):e1979-e1987

5. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. *Nat Rev Neurol.* 2011;8(1):48-58

6. Pestronk A, Cornblath DR, Ilyas AA, et al. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. *Ann Neurol.* 1988;24(1):73-78

7. Goedee HS, Van der Pol WL, Van Asseldonk JTH, et al. Diagnostic value of sonography in treatment-naive chronic inflammatory neuropathies. *Neurology.* 2017;88(2):143-151

8. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. *Neurology.* 2010;75(22):1961-1967

9. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. *J Neuroimmunol.* 2010;229(1-2):256-262

10. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. *Ann Neurol.* 2016;80(1):71-88

11. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. *Neurol Neuroimmunol Neuroinflamm.* 2015;2(4):e119

12. Mathern DR, Heeger PS. Moleculues great and small: the complement system. *Clin J Am Soc Nephrol.* 2015;10(9):1636-1650

13. Noris M, Remuzzi G. Overview of complement activation and regulation. *Semin Nephrol.* 2013;33(6):479-492

14. Ozen A, Comrie WA, Ardy RC, et al. CD55 deficiency, early-onset protein-losing enteropathy, and thrombosis. *N Engl J Med*. 2017;377(1):52-61

15. Hill A, DeZern AE, Kinoshita T, et al. Paroxysmal nocturnal haemoglobinuria. *Nat Rev Dis Primers*. 2017;18:3:17028

16. Haliloglu G, Maluenda J, Sayinbatur B, et al. Early-onset chronic axonal neuropathy, strokes, and hemolysis: inherited CD59 deficiency. *Neurology*. 2015;84(12):1220-1224

17. Michielsen LA, Van Zuilen AD, Kardol-Hoefnagel T, et al. Association between promotor polymorphisms in CD46 and CD59 in kidney donors and transplant outcome. *Front Immunol*. 2018;9;1-9

18. Budding K, Van de Graaf EA, Kardol-Hoefnagel T, et al. A promotor polymorphism in the CD59 complement regulatory protein gene in donor lungs correlates with a higher risk for chronic rejection after lung transplantation. *Am J Transplant*. 2016;16(3):987-998

19. Lin T-Y, Brass AL. Host genetic determinants of influenza pathogenicity. *Curr Opin Virol*. 2013;3(5):531-536

20. Toomey CB, Cauvi DM, Pollard KM. The role of decay accelerating factor in environmentally induced and idiopathic systemic autoimmune disease. *Autoimmune Dis*. 2014;2014:452853

21. Dho SH, Lim JC, Kim LK. Beyond the role of CD55 as a complement component. *Immune Netw.* 2018;18(1):e11

22. Joint Task Force of the EFNS and the PNS. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. *J Peripheral Nerv Syst.* 2010;15(4):295-301

23. Huisman MHB, Seelen M, De Jong SW, et al. Lifetime physical activity and the risk of amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry.* 2013;84(9):976-981s

24. Kuijf, ML, Van Doorn, PA, Tio-Gillen, AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. *J Neurol Sci.* 2005;239(1):37-44

25. Koppers M, Groen EJN, Van Vught PWJ, et al. Screening for rare variants in the coding region of ALS-associated genes at 9p21.2 and 19p13.3. *Neurobiol Aging.* 2013;34(5):1518(e5-e7)

26. Lukacik P, Roversi P, White J, et al. Complement regulation at the molecular level: the structure of decay-accelerating factor. *PNAS.* 2004;101(5):1279-1284

27. Martin BK. Transcriptional control of complement receptor gene expression. *Immunol Res*. 2007;39(1-3):146-159

28. Qian YM, Qin X, Miwa T, et al. Identification and functional characterization of a new gene encoding the mouse terminal complement inhibitor CD59. *J Immunol.* 2000;165(5):2528-2534

29. Ewulonu UK, Ravi L, Medof ME. Characterization of the decay-accelerating factor gene promotor region. *PNAS*. 1991;88(11):4675-4679

30. Thomas DJ, Lublin DM. Identification of 5'-flanking regions affecting the expression of the human decay accelerating factor gene and their role in tissue-specific expression. *J Immunol*. 1993;150(1):151-160

31. Cauvi DM, Cauvi G, Pollard KM. Constitutive expression of murine decay-accelerating factor 1 is controlled by the transcription factor Sp1. *J Immunol*. 2006;177(6):3837-3847

32. Zhou J, To KKW, Dong H, et al. A functional variation in CD55 increases the severity of 2009 pandemic H1N1 influenza A virus infection. *J Infect Dis*. 2012;206(4):495-503

33. Pérez-Rubio G, Ponce-Gallagos MA, Domínguez-Mazzocco BA, et al. Role of the host genetic susceptibility to 2009 pandemic influenza A H1N1. *Viruses*. 2021;13(2):344-365

34. Heckmann JM, Uwimpuhwe H, Ballo R, et al. A functional SNP in the regulatory region of the decay-accelerating factor gene associated with extraocular muscle pareses in myasthenia gravis. *Genes Immun*. 2010;11(1):1-10

35. Budding K, Johansen LE, Van de Walle I, et al. Anti-C2 antibody ARGX-117 inhibits complement in a disease model for multifocal motor neuropathy. *Neurol Neuroimmunol Neuroinflamm*. 2021;9(1):e1107

36. Fitzpatrick AM, Mann CA, Barry S, et al. An open label clinical trial of complement inhibition in multifocal motor neuropathy. *J Peripher Nerv Syst*. 2011;16(2):84-91

37. Mollnes TE, Hogasen K, Hoaas BF, et al. Inhibition of complement-mediated red cell lysis by immunoglobulins is dependent on the Ig isotype and its C1 binding properties. *Scand J Immunol*. 1995;41(5):449-456

38. Basta M, Dalakas MC. High-dose intravenous immunoglobulin exerts its beneficial effect in patients with dermatomyositis by blocking endomysial deposition of activated complement fragments. *J Clin Invest*. 1994;94(5):1729-1735

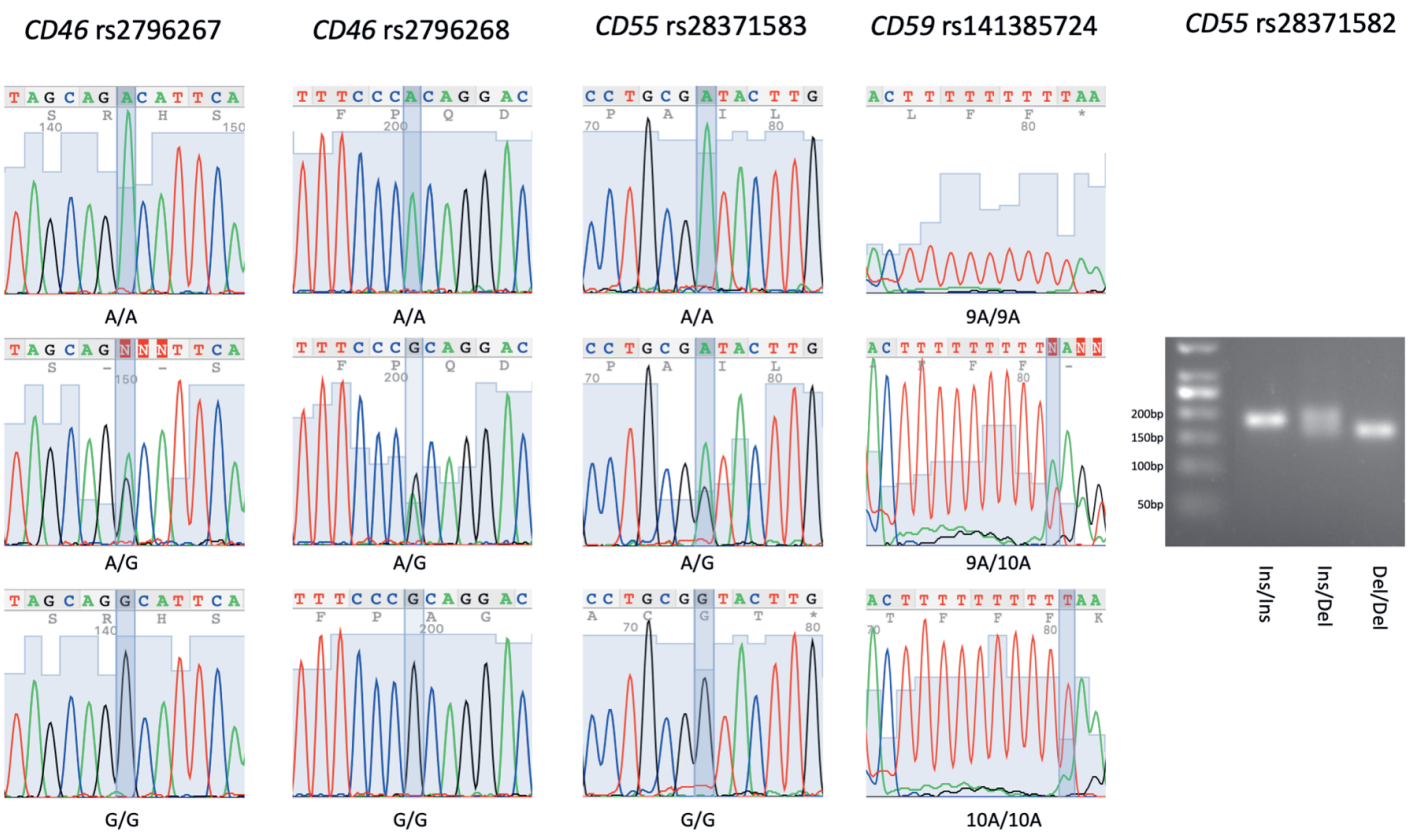
39. Lutz HU, Stammler P, Bianchi V, et al. Intravenously applied IgG stimulates complement attenuation in a complement-dependent autoimmune disease at the amplifying C3 convertase level. *Blood*. 2004;103(2):465-472

40. Boros P, Gondolesi G, Bromberg JS. High dose intravenous immunoglobulin treatment: mechanisms of action. *Liver Transpl*. 2005;11(12):1469-1480

41. Liu X, Cao W, Li T. High-dose intravenous immunoglobulins in the treatment of severe acute viral pneumonia: the known mechanisms and clinical effects. *Front Immunol*. 2020;11:1660

Supplementary materials

	CD46		CD55		CD59
	rs2796267	rs2796268	rs28371582	rs28371583	rs141385724
PCR primers					
Forward	5'-AGGGCCCCAT AGAGTCTCAA-3'	5'-ATACCAAGTA AGGGCCCAGG-3'	5'-GGTCTCTGTTTTC ACATTAGAGAGTG-3'	5'-AGTCTCAGCC CCCGAACT-3'	5'-AGCAAGACGC ACAGAAACG-3'
Reverse	5'-CAGGCAGGGT GGAGAGAAAA-3'	5'-ACGCAACAAT CCCCAACTCA-3'	5'-GAGTTCGGGG GCTGAGACT-3'	5'-ACACAAACGG GGTGTAAACG-3'	5'-AACCCCAGG GAACTGAAAGT-3'
PCR protocol	2 min 96°C	2 min 96°C	2 min 96°C	2 min 96°C	2 min 96°C
	30 sec 96°C	30 sec 96°C	30 sec 96°C	30 sec 96°C	30 sec 96°C
	45 sec 60°C	45 sec 62°C	45 sec 57°C	45 sec 57°C	45 sec 57°C
	1 min 72°C	1 min 72°C	1 min 72°C	1 min 72°C	1 min 72°C
	5 min 72°C	5 min 72°C	5 min 72°C	5 min 72°C	5 min 72°C
Sequence primers					
Forward	5'-AGGGCCCCAT AGAGTCTCAA-3'	5'-ATACCAAGTA AGGGCCCAGG-3'	-	5'-AGTCTCAGCC CCCGAACT-3'	-
Reverse	-	-	-	-	5'-AACCCCAGG GAACTGAAAGT-3'
Sequence protocol	1 min 96°C	1 min 96°C	-	1 min 96°C	1 min 96°C
	10 sec 96°C	10 sec 96°C	-	10 sec 96°C	10 sec 96°C
	5 sec 50°C	5 sec 50°C	-	5 sec 50°C	5 sec 50°C
	4 min 60°C	4 min 60°C	-	4 min 60°C	4 min 60°C



Supplementary figure S1. *Sanger sequencing traces of CD46 rs2796267, CD46 rs2796268, CD55 rs28371583 and CD59 rs141385724, showing each genotype per polymorphism. On the right, a gel electrophoresis example is shown, showing the three CD55 rs28371582 genotypes.*

CHAPTER

Multifocal motor neuropathy is not associated with altered innate immune responses to endotoxin

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Abstract

Objective Antibody- and complement-mediated peripheral nerve inflammation are central in the pathogenesis of MMN. Here, we studied innate immune responses to endotoxin in patients with MMN and controls to further our understanding of MMN risk factors and disease modifiers.

Methods We stimulated whole blood of 52 patients with MMN and 24 controls with endotoxin and collected plasma. With a multiplex assay, we determined levels of the immunoregulating proteins IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L in unstimulated and LPS-stimulated plasma. We compared baseline and stimulated protein levels between patients and controls and correlated concentrations to clinical parameters.

Results Protein level changes after stimulation were comparable between groups ($p > 0.05$). IL-1RA, IL-1β, IL-6 and IL-21 baseline concentrations showed a positive correlation with monthly IVIg dosage (all corrected p-values <0.016). Patients with anti-GM1 IgM antibodies showed a more pronounced IL-21 increase after stimulation (p 0.048).

Conclusions Altered endotoxin-induced innate immune responses are unlikely to be a susceptibility factor for MMN.

Introduction

Multifocal motor neuropathy (MMN) is a rare, asymmetric, chronic and immune-mediated neuropathy. It initially causes distal asymmetrical muscle weakness, which can be reversed partially with regular infusions of intravenously or subcutaneously administered immunoglobulins (IVIg, scIg).[1-4] Despite treatment, accumulating motor nerve axonal damage will eventually lead to refractory deficits, causing permanent and significant disability in about 20% of patients.[5,6] MMN is characterized by the presence of IgM antibodies to paranodal components, primarily the ganglioside GM1.[5,7,8] Upon binding, these antibodies can trigger damage to axons or myelin through activation of the classical pathway of complement.[1,7,9-11] The origin of pathogenic antibodies in MMN is unknown. MMN is not characterized by systemic inflammation as shown by the lack of inflammatory markers in peripheral blood and cerebrospinal fluid.[12,13] In patients with Guillain-Barré syndrome (GBS), an acute inflammatory neuropathy associated with preceding infections that is characterized by the presence of anti-ganglioside IgG autoantibodies, increased TLR4-mediated innate immune responses were shown to be a critical host susceptibility factor.[14] In this study, we aimed to explore the possibility of an altered innate immune response as a susceptibility factor for MMN. To this end, we stimulated blood of patients with MMN and controls with lipopolysaccharide (LPS), a potent endotoxin known to induce an innate immune response after binding to TLR4.[15] We compared levels of secreted immunoregulatory proteins and studied associations with clinical parameters.

Methods

Study population

We enrolled patients with MMN as part of a cross-sectional study in the Netherlands (MAIN study).[2] All patients had been diagnosed at the outpatient clinic of the University Medical Center Utrecht (UMCU), a Dutch national center for MMN and other neuromuscular disorders. All patients fulfilled the most recent 2010 diagnostic criteria for definite, probable or possible MMN and all patients were Dutch.[16] Control subjects were enrolled through the Prospective ALS study in The Netherlands (PAN study), a prospective Dutch population-based case-control study performed at the UMCU.[17,18] Additionally, we obtained blood samples from healthy control subjects via the voluntary in-house donor facility of the UMC Utrecht.

Clinical data

We obtained routine clinical and demographic data from our MMN database; when necessary, these were supplemented with data obtained from the UMCU patient files.[2] We recorded sex, age at onset, age at diagnosis and diagnostic delay, anti-GM1 IgM antibody status, monthly IVIg dosage and muscle strength, displayed as an MRC sum score (see below), for all patients at the time of inclusion.

We defined onset of MMN as the first patient-reported complaint of weakness in arm or leg muscles and diagnostic delay as the time that lapsed between onset and MMN diagnosis. We used a standardized ELISA to document the presence of anti-GM1 IgM antibodies in serum as described previously.[19,20] We calculated the monthly IVIg dosage at the time of inclusion. On the day of inclusion in the MAIN study, we performed a standardized neurological examination that included testing muscle strength, using the 6-point Medical Research Council (MRC) scale,

which ranges from 0 (no muscle contraction) to 5 (normal muscle strength against resistance). We documented MRC scores for left and right shoulder abduction, elbow flexion and extension, wrist flexion and extension, finger flexion and extension, finger spreading, thumb adduction, abduction and flexion, hip flexion, knee flexion and extension, foot flexion and extension, and toe flexion and extension and calculated the MRC sum score (range 0-180).[2]

LPS stimulation experiment

Upon inclusion in the 2015 MAIN study, we collected 10mL of blood in sodium-heparin vacutainers by venipuncture and used it immediately for experimental procedures. For baseline concentrations, we centrifuged 1.5mL of unstimulated blood at 1500 x *g* for 10 minutes at room temperature, collected plasma and stored it immediately at -80^{0}C until further analysis. For whole blood stimulation experiments, we used 24-well plates. Per subject, we filled one well with 1mL of freshly drawn whole blood and added LPS at a final concentration of 1µg/mL (LPS from *Escherichia coli* O127:B8, Sigma L3129, 5mg/mL in millQ (MQ) water). We filled a second well per subject with 1 mL of whole blood and 5µL of MQ, which served as a control. We incubated plates at 37^{0}C for 4 hours in an incubator with 5% CO_2, followed by centrifugation at 1500 x *g* for 10 minutes at room temperature. We collected plasma and stored samples at -80^{0}C until further analysis.

Luminex assay

We used previously validated multiplex immunoassays (Luminex platform) to determine plasma protein levels.[21] All assays were performed at the ISO-certified multiplex core facility of the UMCU. We determined levels of the immunoregulating proteins IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L in plasma of unstimulated, MQ-, and LPS-stimulated whole blood samples.

We considered data that were out of the detection range of the Luminex platform (out of range, OOR), or samples that were extrapolated in the lower or upper ranges from the assay sensitivity to have been measured imprecisely and therefore replaced these values using the following method.[22] Low OOR or extrapolated values in the low range were replaced by either the lower limit of quantification (LLOQ) divided by 2, or, when the lowest measured value of the marker in question was lower than the LLOQ, by the lowest value divided by 2. OOR or extrapolated values at the upper range were, similarly, replaced by the upper limit of quantification, or the highest measured value times 2.

Statistical analysis

For statistical analyses, we used R (version 3.6.1). First, we calculated an LPS-induced change in protein levels by subtracting the change from baseline in the control (MQ) condition from the change from baseline in the LPS stimulation experiment. We compared the LPS-induced change between patients and controls using a Mann-Whitney U test. Second, we correlated clinical parameters to baseline protein levels and their LPS-induced change. We assessed correlations between the baseline protein levels and the LPS-induced change with monthly IVIg dosage, disease duration and MRC sum score using Spearman's correlation coefficient. We compared results between patients with and without anti-GM1 IgM antibodies using the Mann-Whitney U test. Because of sample size assumptions, all analyses of continuous variables, as described above, were only performed for proteins whose above or below OOR outcomes comprised less than 50% of the total data points. If this was not the case, we

compared the percentage of OOR and extrapolated data points between patients and controls using a χ^2 test.
To assess differential clustering of data, we performed a principal component analysis (PCA) using baseline concentrations and concentration changes after stimulation with LPS separately. The in-built PCA function of R was used. Ellipses showing the 95% confidence intervals of the t-distribution were drawn per subgroup.
All p-values were adjusted for multiple testing using the Bonferroni method.

Standard protocol approvals, registrations, and patient consents
The locally appointed ethic committee of the University Medical Center Utrecht approved this study (METC Utrecht, METC.NL.041). All included subjects gave written informed consent prior to inclusion in this study.

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

Results

Study population
We included 52 patients with MMN and 24 control subjects (12 through the PAN study and 12 via the UMCU in-house voluntary healthy donor service). The Luminex assay showed normal standard negative control levels in 47/52 (90%) of the MMN samples and in 24/24 (100%) of the control samples. Baseline characteristics are shown in table 1. IVIg dosage (median 52 grams per month) was positively skewed, ranging from 20 to 212 gram per month. Seven patients with MMN (15%) were not treated with IVIg at the moment of inclusion in this study.

Detection limits
The Luminex platform used for our analyses has a large sensitivity range. However, as detection limits differed per protein included in our analyses, the reliability of the measurements of each protein might also differ. We have, therefore, provided an overview of all out of range (OOR) data in table 2.
Baseline concentrations of IL-1RA, IL-1β and IL-6 were were more often out of range in control subjects than in patients with MMN (χ^2 test, p all <0.001). Baseline levels of IL-10 and TNF-α were below the detection limit in >50% of the subjects, in both the control and MMN group.
OOR data after LPS stimulation were evenly distributed between controls and MMN patients for all proteins. IL-6 and TNF-α data were above detection limits in >50% of the subjects in both groups. Two subjects (one patient and one control) had CD40L levels below the detection limit, at baseline as well as after LPS stimulation.
As sufficient within-range data were available, we decided to compare baseline levels of IL-21, IL-8 and CD40L, and LPS-induced changes of IL-1RA, IL-1β, IL-10, IL-21, IL-8 and CD40L, between control subjects and patients with MMN.

Table 1. Baseline characteristics of patients with MMN and controls			
	MMN (n=47)	**Controls (n=24)**	
		PAN study (n=12)	UMCU (n=12)
Male sex (n, %)	36 (77)	10 (83)	NA
Age at inclusion (years)[$]	58 (17)	70 (10)	NA
Autoimmune disease (n, %)	3/43 (7)	0/12 (0)	
Vitiligo	1/43 (2)	0 (0)	
Astmatic bronchitis	2/43 (5)	0 (0)	
Immunosuppressant therapy (n, %)	0/47 (0)	0/12 (0)	
Disease duration at inclusion (years)[$]	16 (13)		
Age at onset (years)[$]	43 (11)		
Age at diagnosis (years)[$]	48 (8)		
Diagnostic delay (months)[$]	32 (46)		
EFNS MMN diagnosis (n/N, %)			
Definite MMN	32/45 (71)		
Probable MMN	12/45 (27)		
Possible MMN	1/45 (2)		
Anti-GM1 IgM positive (n/N, %)	22/42 (54)		
IVIg treatment at inclusion (n/N, %)	40/47 (85)		
IVIg dosage at inclusion (gr/month)[$]	52 (49)		
MRC sum score at inclusion[$]	168 (11)		

[$] Values displayed as median (IQR)
MMN = multifocal motor neuropathy, MRC = medical research council, NA = not applicable, PAN = prospective ALS study in the Netherlands, UMCU = University Medical Center Utrecht

Table 2. Overview of Out of Range (OOR) data, separately shown for patients with MMN and controls, as determined in plasma obtained at baseline and after stimulation with MQ and with LPS. Uncorrected Chi-squared or Fisher's test p-values are shown.

Protein	**Baseline (t = 0h)**				**MQ stimulation (t = 4h)**				**LPS stimulation (t = 4h)**			
	< OOR	MMN	Controls	p-value	< OOR	MMN	Controls	p-value	> OOR	MMN	Controls	p-value
IL-1RA	19 (27)	3 (6)	16 (67)	<0.001*	9 (13)	2 (4)	7 (29)	0.009*	1 (1)	1 (2)	0 (0)	1.00
IL-1b	42 (59)	19 (40)	23 (96)	<0.001*	42 (59)	20 (43)	22 (92)	<0.001*	27 (38)	18 (38)	9 (38)	1.00
IL-6	23 (32)	5 (11)	18 (75)	<0.001*	9 (13)	1 (2)	8 (33)	<0.001*	57 (80)	37 (79)	20 (83)	0.76
IL-10	41 (58)	26 (55)	15 (63)	0.74	42 (59)	26 (55)	16 (67)	0.51	0 (0)	0 (0)	0 (0)	1.00
IL-21	0 (0)	0 (0)	0 (0)	1.00	0 (0)	0 (0)	0 (0)	1.00	0 (0)	0 (0)	0 (0)	1.00
TNF-α	55 (77)	34 (72)	21 (88)	0.25	12 (17)	9 (19)	3 (13)	0.74	45 (63)	29 (62)	16 (67)	0.88
IL-8	0 (0)	0 (0)	0 (0)	1.00	0 (0)	0 (0)	0 (0)	1.00	10 (14)	4 (9)	6 (25)	0.077
CD40L	2	1 (2)	1 (4)	1.00	2 (3)	1 (2)	1 (4)	1.00	0 (0)	0 (0)	0 (0)	1.00

All values are depicted as n (%)

* Statistically significant after Bonferroni p-value adjustment

Protein concentrations do not correlate with age and sex

We determined the correlation between baseline protein concentrations and the LPS-induced changes to age at inclusion and sex in all subjects for whom these variables were known (n=64; sex and age at inclusion of 12 control subjects included via the voluntary in-house UMCU donor facility were not known). We found no correlations between protein levels at baseline or after stimulation with age (Spearman correlations, corrected p >0.27 in all comparisons, data not shown) or sex (Mann-Whitney U tests, corrected p 1.00 in all comparisons, data not shown). It was, therefore, possible to included in our further analyses the data of 12 control subjects for whom sex and age at inclusion were not known.

Comparison of protein concentrations between patients with MMN and controls

Baseline and LPS-stimulated protein concentrations of IL-1RA, IL-1β, IL-10, IL-21, IL-8 and CD40L are shown in table 3. Baseline IL-21 and IL-8 concentrations were higher in patients with MMN than in controls (Mann-Whitney U test, p <0.001 in both comparisons). Baseline CD40L concentration did not differ between groups (Mann-Whitney U test, p 0.62).

LPS-induced protein concentration changes did not differ between patients with MMN and controls (Mann-Whitney U test, p all >0.05). Interestingly, in both patients and controls, we observed a decrease in IL-21 levels after LPS stimulation in some subjects, and no or only a moderate increase in others (IL-21 fold change range 0.81 – 1.51). As only one subject had a change exceeding 1.5-fold, we conclude that, at a group level, IL-21 increase did not occur consistently within four hours of LPS stimulation.

Correlation of immunoregulating protein levels with patient characteristics

IVIg therapy

We correlated baseline protein concentrations and associated LPS-induced changes to the monthly IVIg dosage and found a positive and statistically significant correlation with baseline concentrations of IL-1RA, IL-1β, IL-6, and IL-21 (Fig. 1).

We hypothesized that IVIg dosage might be a proxy for disease severity, where patients with more severe MMN would receive a higher IVIg dosage. However, when we correlated IL-1RA, IL-1β, IL-6 and IL-21 baseline concentrations to disease duration and the MRC sum score at inclusion, we did not observe significant associations (Spearman correlation, p >0.05 in all correlations, data not shown). Moreover, none of the proteins' LPS-induced change was correlated with monthly IVIg dosage (Fig. 1).

Table 3. Baseline (T0) concentrations, concentrations after stimulation with LPS (T=4h) and concentration increase (Δ) of IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L in patients with MMN and controls. All concentrations were measured in pg/mL. The uncorrected Mann-Whitney U test p-value is shown for comparisons where <50% of data points were measured out of the detection limits in both groups.

Protein	**MMN**			**Controls**				
	T0	T=4h	Δ	T0	T=4h	Δ	p T0	p Δ
IL-1RA	353 (210)	10009 (6225)	9557 (6168)	4.20 (186)	8866 (3526)	8862 (3503)	-	0.73
IL-1β	3.04 (4.45)	3142 (9223)	3138 (9223)	0.105 (0)	3192 (9115)	3188 (9115)	-	0.69
IL-6	34.8 (40.3)	23559 (106)	23526 (116)	1.40 (0.865)	23559 (13)	23557 (18)	-	-
IL-10	0.965 (2.45)	62.3 (52.6)	57.4 (51.5)	0.965 (2.80)	78.7 (67.4)	75.6 (65.7)	-	0.14
IL-21	460 (227)	461 (227)	2.49 (72.4)	273 (36.3)	277 (52.9)	4.60 (31.2)	<0.001*	0.86
TNF-α	0.250 (0.780)	21778 (17291)	21777 (17292)	0.250 (0)	21778 (16699)	21777 (16699)	-	-
IL-8	35.2 (25.2)	3777 (2175)	3757 (2191)	11.6 (5.36)	4530 (11596)	4502 (11596)	<0.001*	0.08
CD40L	137 (201)	506 (433)	203 (275)	262 (109)	530 (706)	186 (452)	0.62	0.86

All values are depicted as median (IQR)

*Statistically significant after Bonferroni p-value adjustment

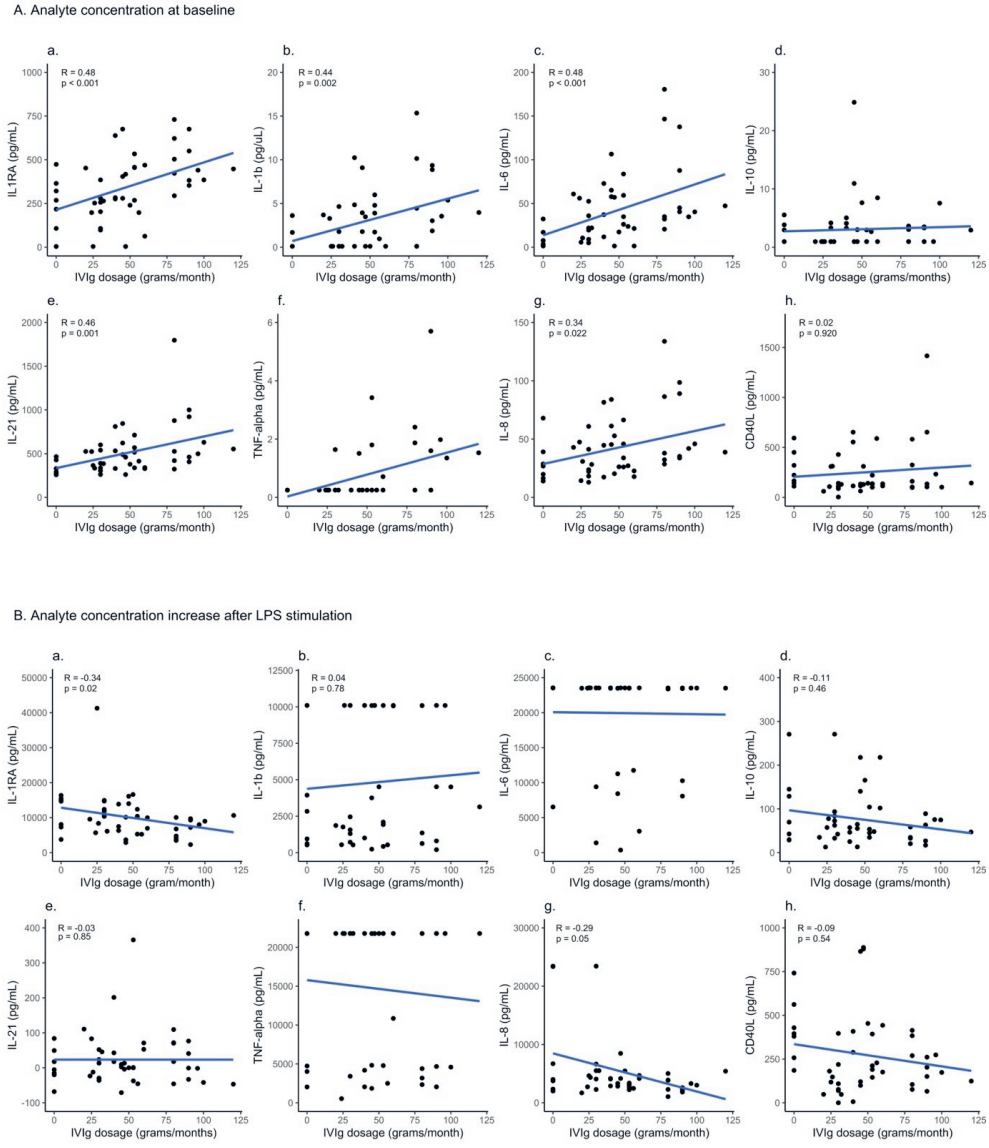

Figure 1. *IVIg dosage correlated to baseline concentrations (panel A) and concentration changes after LPS stimulation (panel B), of IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L (plots a-h in both panels). In the upper left corner, Spearman's rho correlation coefficient and its uncorrected p-value are shown for proteins for which more than 50% of the data points were measured within detection limits.*

Next, to further study the association between IVIg treatment and protein concentrations at baseline and after LPS stimulation, we compared protein concentrations between controls and patients with MMN who were treatment-naïve at the time of inclusion in this study (n=7). Baseline IL-8 concentration was higher in treatment-naïve patients than in controls (Mann-Whitney U test, p 0.0049), but none of the other proteins' concentrations, either at baseline or after LPS stimulation, differed between groups (Mann-Whitney U test, unadjusted p all >0.05, data not shown).
Next, as baseline concentrations of IL-8 and IL-21 were higher in MMN patients, but were also associated with IVIg dosage, we corrected these for monthly IVIg dosage using a linear regression analysis, including all subjects. We found that the difference in IL-21 baseline concentration was dependent on IVIg dosage only (monthly IVIg dosage: beta = 2.55, t = 3.17, p 0.002; subject group: beta = 81.7, t = 1.21, p 0.229), whereas baseline IL-8 concentrations remained higher in MMN patients, also after correcting for IVIg dosage (monthly IVIg dosage: beta = 0.20, t = 2.46, p 0.016; subject group: beta = 16.1, t = 2.36, p 0.021).
In summary, these results indicate that patients with MMN have higher baseline IL-8 concentrations than controls, independent of IVIg therapy or dosage. However, the observed increased baseline concentrations of IL-1RA, IL-1β, IL-6 and IL-21 in patients may be a consequence of IVIg therapy, rather than a reflection of the immunopathology underlying MMN. Finally, the protein concentration changes after LPS stimulation prove to be independent of IVIg therapy.

Anti-GM1 IgM antibody status
Since patients with anti-GM1 antibodies might have a different immunopathology underlying their disease than patients without these antibodies, we studied possible differences in the innate immune response by comparing baseline concentrations and LPS-induced changes between patients with and without anti-GM1 IgM antibodies (Fig. 2). Baseline concentrations of IL-1RA were increased in anti-GM1 IgM positive patients (Mann-Whitney U test, corrected p 0.030). IL-1β and IL-21 showed a similar trend. When corrected for monthly IVIg dosage using linear regression analysis, we found that the difference between groups depended on monthly IVIg dosage only (monthly IVIg dosage: beta = 2.1, t = 2.627, p 0.012; anti-GM1 IgM antibody status: beta = 95.3, t = 1.829, p 0.075). Therefore, baseline concentrations of all analytes did not differ between patients with or without anti-GM1 IgM antibodies.
When comparing protein concentrations between anti-GM1 IgM antibody groups after LPS stimulation, we did not identify differences in the increase of IL-1RA, IL-1β, IL-10, IL-8 and CD40L (Fig. 2). Although we concluded that at a group level unequivocal IL-21 stimulation (i.e. at least a 1.5-fold concentration increase after LPS stimulation) did not occur, we observed a concentration increase in patients with anti-GM1 IgM antibodies more often than in patients without anti-GM1 IgM antibodies. The absolute increase after LPS stimulation was higher in patients with anti-GM1 IgM antibodies (Mann-Whitney U test, p 0.048). The percentage of patients that showed an increase of at least 10% from baseline was also higher in patients with anti-GM1 IgM antibodies than in patients without (8/22 (36%) vs. 1/19 (5%), χ^2 test p 0.016).

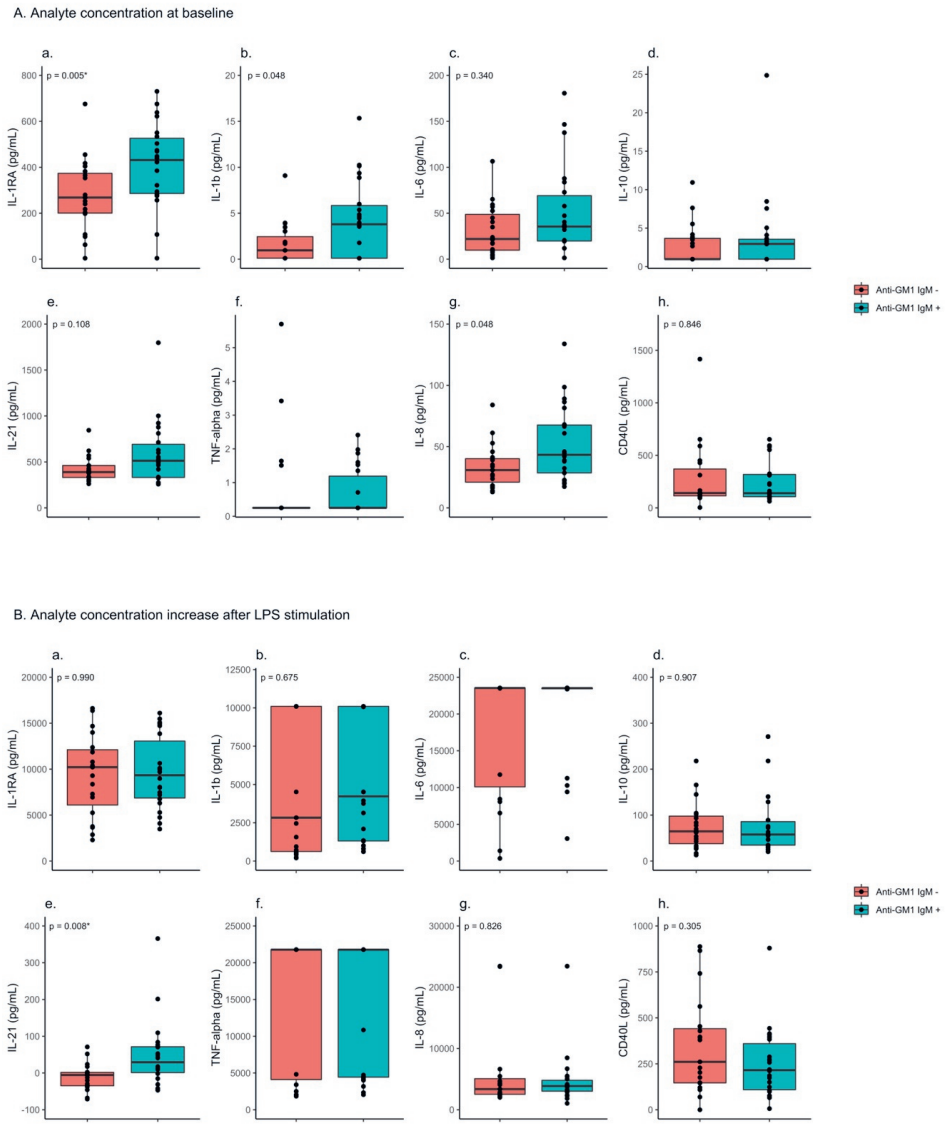

Figure 2. *Baseline concentrations (panel A) and concentration changes after LPS stimulation (panel B) of IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L (plots a-h in both panels), stratified by anti-GM1 IgM antibody status in patients with MMN. In the upper left corner, the Mann-Whitney U test's uncorrected p-value is shown for proteins for which at least 50% of the data points were measured within detection limits in both groups.*

Integrated comparisons using PCA reveals no clustering of data

Since the analyses described above focused on each protein separately, we decided to use a combined approach to study possible clustering of data by performing a principal component analysis (PCA, Fig. 3). For baseline concentrations, controls showed less scattering of data than patients, in line with data presented in table 1 and 2, but no unequivocal clustering occurred. After LPS stimulation, we observed no clustering of data when comparing patients and controls. Likewise, no differential clustering was found in either comparison for patients with or without IVIg treatment, or for patients with or without anti-GM1 IgM antibodies.

In conclusion, in addition to comparing each protein separately, no immunological signatures were found for patients with MMN, patients treated with IVIg or patients with anti-GM1 IgM antibodies in this combined approach using PCA.

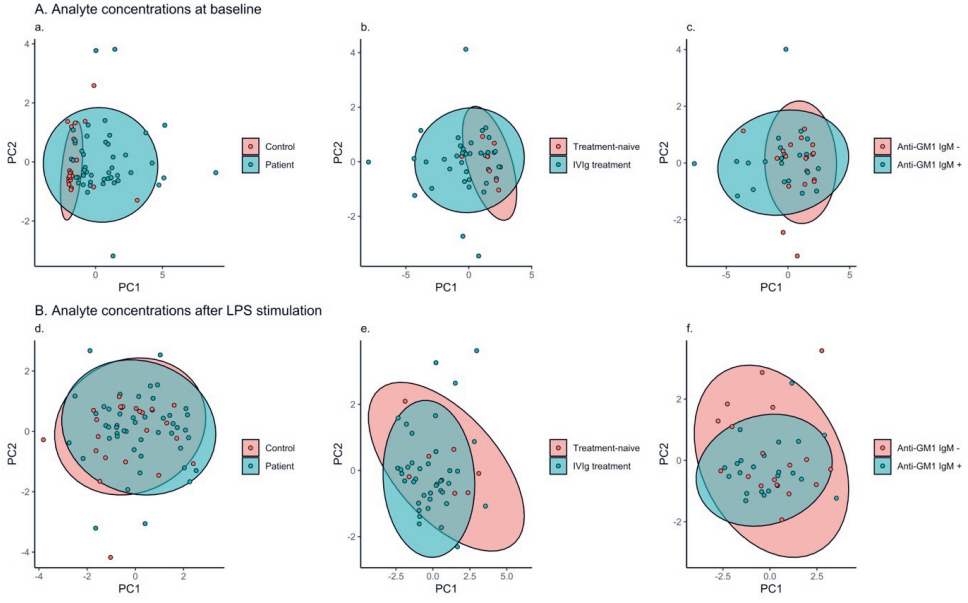

Figure 3. *Principal component analysis (PCA) for baseline concentrations (panel A) and concentration changes after stimulation with LPS (panel B), stratified for patients and controls (plots a. and d.), IVIg treatment status (plots b. and e.) and anti-GM1 IgM antibody status (c. and f.). Data for IL-1RA, IL-1β, IL-6, IL-10, IL-21, TNF-α, IL-8 and CD40L were included in all PCA's.*

Discussion

Our understanding of the role of the innate immune system in the immunobiology underlying multifocal motor neuropathy is limited. Therefore, we have explored innate immune responses in whole blood samples from patients with MMN and controls following stimulation with the endotoxin LPS. At baseline, patients with MMN had higher IL-8 concentrations than controls. After endotoxin activation, the levels of a range of immunoregulating proteins increased, but these increases did not differ between patients and controls. Hence, our results do not support variation in the innate immune response as a susceptibility factor for MMN.

Previous studies have shown the value of *ex vivo* stimulation experiments using endotoxins in uncovering altered innate immune responses and their association with susceptibility to various infectious, autoinflammatory and autoimmune diseases.[23-27] Interestingly, altered innate immune responses have been shown to be a critical host susceptibility factor underlying the loss of immunotolerance to anti-ganglioside antibodies in Guillain-Barré syndrome (GBS), an acute, monophasic autoimmune neuropathy, characterized by IgG antibodies to gangliosides, including GM1.[28,29] In GBS, sialylation of *Campylobacter jejuni* endotoxin augments the TLR4-mediated innate immune response by dendritic cells, leading to increased T-cell independent B-cell stimulation.[15] Moreover, this study showed that the innate immune response triggered by *C. jejuni* LPS is a host susceptibility factor in patients with GBS and associated with GBS severity.[14] Although MMN is not associated with preceding *C. jejuni* infection, the multiple phenotypic similarities between the acute motor axonal neuropathy (AMAN) variant of GBS and MMN, i.e. a pure motor neuropathy associated with the presence of conduction block, anti-GM1 antibodies and a response to IVIg but not corticosteroids, suggested the possibility of specific innate immune responses as a susceptibility factor for MMN.[30-33] The lack thereof, combined with the absence of inflammatory markers in blood and cerebrospinal fluid in patients with MMN may suggest that local rather than systemic inflammation is a feature of MMN, in contrast with findings in patients with GBS.[12,13,34]

Despite the overall lack of association between innate immune responses and MMN, we observed some interesting patterns for specific proteins. Although the results of the comparison between treatment-naïve patients with MMN and controls should be interpreted with care given the small group of treatment-naïve patients (n=7), we found higher IL-8 baseline concentrations in patients with MMN, independent of IVIg treatment. IL-8 is a pro-inflammatory, neutrophil chemotactic factor produced by a variety of cells after stimulation with other cytokines, such as IL-1β and TNF-α.[35] When comparing patients with MMN and controls, drawing definite conclusions is complicated because of the complex interplay of immunoregulating proteins, in which baseline IL-8 levels could serve as a proxy for other factors, and the limited number of treatment-naïve patients in our study (n=7). Moreover, within four hours of stimulation with LPS, we found that patients with anti-GM1 IgM antibodies showed an IL-21 increase more often than to patients without anti-GM1 IgM antibodies. The fact that IL-21, a B-cell stimulating and regulating factor, is produced by T cells could explain why not all samples showed a clear IL-21 increase after four hours of stimulation.[36,37] Indeed, other B-cell stimulating or regulating proteins, such as APRIL and BAFF, have previously been shown to emerge only after longer periods of stimulation.[15] Therefore, whether this finding reflects a difference in the timing of the onset of IL-21 increase

or preludes a difference in maximum IL-21 production after longer periods of stimulation remains to be uncovered.

To the best of our knowledge, we have performed the first study on innate immune responses to endotoxin of peripheral blood cells in MMN. Considering the rarity of MMN, our patient cohort should be considered large; this allowed us to correlate data on a variety of immunoregulating proteins to detailed clinical parameters, measured at the time of sampling. We believe that our results, obtained after four hours of stimulation with LPS, essentially rule out major general differences in the innate immune response between patients with MMN and controls. However, the results of our study suggest that further research, focusing on products of the innate immune response emerging after longer periods of stimulation with endotoxin, such as IL-21, APRIL of BAFF, may be necessary. The correlation between IVIg dosage at the time of sampling and baseline levels of specific immunomodulating proteins indicates biomarker potential for efficacy of IVIg treatment, but this would need to be addressed further in future studies.

In summary, we demonstrate that altered innate immune responses to LPS are not a susceptibility factor for MMN. As innate immune responses were not associated with the presence of anti-GM1 IgM antibodies, the results of our study indicate that differences in the innate immune response to endotoxin are unlikely to form part of the immunopathology underlying anti-ganglioside antibody production in MMN.

References

1. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

2. Herraets I, Van Rosmalen M, Bos J, et al. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. Neurology 2020;95(14):1979-1987

3. Briani C, Cocito D, Campagnolo M, et al. Update on therapy of chronic immune-mediated neuropathies. Neurol Sci 2021;43(2):605-614

4. Beadon K, Guimarães-Costa R, Léger J-M. Multifocal motor neuropathy. Curr Opin Neurol 2018;31(5):559-564

5. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology 2010;75(9):818-825

6. Van Asseldonk JTH, Van den Berg LH, Kalmijn S, et al. Axon loss is an important determinant of weakness in multifocal motor neuropathy. J Neurol Neurosurg Psych. 2006;77(6):743-747

7. Pestronk A, Cornblath DR, Ilyas AA, et al. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. Ann Neurol 1988;24(1):73-78

8. Mathey EK, Garg N, Park SB, et al. Autoantibody responses to nodal and paranodal antigens in chronic inflammatory neuropathies. J Neuroimmunol 2017;309:41-46

9. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol 2016;80(1):71-88

10. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2015;2(4):e119

11. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol 2010;229(1-2):256-262

12. Furukawa T, Matsui N, Fujita K, et al. CSF cytokine profile distinguishes multifocal motor neuropathy from progressive muscular atrophy. Neurol Neuroimmunol Neuroinflamm 2015;2(5):e138

13. Vlam L, Stam M, De Jager W, et al. Cytokine profiles in multifocal motor neuropathy and progressive muscular atrophy. J Neuroimmunol 2015;286:1-4

14. Huizinga R, Van den Berg B, Van Rijs W, et al. Innate immunity to campylobacter jejuni in Guillain-Barré syndrome. Ann Neurol 2015;78(3):343-354

15. Huizinga R, Van Rijs W, Bajramovic JJ, et al. Sialylation of Campylobacter jejuni endotoxin promotes dendritic cell-mediated B cell responses through CD14-dependent production of IFN-beta and TNF-alpha. J Immunol 2013;191(11):5636-5645

16. Van Schaik IN, Leger JM, Nobile-Orazio E, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripheral Nerv Syst 2010;15(4):295-301

17. Huisman, MHB, Seelen, M, De Jong, SW, et al. Lifetime physical activity and the risk of amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry 2013; 84(9):976-981s

18. De Jongh AD, van Eijk RPA, Peters SM, et al. Incidence, prevalence and geographical clustering of motor neuron disease in the Netherlands. Neurology 2021;96:e1227-1236

19. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010;75(22):1961-1967

20. Kuijf ML, Van Doorn PA, Tio-Gillen AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci 2005;239(1):37-44

21. Scholman R, Giovannone B, Hiddingh S, et al. Effect of anticoagulants on 162 circulating immune related proteins in healthy subjects. Cytokine 2018;106:114-124

22. Delemarre EM, Van Hoorn L, Bossink AWJ, et al. Serum biomarker profile including CCL1, CXCL10, VEGF and adenosine deaminidase activity distinguishes active from remotely acquired latent tuberculosis. Front Immunol 2021;12:725447

23. Westendorp RG, Langermans JA, Huizinga TW, et al. Genetic influence on cytokine production and fatal meningococcal disease. Lance 1997;349(9046):170-173

24. Regnault MM, Frouin E, Jéru I, et al. Cytokine signature in Schnitzler syndrome: proinflammatory cytokine production associated to Th suppression. Front Immunol 2020;11:588322

25. Van der Linden MW, Westendorp RG, Sturk A, et al. High interleukin-10 production in first-degree relatives of patients with generalized but not cutaneous lupus erythematosus. J Investig Med 2000;45(5):327-334

26. De Jong BA, Schrijver HM, Huizinga TW, et al. Innate production of interleukin-10 and tumor necrosis factor affects the risk of multiple sclerosis. Ann Neurol 2000;48(4):641-646

27. Wirtz PW, Huizinga TW, Stoeken D, et al. High innate production of interleukin-10 and tumor necrosis factor-alpha contributes to susceptibility for non-paraneoplastic Lambert-Eaton myasthenic syndrome. J Neuroimmunol 2003;140(1-2):194-197

28. Kusunoki S, Willison HJ, Jacobs BC. Antiglycolipid antibodies in Guillain-Barré and Fisher syndromes: discovery, current status and future perspectives. J Neurol Neurosurg Psychiatry 2021;92(3):311-318

29. Willison HJ, Goodyear CS. Glycolipid antigens and autoantibodies in autoimmune neuropathies. Trend Immunol 2013;34(9):453-459

30. Terenghi F, Allaria S, Scarlato G, et al. Multifocal motor neuropathy and Camyplobacter jejuni reactivity. Neurology 2002;59(2):282-284

31. Kokubun N, Nishibayashi M, Uncini A, et al. Conduction block in acute motor axonal neuropathy. Brain 2010;133(10:2897-2908

32. Kuwabara S, Yuki N. Axonal Guillain-Barré syndrome: concept and controversies. Lancet Neurol 2013;12(12):1180-1188

33. Leonhard SE, Mandarakas MR, Gondim FAA, et al. Diagnosis and management of Guillain-Barré syndrome in ten steps. Nat Rev Neurol 2019;15(11):671-683

34. Sun T, Chen X, Shi S, et al. Peripheral blood and cerebrospinal fluid cytokine levels in Guillain Barré syndrome: a systematic review and meta-analysis. Front Neurosci 2019;13:717

35. Akdis M, Aab A, Altunbulakli C, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. J Allergy Clin Immunol 2016;138(4):984-1010

36. Konforte D, Simard N, Paige CJ. IL-21: an executor of B cell fate. J Immunol 2009;182(4):1781-178

37. Spolski R, Leonard WJ. Interleukin-21: a double-edged sword with therapeutic potential. Nat Rev Drug Discov 2014;13(5):379-395

CHAPTER

IgM anti-GM2 antibodies in patients with multifocal motor neuropathy target Schwann cells and are associated with early onset

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Abstract

Background Multifocal motor neuropathy (MMN) is a rare, chronic immune-mediated polyneuropathy characterized by asymmetric distal extremity weakness. An important feature of MMN is the presence of IgM antibodies against gangliosides, in particular GM1 and less often GM2. Antibodies against GM1 bind to motor neurons (MNs) and cause damage through the activation of complement. The involvement of Schwann cells (SCs), expressing GM1 and GM2, in the pathogenesis of MMN is unknown.

Methods Combining the data of our 2007 and 2015 combined cross-sectional and follow-up studies in Dutch patients with MMN, we evaluated the presence of IgM antibodies against GM1 and GM2 in serum from 124 patients with MMN and investigated their binding to SCs and complement-activating properties. We assessed IgM binding, complement deposition, and their association with clinical characteristics.

Results Thirteen out of 124 patients (10%) had a positive ELISA titer for IgM anti-GM2. Age at onset of symptoms was significantly lower in patients with MMN with anti-GM2 IgM. IgM binding to SCs, but not to MNs, correlated with IgM anti-GM2 titers and nor with IgM anti-GM1 titers. IgM binding to SCs decreased upon pre-incubation of serum with soluble GM2, but not with soluble GM1. IgM anti-GM2 binding to SCs correlated with complement activation, as reflected by increased C3 fixation on SCs and C5a formation in the supernatant.

Conclusion Circulating IgM anti-GM2 antibodies define a subgroup of patients with MMN that has an earlier onset of disease. These antibodies probably specifically target SCs and activate complement, similarly as IgM anti-GM1 on MNs. Our data support that complement activation by IgM antibodies bound to SCs and MNs underlies MMN pathology.

Background

Multifocal motor neuropathy (MMN) is a rare, chronic motor neuropathy characterized by slowly progressive asymmetric weakness of distal limbs, that responds to treatment with intravenous or subcutaneous immunoglobulins (IVIg; ScIg).[1-6] (Multi)focal motor conduction block with normal sensory function is considered the hallmark of MMN, but imaging studies have shown a more generalized pattern of nerve pathology.[7-9] Serum from patients with MMN often contains IgM antibodies against ganglioside GM1 and occasionally GM2.[3,10,11] The pathophysiological mechanisms underlying MMN are incompletely understood due to the few pathological studies performed in MMN and the lack of a representative animal model, but the available evidence suggests immune-mediated abnormalities of (perinodal and perisynaptic) Schwann cells (SCs), myelin sheath and the (peri)nodes of Ranvier.[10,12-14]
GM1 is a glycosphingolipid that is highly expressed in perinodal regions of peripheral nerves and a target for antibodies, found in patients with MMN and acute motor axonal neuropathy (AMAN).[11] Anti-GM1 IgM binds to axons and neurites of induced pluripotent stem cell (iPSC) derived motor neurons (MNs) and induces cellular damage through the activation of the classical complement pathway.[10,14] Hence, complement activation by IgM anti-GM1 antibodies may underlie disease progression and permanent weakness due to accumulating axonal damage.[15] Higher titers of anti-GM1 are associated with both more complement deposition in vitro and more pronounced weakness in patients.[3,16,17]
Initial discrepancies of anti-GM1 IgM prevalence reports in MMN were caused by differences in methodology, but recent studies showed that IgM anti-GM1 are present in serum of approximately 50% of patients.[3,4,12,13,18] This is an underestimation due to limited sensitivity of detection techniques, but it is likely that serum from a subgroup of patients with MMN does not contain IgM anti-GM1, but IgM auto-antibodies with other specificities, such as NS6S heparin disaccharide and other gangliosides, such as GM2, GD1b, and GD1a.[3,10,19-22]
We previously described the pathogenic effects of MMN-associated antibodies using an iPSC-MN model.[10,14] The goal of this study was to study binding of IgM antibodies against gangliosides using a SC-line as well as iPSC-derived MNs and their potency to activate complement in a cohort of 124 well characterized patients with MMN.

Methods

Standard protocol approvals, registrations, and patient consents

The Ethics Committee of the University Medical Center Utrecht approved the collection of patient sera as part of a national cross-sectional study (UMCU, METC protocol nr: 14-528).[4] Written informed consent was obtained from all study participants prior to inclusion in this study.

Study populations

All patients with MMN had been diagnosed at the outpatient clinic of the UMCU and met the 2010 EFNS diagnostic criteria for definite, probable or possible MMN.[23] We only included patients of whom clinical data were available. We obtained serum samples of healthy controls (HC) through the in-house donor facility of the UMCU. Serum samples of all subjects were heat-inactivated for 30 minutes at 56°C and stored in aliquots at -80°C until used.

Clinical data

We retrieved clinical data from the UMCU MMN database. This registry contains data collected during the 2007 and 2015 Dutch national combined cross-sectional and follow-up studies on MMN, complemented with data from patients' UMCU patient files.[3,4,24] Age at onset was defined as the age at which a patient first noticed signs of muscle weakness, and disease duration was defined as the time that lapsed since disease onset. Nerve conduction studies (NCS) were done as described previously.[4,24] We recorded the presence of abnormal brachial plexus MR imaging (nerve thickening or nerve hyperintensity), postural hand tremor and vibration sense abnormalities.[3,4] We calculated an MRC sum score (MRCss) of shoulder abduction, elbow, wrist and finger flexion and extension, finger spreading, hip and knee flexion, knee extension and foot dorsal and plantar flexion bilaterally as a measure for total muscle strength (maximum score 130) at patients' first visit to our hospital.[25] The difference in MRCss (ΔMRCss) as measured during the Dutch national cross-sectional studies on MMN in 2007 and 2015 was used as a measure of disease progression.[3,4] We listed the presence of IgG and/or IgM monoclonal gammopathy as determined in serum, cerebrospinal fluid (CSF) analysis results and anti-GM1 and anti-GM2 IgM antibody status, which were determined by ELISA as described previously.[3,26] For IVIg analyses, we determined patients' IVIg treatment status and IVIg dosage in grams/month.

Cell culture

The iPSC-derived model for MMN was modified from protocols described previously.[10,14] Human SC-line sNF96.2 (derived from a malignant peripheral nerve sheath tumor) was obtained from ATCC (CRL-2884) and cultured in T75 or T175 flasks (Greiner) in DMEM medium (Life Technologies) supplemented with 100 U/mL penicillin (Life Technologies), 100µg/µL streptomycin (Life Technologies), and 10% v/v (volume/volume) fetal calf serum (FCS, Bodinco) at 37°C and 5%, v/v, CO2. Cells were passaged at 80% confluency, first washed in PBS, following detachment using Accutase cell detachment solution (Sigma-Aldrich). Cell numbers and viability (typically >80%) were assessed via trypan blue (Sigma-Aldrich) exclusion assay using an automated cell counter (Countess, Invitrogen).

Flow cytometry

sNF96.2 SCs were transferred to V-bottom plates (Greiner) at a density of 50,000 cells/well. To assess IgM binding, cells were opsonized with heat-inactivated (HI) MMN serum or HC serum (1:20 diluted in veronal buffer (VB, Lonza)) for 1 hour at room temperature (RT). Between every incubation step, cells were washed with 100µL FACS buffer (FB, which is phosphate buffered saline, pH 7.4 (PBS, Sigma)-0.1%, w/v, bovine serum albumin (BSA, BSA Fraction V, Roche)-0.01%, w/v, sodium azide) and centrifuged for 5 minutes at 125g. Next, cells were stained (20µl, diluted in FB, 45 min on ice in the dark) with a primary detection antibody (goat anti-human IgM biotin, 1:50, Sigma) followed by incubation with a secondary detection antibody (streptavidin-APC, 1:100, ThermoFisher). To correct for day-to-day variation, the mean fluorescent intensity (MFI) of each sample was divided by the average MFI of a set of HC sera (n=6) that we tested simultaneously and expressed as fold change (FC). To assess IgM anti-GM1 and/or anti-GM2 antibody specificity, MMN patient sera were pre-incubated with soluble GM1 (Enzo Life Sciences) or GM2 (Sigma) at 100µg/µL for 30 min at RT, prior to opsonization. The % inhibition of IgM binding was defined in ELISA as the reduction in antibody activity defined by OD in a serum sample preincubated with GM1/GM2 compared to a serum sample without such preincubation. To evaluate complement activation by bound IgM antibodies,

following opsonization with MMN patient or HC serum cells were incubated with 5%, v/v, pooled complement active serum (InnovativeResearch), diluted in VB. Fixation of C3 to the cells was then measured by a subsequent inhibition with mouse anti-human C3 biotin (1:00 in FB, LSBio). Cells were analyzed using flow cytometry (FACS Canto II, and accompanying software, FACS DIVA, BD Biosciences).

Anti-GM2 specific ELISA

To assess GM2 specificity, we utilized a modified version of the previously published anti-GM2 ELISA.[3,26] Wells of a MaxiSorp plate (NUNC) were coated with 70µL 0.1µg/mL GM2 (Sigma), diluted in methanol, and left to evaporate O/N in a laminar flow. Wells were blocked with 200 µL 1% BSA-PBS for 1 hour at RT. Patient sera were diluted 1:200 in 1% BSA-PBS, either or not pre-incubated with GM1 or GM2 (50µg/µL for 30 min at RT), and incubated in GM2 coated wells 1 hour at RT. Next, wells were washed 3 times with PBS, and IgM binding was detected using goat anti-human IgM (Sigma, 70µL 1:10000 in 1% BSA-PBS, 1 hour at RT), followed by a 3-time wash in PBS, and incubation with streptavidin-POD (Sigma, 70µL 1:1000 in 1% BSA-PBS, 30 min at RT), and a final 3-time wash in PBS. For detection 100µL TMB (Invitrogen) was added to each well. Finally, the reaction was stopped using 1M HCL (Fisher Chemical). All measurements were conducted in triplicate and OD_{450nm} (read-out at a wave length of 450 nm) was analyzed using a SpectraMax M3 (Molecular devices). The d-OD_{450nm} was obtained by subtracting the OD_{450nm} from an uncoated well of the respective OD_{450nm} of a GM2 coated well. The % inhibition of IgM binding was calculated by setting the OD_{450nm} of the serum sample without GM1/GM2 preincubation at 0% inhibition.

Microscopy and live cell complement activation

sNF96.2 SCs were seeded (50.000 cells/well) on coverslips (VWR) in a 24-well plate (Greiner) for 2 days prior to experimental analysis. Cells were opsonized with HI MMN patient serum (150 µL, 1:50 in VB) for 1 hour at RT. Next, 150 µl 15% pooled complement active serum (pre-incubated with complement inhibitors for 15 min at RT when indicated) was added to each well and incubated for 1 hour at 37°C. Supernatant was collected after incubation with complement-active serum for the measurement of complement activation products. For microscopy, cells were fixed in 4% paraformaldehyde (Klinipath) 10 min at RT, the coverslips were removed from the 24 wells plate, washed with PBS and quenched for autofluorescence using NH¬4Cl (5 min RT). Subsequent incubation steps were performed top-down in 100 µL droplets on parafilm. Coverslips were blocked in 2% BSA-PBS for 1 hour RT, and subsequently stained with a primary (biotinylated goat anti-human C3, 1:2000, MyBioSource) and secondary detection antibody (Streptavidin APC, 1:100, eBioscience), both diluted in 2% BSA-PBS, for 1 hour at RT in the dark and washed with PBS. After the last antibody incubation step coverslips were washed in PBS and distilled water (Milli-Q). After removal of excessive liquid, the coverslip was mounted on an object glass in 7µL ProLong Diamond Antifade Mountant with DAPI (Invitrogen) and dried overnight at RT. Samples were analyzed at 20x magnification using a Zeiss Z1 microscope (Carl Zeiss Microscopy) with Colibri LEDs and the following settings: 25% LED 400 ms for Alexa Fluor 488, 25% LED 100 ms for APC, 25% LED 50 ms for DAPI. To prevent bias, 4 pictures were taken throughout the image field. Pictures were exported in single and merged channel to non-compressed TIFF-format using ZEN 2 software (Carl Zeiss Microscopy) and mean grey values were calculated for each single channel using ImageJ (Fiji 1.53).

Other analyses
C5a in the supernatant samples after complement activation was measured with the Human Complement Component C5a DuoSet ELISA (R&D Systems) according to manufacturer's instructions. For all complement read-outs, values were normalized by calculating the FC relative to the non-opsonized serum control, which was set at 1 from each experiment set at 1.

Statistical analysis
GraphPad Prism 9 was used for data analysis and visualization of experimental data. Correlation analyses were conducted using the Spearman's rank correlation coefficient with the following rs grading: 0.00-0.10 negligible; 0.10-0.39 weak; 0.40-0.69 moderate; 0.70-1.00 strong. Clinical data were analyzed using R version 4.2.0. We compared categorical data using a Chi-squared test. Continuous variables were compared between groups using a Student's t-test or Mann-Whitney U test, as appropriate. Multiple-group comparisons were performed using a Kruskal-Wallis test with Dunn's test as a post-hoc analysis. In analyses concerning IVIg dosage (grams/month) and MRC sum score, i.e., the MRCss at patients' first visit and the change in muscle strength between 2007 and 2015 which we termed ΔMRCss, comparisons between groups were corrected for disease duration using a linear regression model. Analyses involving patients' MRCss at their first visit to the UMCU were performed in IVIg treatment-naïve patients only. For nerve conduction comparisons, we included a subset of patients whose samples were used in the complement activation assay (see above), of whom detailed nerve conduction data were available. A p-value <0.05 was considered statistically significant. When appropriate, we corrected p-values for multiple testing using the Bonferroni method.

Results

Study population
We included 124 patients with MMN in the study. We previously assessed anti-GM1 and anti-GM2 titers with ELISA in serum samples from 87 patients collected during the 2007 cross-sectional study, and we used the same methodology for 37 additional patients in the present study, whose samples were collected during the 2015 cross-sectional study.[3,24,26] Clinical characteristics are summarized in Table 1. IgM anti-GM1 and anti-GM2 antibodies as detected by ELISA were present in 58% and 10% of the patients, respectively, while 39% was negative for either one of these antibodies. IgM anti-GM1 and IgM anti-GM2 titers did not correlate (Figure 1A; r_s=0.1099, *p*=0.2244), suggesting that these are two different types of antibodies rather than one cross-reacting antibody.

We next investigated specific clinical characteristics of patients with IgM anti-GM2. To this end, we stratified patients by the presence or absence of IgM anti-GM1 and GM2: IgM anti-GM2 positive, either or not with anti-GM1 (group A); IgM anti-GM1 positive and anti-GM2 negative (group B); and double negative (group C). We did not analyze patients with IgM anti-GM2 without GM1 antibodies (n=4) separately due to small numbers. Comparisons between groups are shown in Table 2.

Table 1. Baseline characteristics of 124 patients with MMN included in this study. A separate column shows the baseline characteristics of 98 patients with MMN of whom serum was available for SC IgM binding experiments. Comparisons were made between this group of 98 patients and the remainder of patients (n=26), the p-values of which are shown in the right column.

	MMN	**IgM binding experiments**		
	N=124	Serum available (n=98)	No serum available (n=26)	p
Male sex[$]	93 (75)	76 (77)	17 (65)	0.31
Age at onset (years)[#]	42 (16)	41 (13)	43 (24)	0.45
Diagnostic delay (years)[#]	6.8 (12)	7.5 (16)	5.7 (5.3)	0.22
MMN EFNS 2010 diagnosis[$]				0.69
Definite	88 (72)	68 (70)	20 (77)	
Probable	26 (21)	21 (22)	5 (19)	
Possible	9 (7)	8 (8)	1 (4)	
IgM Anti-GM1 positive[$]	72 (58)	52 (53)	20 (77)	0.049[*]
IgM Anti-GM2 positive[$]	13 (10)	11 (11)	2 (8)	0.60
IVIg treatment at sampling[$]	-	85 (87)	-	
IVIg dosage (grams/week)[#]	-	13	-	

[*]Statistically significant at p-value <0.05

[$]Values displayed as n (%)

[#]Values displayed as median (IQR)

SC Schwann cell, EFNS European federation of neurological societies, IVIg intravenous immunoglobulin, MMN Multifocal motor neuropathy

Table 2. Clinical parameters of patients with MMN (N=124), stratified by IgM anti-GM1 and anti-GM2 antibody status.

	Patient groups			**p-values**	
	(A) Anti-GM2 +	(B) Anti-GM1+	(C) Anti-GM2/GM1-	A vs. B	A vs. C
	N = 13	N = 63	N = 48		
IgM anti-GM1 IgM positive (n (%))	9 (69)	63 (100)	0 (0)	-	-
IgM anti-GM2 IgM positive (n (%))	13 (100)	0 (0)	0 (0)	-	-
Male sex (n (%))	10 (77)	47 (75)	36 (75)	1.00	1.00
EFNS MMN diagnosis (n (%))				0.88	0.54
Definite	10 (77)	42 (67)	36 (77)		
Probable	3 (23)	16 (25)	7 (15)		
Possible	0 (0)	5 (8)	4 (8)		
Age at onset (median years (IQR))	31 (16)	43 (13)	42 (14)	**0.015***	**0.034***
Inclusion MAIN 2007 (n (%))	12 (92)	50 (79)	25 (52)	-	-
Inclusion MAIN 2015 (n (%))	10 (77)	43 (68)	41 (85)	-	-
Inclusion MAIN 2007 & MAIN 2015 (n (%))	9 (69)	30 (47)	20 (42)	-	-
Disease duration (median months (IQR))					
At first UMCU visit	57 (96)	49 (80)	32 (63)	0.36	**0.037***
MAIN 2007	216 (120)	132 (141)	120 (96)	0.13	**0.042***
MAIN 2015	308 (170)	216 (182)	166 (122)	0.16	**0.008***
Nerve conduction studies at diagnosis					
Conduction block (n (%))	12 (93)	50 (93)	43 (96)	1.00	0.54
Definite conduction block (n (%))	9 (69)	34 (63)	36 (80)	0.76	0.46
Axonal damage (n (%))	7 (54)	24 (44)	13 (29)	0.55	0.11
Nerves with CB (median (range))	2 (0-9)	3 (0-12)	3 (0-14)	0.94	0.90
Nerves with definite CB (median (range))	1 (0-5)	1 (0-5)	1 (0-8)	0.68	0.84
Nerves with axonal damage (median (range))	1 (0-7)	0 (0-7)	0 (0-3)	0.73	0.31

IVIg treatment (n (%))					
At first UMCU visit	5 (42)	16 (25)	4 (9)	0.30	**0.015***
MAIN 2007	10 (83)	39 (78)	17 (68)	1.00	0.44
MAIN 2015	10 (100)	39 (91)	15 (75)	1.00	0.14
IVIg dosage (median gr/month (IQR))					
MAIN 2007	18 (9)	14 (8)	12 (7)	0.33	0.16
MAIN 2015	20 (8)	15 (10)	10 (7)	0.85	0.08
Monoclonal gammopathy (n (%))					
IgM	1 (8)	6 (10)	3 (7)	1.00	1.00
IgG	0 (0)	2 (3)	2 (5)	1.00	1.00
CSF studies performed (n (%))	2 (15)	15 (26)	15 (31)	0.72	0.48
CSF elevated protein level	2 (100)	15 (100)	9 (60)	1.00	0.51
CSF leukocytosis	0 (0)	0 (0)	1 (14)	-	-
Brachial plexus MRI abnormalities (n (%))	7 (78)	17 (45)	10 (33)	0.14	**0.026***
MRC sum score (median (IQR))					
At first UMCU visit	115 (4)	121 (9)	124 (8)	0.12	**0.0003***
ΔMRCss (MAIN 2007-2015)	-8 (9)	-11 (10)	-4 (7)	0.41	0.13
Postural tremor (n (%))	8 (80)	27 (64)	25 (63)	0.47	0.46
Sensory abnormalities (n (%))					
Hypesthesia/paresthesia	4 (33)	3 (6)	4 (16)	**0.023***	0.39
Vibration sense abnormalities	3 (25)	13 (26)	3 (12)	1.00	0.37

* Statistically significant at p-value <0.05

All analyses concerning IVIg dosage and MRC sum scores were corrected for disease duration. Analyses concerning the MRC sum score at patients' first visit to the UMCU were performed in treatment-naïve patients only.

CB = conduction block, CSF = cerebrospinal fluid, IVIg = intravenous immunoglobulins, MMN = multifocal motor neuropathy, MRCss = MRC sum score, MRI = magnetic resonance imaging

Table 3. Comparing detailed NCS data between patients with MMN with IgM anti-GM2 antibodies (n=7) and without (n=9).

CMAPs/SNAPs/F-waves		GM2 +	GM2 -	p	Velocities (m/s)	GM2 +	GM2 -	p
Median nerve								
DML		4.2 (0.65)	4.1 (0.83)	0.86	-	-	-	-
CMAP	Wrist	8.7 (4.2)	7.5 (11.3)	0.8	-	-	-	-
	Elbow	6.0 (7.2)	6.0 (7.8)	0.92	Forearm	48 (15.5)	51 (19.8)	0.85
	Axilla	2.9 (4.4)	6.0 (6.9)	0.46	Upper arm	50 (11)	55 (20.5)	0.40
	Erb's point	3.3 (6.4)	6.5 (6.5)	0.59	Erb's point	68 (23.8)	67 (10)	0.63
SNAP	Wrist	17.1 (13.2)	20.8 (15.9)	0.88	SNAP velocity	52 (3)	55 (7)	0.25
F-wave latency		35.1 (15.1)	33.2 (7.1)	0.70	-	-	-	-
Ulnar nerve								
DML		3.5 (1.1)	3.4 (0.53)	0.55	-	-	-	-
CMAP	Wrist	6.4 (3.0)	7.9 (9.7)	0.58	-	-	-	-
	Sulcus (distal)	2.9 (4.9)	7.8 (9.0)	0.58	Forearm	52 (25.8)	54 (9)	0.31
	Sulcus (proximal)	3.0 (5.5)	7.5 (9.0)	0.49	Sulcus	47 (12.3)	50 (12.3)	0.43
	Axilla	2.7 (4.7)	4.4 (6.9)	0.47	Upper arm	49 (25)	56 (33.5)	0.80
	Erb's point	1.9 (4.1)	4.5 (6.2)	0.42	Erb's point	63 (23)	64 (12)	0.42
SNAP	Wrist	25 (22.9)	10 (6.7)	**0.0074***	SNAP	51 (2.8)	48 (6.3)	0.38
F-wave latency		36.1 (26.9)	31.7 (7.3)	0.65	-	-	-	-
Radial nerve								
SNAP	Wrist	17 (15.4)	14.9 (13.5)	0.27	SNAP velocity	53 (4)	52 (4.3)	0.66

Deep peroneal nerve								
DML		4.1 (0.7)	5.1 (1.9)	**0.007**	-	-	-	-
CMAP	Ankle	3.6 (5.6)	3.7 (4.1)	0.98	-	-	-	-
	Fibular head (distal)	2.8 (4.4)	2.9 (4.2)	0.63	Lower leg	46 (7)	42 (6)	0.26
	Fibular head (proximal)	2.7 (3.7)	2.8 (4.1)	0.63	Fibular head	46 (5)	47 (6)	0.79
F-wave latency		57.3 (11.9)	59.7 (9.3)	0.24	-	-	-	-
Tibial nerve								
DML		5.1 (1.0)	4.9 (1.8)	0.65	-	-	-	-
CMAP	Ankle	6.5 (6.2)	9 (8.2)	0.65	-	-	-	-
	Knee	3.4 (5.4)	4.4 (6.2)	0.59	Lower leg	44 (4)	45 (2)	1.00
F-wave latency		58.9 (18)	60.3 (5.0)	0.89	-	-	-	-
Sural nerve								
SNAP		9 (3.5)	12 (9.5)	0.52	SNAP velocity	46 (8)	48 (7)	0.28

* Statistically significant at p-value <0.05

CMAP Compound muscle action potential, DML Distal motor latency, MMN Multifocal motor neuropathy, NCS Nerve conduction studies, SNAP Sensory nerve action potential

Patients with IgM anti-GM2 had a significantly lower age at onset of disease, with a median difference of 12 years. Patients with IgM anti-GM2 antibodies had significantly lower MRCss than patients without antibodies, but this difference must probably be attributed to the concomitance of IgM anti-GM1, which was associated with more pronounced weakness in a previous study.[3] Indeed, comparison of patients with only IgM anti-GM1 antibodies and those without antibodies showed significantly lower MRCss at their first visit to the UMC Utrecht (p=0.046).[4,24] Finally, reported sensory symptoms such as hypesthesia or paresthesia were more frequent in the group with IgM anti-GM2 (group A) (33% vs. 6%, p=0.023).
Finally, we compared available nerve conduction studies performed at diagnosis of 16 patients with MMN, of whom 7 had IgM anti-GM2 antibodies. Results are shown in Table 3. There were no differences in nerve conduction velocities of motor nerves between patients with or without IgM anti-GM2 antibodies. Patients with IgM anti-GM2 antibodies did not have lower sensory nerve action potential (SNAP) amplitudes of altered sensory conduction velocities as compared to patients without IgM anti-GM2 antibodies. F-wave latencies in all investigated nerves were comparable between groups.

IgM anti-GM2 antibodies from patients with MMN bind to SCs.
To investigate binding of IgM anti-GM1 or -GM2 to SCs and iPSC-MNs, we used 98 available sera. Apart from a minor difference in IgM anti-GM1 positivity, clinical characteristics of these patients were similar to those of the other 26 patients (Table 1). FC of IgM antibody against SCs in MMN patient's sera varied between 0.28 and 74.54. Moreover, IgM anti-SC FC differed from IgM anti-iPSC-MNs measured and expressed in a similar way (Figure 1B).
Next, we investigated differences in IgM binding to SCs and iPSC-MNs among patients stratified by their IgM anti-GM1/2 status as determined with ELISA. Regarding IgM binding to SCs, we observed a significantly higher FC for patients positive for anti-GM2 compared to anti-GM2 negative patients. FC of IgM did not differ between patients with or without IgM anti-GM1 (Figure 1C, and Supplemental Figure 1). In contrast, binding of IgM to the iPSC-MNs was significantly higher in the patients who had IgM anti-GM1 antibodies compared to patients negative for these antibodies (Figure 1D). FC on SCs moderately correlated with IgM anti-GM2 titers measured with ELISA (r_s = 0.4983, p < 0.0001), but not with IgM anti-GM1 titers (Figure 2A and B).

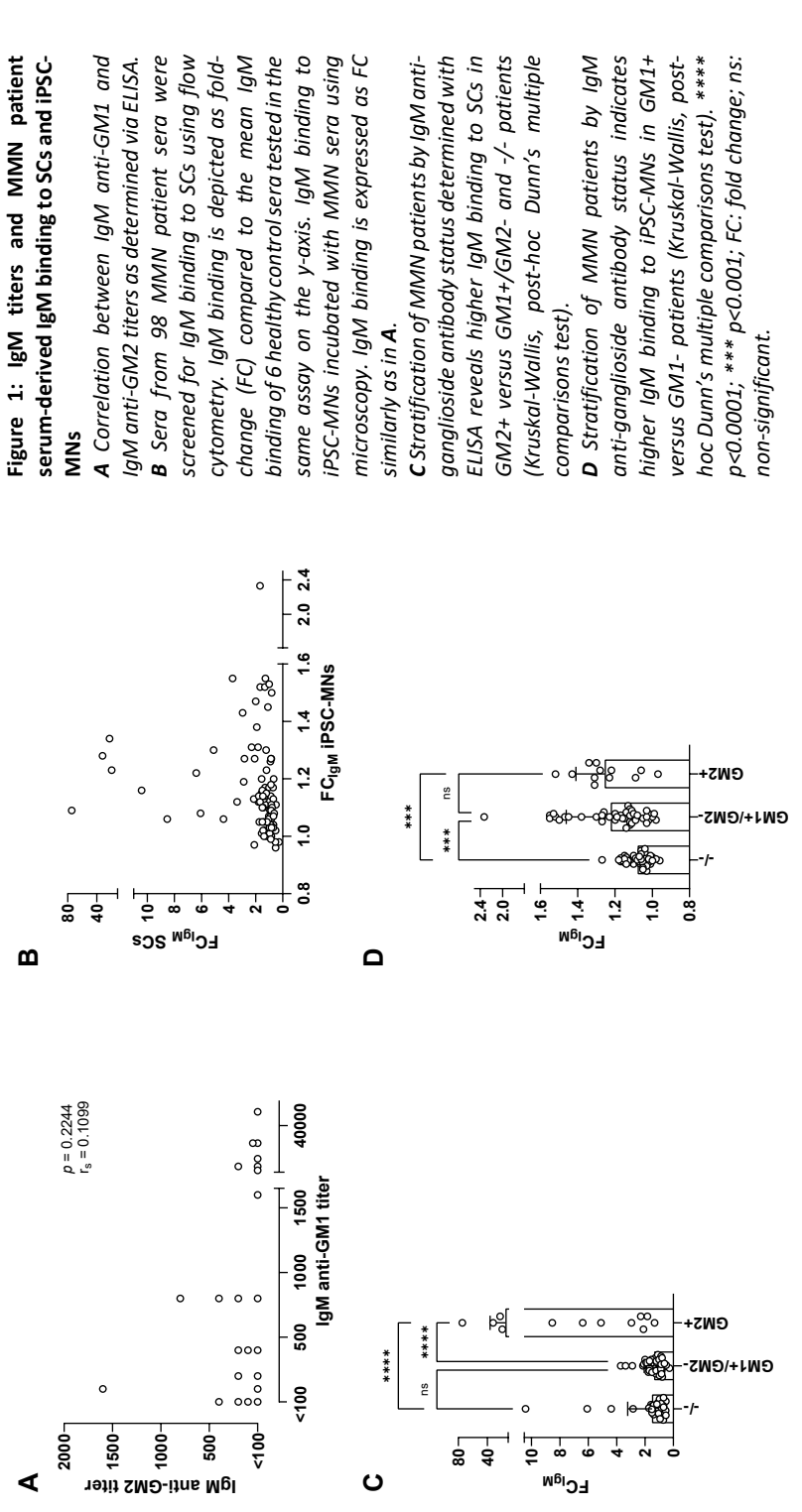


Figure 1: IgM titers and MMN patient serum-derived IgM binding to SCs and iPSC-MNs

A *Correlation between IgM anti-GM1 and IgM anti-GM2 titers as determined via ELISA.* ***B*** *Sera from 98 MMN patient sera were screened for IgM binding to SCs using flow cytometry. IgM binding is depicted as fold-change (FC) compared to the mean IgM binding of 6 healthy control sera tested in the same assay on the y-axis. IgM binding to iPSC-MNs incubated with MMN sera using microscopy. IgM binding is expressed as FC similarly as in* ***A****.*

C *Stratification of MMN patients by IgM anti-ganglioside antibody status determined with ELISA reveals higher IgM binding to SCs in GM2+ versus GM1+/GM2- and -/- patients (Kruskal-Wallis, post-hoc Dunn's multiple comparisons test).*

D *Stratification of MMN patients by IgM anti-ganglioside antibody status indicates higher IgM binding to iPSC-MNs in GM1+ versus GM1- patients (Kruskal-Wallis, post-hoc Dunn's multiple comparisons test). **** p<0.0001; *** p<0.001; FC: fold change; ns: non-significant.*

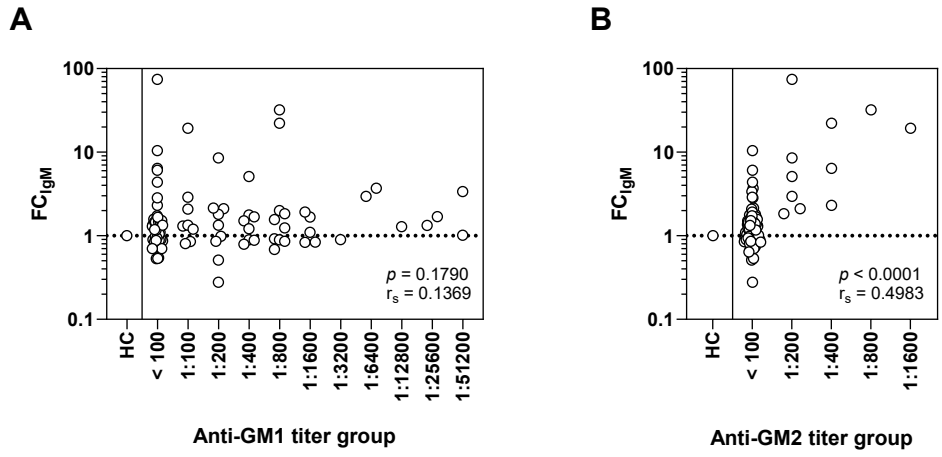


Figure 2: IgM binding to SCs stratified by IgM anti-GM2 and anti-GM1 antibody titer
A *IgM binding to SCs stratified for IgM anti-GM1 titer. No correlation was found between* FC_{IgM} *and IgM anti-GM1 titers (Spearman's rho* r_s*=0.1369, p=0.1790).*
B *IgM binding to SCs stratified for IgM anti-GM2 antibody titer group shows a moderate correlation between IgM binding (*FC_{IgM}*) and anti-GM2 titer (Spearman's rho* r_s*=0.4983, p<0.0001). FC: fold change.*

IgM binding of MMN patients to SCs is GM2 specific

To confirm anti-GM2 antibody specific binding to SCs, we pre-incubated MMN sera positive for anti-GM2 IgM with soluble GM1 and GM2 and tested residual IgM binding to membrane bound or solid-phase GM2 using flow cytometry and a GM2-specific ELISA. Pre-incubation of anti-GM2-positive MMN patient serum with soluble GM1 resulted in a modest reduction in FC of IgM binding to SCs (Figure 3A), whilst pre-incubation with soluble GM2 strongly reduced residual FC IgM binding (Figure 3B). When plotted as % inhibition of IgM binding, setting the non-treated serum sample at 0% inhibition, we observed a significant inhibition of IgM binding to SCs upon pre-incubation of the IgM anti-GM2 positive sera with soluble GM2. Under the same conditions, we did not observe significant inhibition upon pre-incubation of these sera with soluble GM1 (Figure 3C). Upon preincubation of anti-GM2-positive MMN patient sera with soluble GM1, OD_{450nm} did not decrease, whereas pre-incubation with soluble GM2 resulted in a significant inhibition of IgM binding (Figures 3D and E) thereby confirming the flow cytometry results and specificity of the IgM antibodies towards GM2 expressed on SCs.

IgM anti-GM2 binding on SCs results in complement activation

Complement activation by IgM anti-GM1 antibody bound to MNs is probably a major mechanism in the pathogenesis of MMN (3, 10, 16). We therefore investigated whether IgM anti-GM2 antibody binding induced complement activation on SCs. SCs were incubated with HI sera from patients with MMN either or not positive for IgM anti-GM2, and with fresh pooled human serum as a source of active complement. Since the majority of IgM anti-GM2 positive

sera also contained IgM anti-GM1, we compared sera with IgM anti-GM2 to sera without anti-GM2 IgM, with and without IgM anti-GM1. A detailed overview of antiganglioside antibody titers for each patient can be found in Supplemental Table 1. Results were expressed similarly as for IgM binding (Figure 4A). C3 fixation (Figure 4B) to SCs opsonized with IgM anti-GM2 positive patient sera was significantly increased compared to that seen with IgM anti-GM2-negative sera (Figure 4C). Moreover, FC_{IgM} and FC_{C3} correlated strongly (Figure 4D).

We further assessed complement activation on SCs in culture using membrane-bound and soluble complement activation markers as read-out. We confirmed C3 fixation upon opsonization of SCs with IgM anti-GM2 positive patient sera and complement active serum microscopically. The level of C3 fixation observed on opsonized SCs following incubation with HI serum was similar to the non-opsonized serum control. Both pre-incubation of complement active serum with an irrelevant control antibody or an anti-C5 antibody did not decrease C3 fixation (representative microscopic images depicted in Figure 5A, quantified as FC to the non-opsonized serum control for multiple experiments in Figure 5B). To assess down-stream complement activation, we measured C5a in the culture medium of opsonized and complement-exposed SCs. C5a increased upon the addition of complement active serum to opsonized SCs. This increase was significantly inhibited by pre-treating the complement active serum with an anti-C5 antibody which reduced C5a levels to those observed in the non-opsonized serum control (Figure 5C).

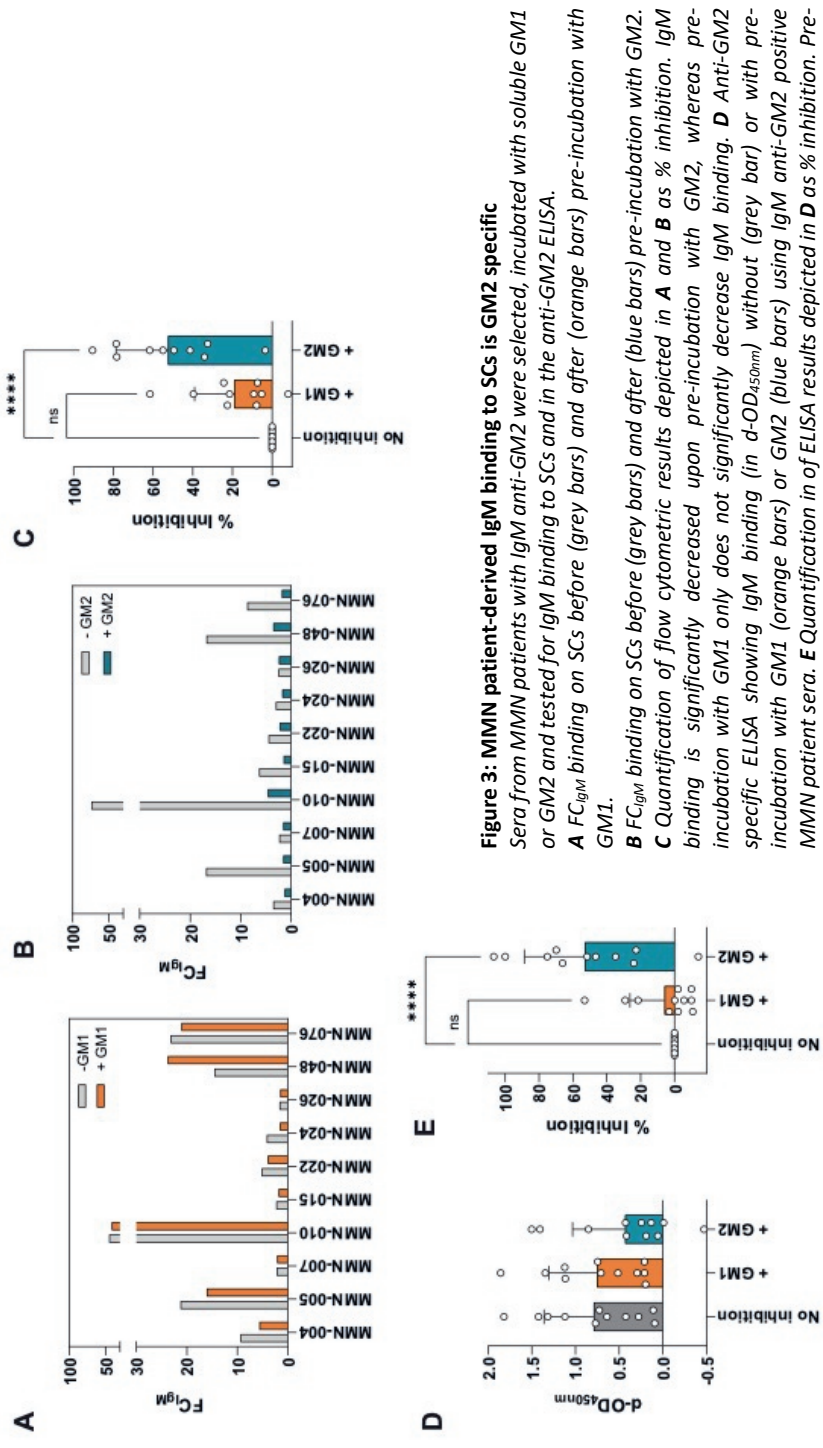


Figure 3: MMN patient-derived IgM binding to SCs is GM2 specific

Sera from MMN patients with IgM anti-GM2 were selected, incubated with soluble GM1 or GM2 and tested for IgM binding to SCs and in the anti-GM2 ELISA.

A *FC_{IgM} binding on SCs before (grey bars) and after (orange bars) pre-incubation with GM1.*

B *FC_{IgM} binding on SCs before (grey bars) and after (blue bars) pre-incubation with GM2.*

C *Quantification of flow cytometric results depicted in **A** and **B** as % inhibition. IgM binding is significantly decreased upon pre-incubation with GM2, whereas pre-incubation with GM1 only does not significantly decrease IgM binding.* ***D*** *Anti-GM2 specific ELISA showing IgM binding (in d-OD_{450nm}) without (grey bar) or with pre-incubation with GM1 (orange bars) or GM2 (blue bars) using IgM anti-GM2 positive MMN patient sera.* ***E*** *Quantification in of ELISA results depicted in **D** as % inhibition. Pre-incubation with GM2 significantly lowers IgM binding.*

*Mean + SD, Kruskal-Wallis, post-hoc Dunn's multiple comparisons test. **** p<0.0001; ns: non-significant.*

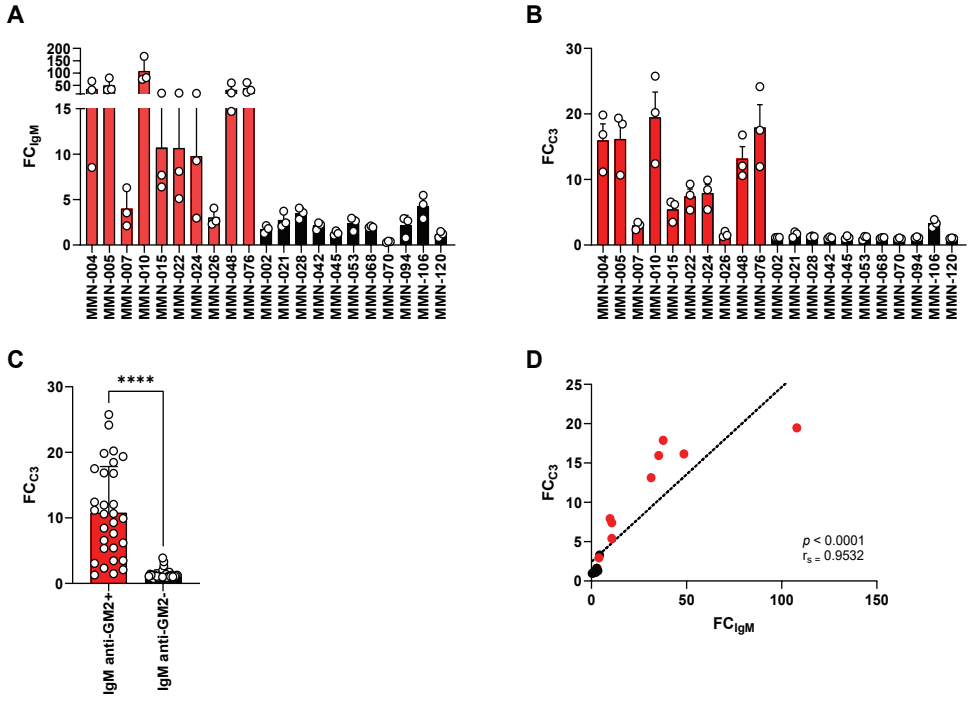


Figure 4: IgM anti-GM2 binding on SCs results in complement activation

*IgM binding (FC_{IgM}, **A**) and C3 fixation (FC_{C3}, **B**) to SCs opsonized with IgM anti-GM2 positive (red bars) or negative (black bars) MMN patient serum and incubated with fresh serum as complement source. Opsonization with anti-GM2 MMN patient serum results in increased IgM binding and increased complement activation. Data are mean + SD of different assays **C** Pooled results of FC_{C3} data depicted in **B**, Mann-Whitney test. **D** Highly significant strong correlation between IgM anti-GM2 binding to SCs and subsequent complement activation. Red dots IgM anti-GM2+ sera, black dots IgM anti-GM2- sera. Mean + SD, Spearman's rho r_s=0.9532, p<0.0001. **** p<0.0001; FC: fold change.*

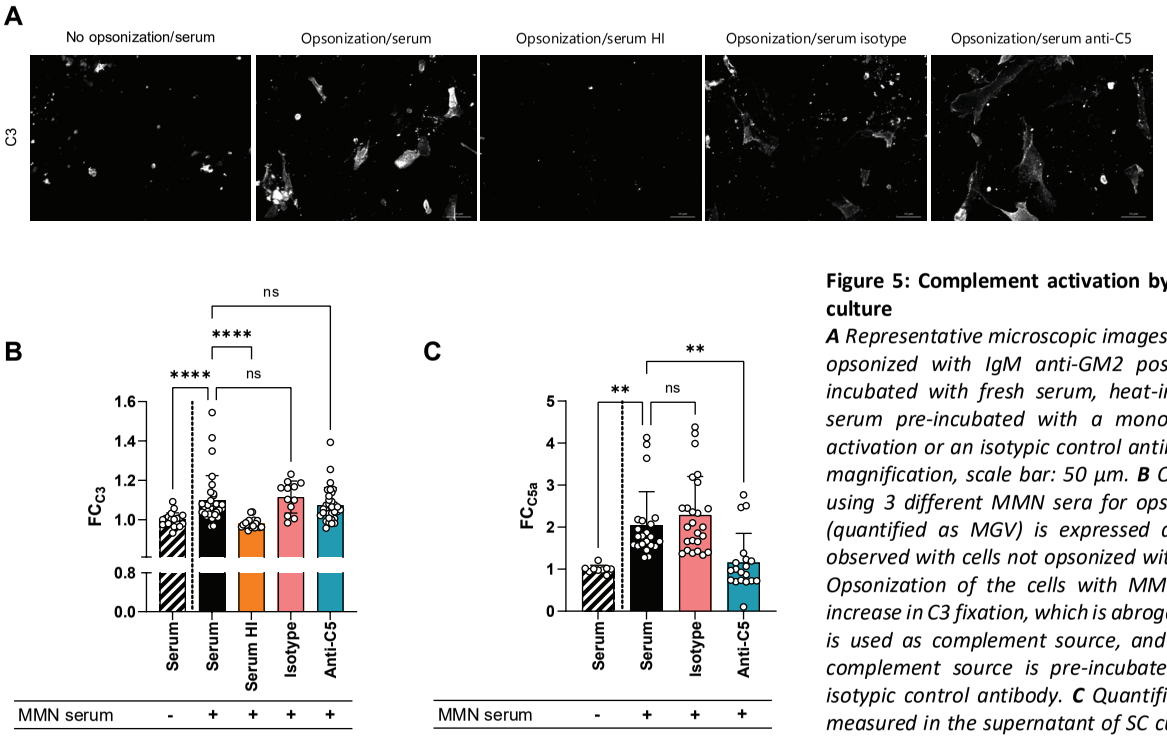


Figure 5: Complement activation by IgM anti-GM2 bound to SCs in culture

A *Representative microscopic images of complement activation on SCs opsonized with IgM anti-GM2 positive MMN serum or not, and incubated with fresh serum, heat-inactivated serum (serum HI), or serum pre-incubated with a monoclonal antibody that blocks C5 activation or an isotypic control antibody, as complement source. 20x magnification, scale bar: 50 µm.* ***B*** *Quantification of microscopy data using 3 different MMN sera for opsonization.C3 fixation to the cells (quantified as MGV) is expressed as FC (FC_{C3}) setting the fixation observed with cells not opsonized with MMN serum (striped bar) as 1. Opsonization of the cells with MMN serum results in a significant increase in C3 fixation, which is abrogated when heat inactivated serum is used as complement source, and which is not reduced when the complement source is pre-incubated with anti-C5 antibody, or an isotypic control antibody.* ***C*** *Quantification of C5a, depicted as FC_{C5a}, measured in the supernatant of SC cultures. C5a generation in culture medium is increased upon opsonization of the cells with IgM anti-GM2 positive MMN patient serum and incubation with fresh serum. This C5a generation is inhibited by an anti-C5 antibody added to the complement source but not by a control antibody. Mean + SD, Kruskal-Wallis, post-hoc Dunn's multiple comparisons test, ** p<0.01; **** p<0.0001; FC: fold change; HI: heat inactivated; MMN: multifocal motor neuropathy; ns: non-significant.*

Discussion

In this study, we show that 10% of 124 patients with MMN have circulating IgM antibodies against the ganglioside GM2, which is mainly expressed on SCs. Disease symptoms of this subgroup of patients, some of whom did not have detectable IgM anti-GM1 antibodies, were indiscernible from those of the other MMN patients, except for an earlier onset of muscle weakness, and the presence of subjective sensory disturbances. Our findings suggest a pathogenic role of IgM anti-GM2 antibodies and involvement of SCs in at least a subgroup of patients.

Gangliosides are a group of sialic acid containing glycosphingolipids that are expressed in the plasma membrane of cells of both the peripheral and central nervous system, and by ensuring myelin integrity contribute to optimal saltatory conduction.[11,13] GM1, GD1a, GD1b and GT1b are the predominant gangliosides in neural tissue. GM1 is localized around the nodal axolemma and nodal Schwann cell membranes. GM2 is less abundant than other gangliosides. On peripheral nerves, GM2 is localized at the abaxonal site of SCs, and to a lesser extent on the abaxonal membranes and the axonal area.[20,27-29] Here, we show that IgM anti-GM2 antibodies in sera from MMN patients specifically bind to GM2 on a SC line.

GM1 and GM2 are structurally related and differ in only one galactose residue with is added to GM1 during its synthesis from GM2.[30] Although this structural similarity between GM1 and GM2 raises the possibility of cross-reactivity of IgM anti-GM1 antibodies with GM2, and vice versa, our results show that binding is likely to be specific and not cross-reactive.[3] Both in ELISA as well as in the SC model, binding of IgM from most patients with anti-GM2 antibodies was inhibited by pre-incubation with soluble GM2 and not, or only minimally, by soluble GM1. In order to confirm target-specificity, antibody isolation and sequence analysis is key to understand the complex interaction between IgM anti-ganglioside antibodies and their respective targets.[31]

The prevalence of IgM antibodies against GM2 of 10% in our large MMN cohort is in line with the 6-10% found in previous studies.[3,21,32,33] IgM anti-GM2 antibodies have been described throughout the spectrum of immune-mediated neuropathies, including Guillain-Barré syndrome (GBS) and its acute motor axonal variants, chronic inflammatory demyelinating polyneuropathy, and sensory demyelinating neuropathy with ataxia.[20,21,32,34-38] IgG anti-GM2 antibodies are a biomarker for immune-mediated polyneuropathies in cats.[39] Interestingly, the presence of IgM anti-GM2 antibodies is associated with preceding cytomegalovirus (CMV) infection in patients with GBS.[40] The relationship between MMN susceptibility and preceding CMV infections is unknown, but, given the chronic course, not likely.

We found IgM anti-GM2 antibodies to be associated with earlier onset of muscle weakness. Interestingly, IgM anti-GM2 antibodies have been reported in case-reports of children diagnosed with MMN, and in an Indian cohort of childhood-onset GBS.[35,41,42] In a selected subgroup of patients with MMN whose samples were included in the complement activation assays (see Fig. 4), the presence of IgM anti-GM2 antibodies was also associated with the presence of subjective sensory complaints but normal sensory nerve conduction studies. Whether sensory complaints in the group with IgM anti-GM2 antibodies are a mere reflection of longer disease duration, or reflect a specific pathological effect of IgM anti-GM2 antibodies

on sensory neurons remains to be determined.[4,23,24,43] Importantly, patients with MMN with IgM anti-GM2 antibodies had similar disease characteristics, response to IVIg treatment, and disease trajectories as patients without these antibodies, including the 4 patients with only IgM anti-GM2 antibodies, indicating that the presence of IgM anti-GM1 antibodies is not a prerequisite in MMN. This suggests that IgM anti-GM1 and anti-GM2 antibodies trigger a similar pathological mechanism.

Although IgM anti-GM2-induced complement-mediated cytotoxicity has been described previously using a neuroblastoma cell line that expresses GM2, we did not observe lysis of SCs upon complement activation by bound IgM anti-GM antibodies.[20,44] This is presumably due to the protective effects of membrane complement regulatory proteins, including CD59, which we also found to be highly expressed in our SC model.[14,45-47] We hypothesize that the contribution of complement activation by IgM anti-GM2 antibodies bound to SCs in MMN pathology is the deposition of other complement components than the membrane attack complex and the production of soluble activation products. We detected C5a generation by IgM bound to SCs in supernatants (Figure 5). Similar mechanisms may be relevant for MNs, which are also well protected by complement regulatory membrane proteins.[14] Receptors for C3a and C5a are expressed by motor neurons and glial cells and their engagement results in increased inflammation.[14,48] SCs upon stimulation produce inflammatory cytokines, including IL-1β, IL-6, and TNF-α, which could amplify immune activation and inflammation.[49-51] The importance of crosstalk was suggested in an *in vitro* model where SCs were activated by neurons upon complement-activation by an anti-GQ1b antibody.[52] Therefore, we postulate that the pathologic mechanism shared by IgM anti-GM1 bound to MNs and anti-GM2 antibodies bound to SCs underlying MMN is the generation and deposition of upstream complement activation products. Generation of these activation products could induce an inflammatory interplay between MNs and SCs resulting in MN dysfunction and thickening of affected nerves.

We acknowledge that the use of two separate cell lines is a limitation of this study, as is the non-myelinating nature of the SC line, since this could affect the ganglioside distribution in comparison to myelinated nerve tissue. Additionally, there could be a difference in overall ganglioside distribution between the sNF96.2 Schwann cell line and Schwann cells in patients. Therefore, it is important to ultimately reproduce some of the key findings using primary SCs or nerve tissue. Nevertheless, the current model allowed us to investigate anti-ganglioside antibody interactions and subsequent immunological effector mechanisms in more detail than before.

In conclusion, we show that IgM anti-GM2 antibodies that target SCs are found in 10% of patients with MMN, sometimes in the absence of detectable anti-GM1 antibodies. Anti-GM2 antibodies are associated with a clinical phenotype of MMN that except for an early onset is indiscernible from the disease associated with anti-GM1 antibodies, suggesting a common pathogenic mechanism shared by either type of antibody. We postulate that this mechanism includes the generation of fluid-phase complement activation products that interact with receptors on SCs and MNs.

References

1. Beadon K, Guimaraes-Costa R, Leger JM. Multifocal motor neuropathy. Curr Opin Neurol. 2018;31(5):559-64.

2. Nobile-Orazio E. Multifocal motor neuropathy. J Neuroimmunol. 2001;115(1-2):4-18.

3. Cats EA, Jacobs BC, Yuki N, Tio-Gillen AP, Piepers S, Franssen H, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology. 2010;75(22):1961-7.

4. Herraets I, van Rosmalen M, Bos J, van Eijk R, Cats E, Jongbloed B, et al. Clinical outcomes in multifocal motor neuropathy: A combined cross-sectional and follow-up study. Neurology. 2020;95(14):e1979-e87.

5. Pestronk A. Multifocal motor neuropathy: diagnosis and treatment. Neurology. 1998;51(6 Suppl 5):S22-4.

6. Hahn AF, Beydoun SR, Lawson V, Team IiMS, Oh M, Empson VG, et al. A controlled trial of intravenous immunoglobulin in multifocal motor neuropathy. J Peripher Nerv Syst. 2013;18(4):321-30.

7. Goedee HS, van der Pol WL, Hendrikse J, van den Berg LH. Nerve ultrasound and magnetic resonance imaging in the diagnosis of neuropathy. Curr Opin Neurol. 2018;31(5):526-33.

8. Herraets IJT, Goedee HS, Telleman JA, van Eijk RPA, Verhamme C, Saris CGJ, et al. Nerve ultrasound for diagnosing chronic inflammatory neuropathy: A multicenter validation study. Neurology. 2020;95(12):e1745-e53.

9. Jongbloed BA, Haakma W, Goedee HS, Bos JW, Bos C, Hendrikse J, et al. Comparative study of peripheral nerve Mri and ultrasound in multifocal motor neuropathy and amyotrophic lateral sclerosis. Muscle Nerve. 2016;54(6):1133-5.

10. Harschnitz O, van den Berg LH, Johansen LE, Jansen MD, Kling S, Vieira de Sa R, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol. 2016;80(1):71-88.

11. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain. 2002;125(Pt 12):2591-625.

12. Yeh WZ, Dyck PJ, van den Berg LH, Kiernan MC, Taylor BV. Multifocal motor neuropathy: controversies and priorities. J Neurol Neurosurg Psychiatry. 2020;91(2):140-8.

13. Vlam L, van der Pol WL, Cats EA, Straver DC, Piepers S, Franssen H, van den Berg LH. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol. 2011;8(1):48-58.

14. Budding K, Johansen LE, Van de Walle I, Dijkxhoorn K, de Zeeuw E, Bloemenkamp LM, et al. Anti-C2 Antibody ARGX-117 Inhibits Complement in a Disease Model for Multifocal Motor Neuropathy. Neurol Neuroimmunol Neuroinflamm. 2022;9(1).

15. Van Asseldonk JT, Van den Berg LH, Kalmijn S, Van den Berg-Vos RM, Polman CH, Wokke JH, Franssen H. Axon loss is an important determinant of weakness in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry. 2006;77(6):743-7.

16. Vlam L, Cats EA, Harschnitz O, Jansen MD, Piepers S, Veldink JH, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm. 2015;2(4):e119.

17. Piepers S, Jansen MD, Cats EA, van Sorge NM, van den Berg LH, van der Pol WL. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol. 2010;229(1-2):256-62.

18. van Schaik IN, Bossuyt PM, Brand A, Vermeulen M. Diagnostic value of GM1 antibodies in motor neuron disorders and neuropathies: a meta-analysis. Neurology. 1995;45(8):1570-7.

19. Pestronk A, Chuquilin M, Choksi R. Motor neuropathies and serum IgM binding to NS6S heparin disaccharide or GM1 ganglioside. J Neurol Neurosurg Psychiatry. 2010;81(7):726-30.

20. O'Hanlon GM, Veitch J, Gallardo E, Illa I, Chancellor AM, Willison HJ. Peripheral neuropathy associated with anti-GM2 ganglioside antibodies: clinical and immunopathological studies. Autoimmunity. 2000;32(2):133-44.

21. Nobile-Orazio E, Giannotta C, Musset L, Messina P, Leger JM. Sensitivity and predictive value of anti-GM1/galactocerebroside IgM antibodies in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry. 2014;85(7):754-8.

22. Martinez-Thompson JM, Snyder MR, Ettore M, McKeon A, Pittock SJ, Roforth MM, et al. Composite ganglioside autoantibodies and immune treatment response in MMN and MADSAM. Muscle Nerve. 2018;57(6):1000-5.

23. Joint Task Force of the E, the PNS. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripher Nerv Syst. 2010;15(4):295-301.

24. Cats EA, van der Pol WL, Piepers S, Franssen H, Jacobs BC, van den Berg-Vos RM, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology. 2010;75(9):818-25.

25. Bos JW, Groen EJN, Wadman RI, Curial CAD, Molleman NN, Zegers M, et al. SMN1 Duplications Are Associated With Progressive Muscular Atrophy, but Not With Multifocal Motor Neuropathy and Primary Lateral Sclerosis. Neurol Genet. 2021;7(4):e598.

26. Kuijf ML, van Doorn PA, Tio-Gillen AP, Geleijns K, Ang CW, Hooijkaas H, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci. 2005;239(1):37-44.

27. Rupp A, Galban-Horcajo F, Bianchi E, Dondi M, Penderis J, Cappell J, et al. Anti-GM2 ganglioside antibodies are a biomarker for acute canine polyradiculoneuritis. J Peripher Nerv Syst. 2013;18(1):75-88.

28. Chiba A, Kusunoki S, Obata H, Machinami R, Kanazawa I. Ganglioside composition of the human cranial nerves, with special reference to pathophysiology of Miller Fisher syndrome. Brain Res. 1997;745(1-2):32-6.

29. Svennerholm L, Bostrom K, Fredman P, Jungbjer B, Lekman A, Mansson JE, Rynmark BM. Gangliosides and allied glycosphingolipids in human peripheral nerve and spinal cord. Biochim Biophys Acta. 1994;1214(2):115-23.

30. Regier DS, Proia RL, D'Azzo A, Tifft CJ. The GM1 and GM2 Gangliosidoses: Natural History and Progress toward Therapy. Pediatr Endocrinol Rev. 2016;13 Suppl 1(Suppl 1):663-73.

31. Paterson G, Wilson G, Kennedy PG, Willison HJ. Analysis of anti-GM1 ganglioside IgM antibodies cloned from motor neuropathy patients demonstrates diverse V region gene usage with extensive somatic mutation. J Immunol. 1995;155(6):3049-59.

32. Nobile-Orazio E, Gallia F, Terenghi F, Allaria S, Giannotta C, Carpo M. How useful are anti-neural IgM antibodies in the diagnosis of chronic immune-mediated neuropathies? J Neurol Sci. 2008;266(1-2):156-63.

33. Galban-Horcajo F, Vlam L, Delmont E, Halstead SK, van den Berg L, van der Pol WL, Willison HJ. The Diagnostic Utility of Determining Anti-GM1: GalC Complex Antibodies in Multifocal Motor Neuropathy: A Validation Study. J Neuromuscul Dis. 2015;2(2):157-65.

34. Kim JK, Kim YH, Yoon BA, Cho JY, Oh SY, Shin HY, et al. Clinical Heterogeneity of Anti-GM2-Ganglioside-Antibody Syndrome. J Clin Neurol. 2018;14(3):401-6.

35. Kannan MA, Ch RK, Jabeen SA, Mridula KR, Rao P, Borgohain R. Clinical, electrophysiological subtypes and antiganglioside antibodies in childhood Guillain-Barre syndrome. Neurol India. 2011;59(5):727-32.

36. Fan C, Jin H, Hao H, Gao F, Sun Y, Lu Y, et al. Anti-ganglioside antibodies in Guillain-Barre syndrome and chronic inflammatory demyelinating polyneuropathy in Chinese patients. Muscle Nerve. 2017;55(4):470-5.

37. Lopate G, Choksi R, Pestronk A. Severe sensory ataxia and demyelinating polyneuropathy with IgM anti-GM2 and GalNAc-GD1A antibodies. Muscle Nerve. 2002;25(6):828-36.

38. Stork AC, van der Pol WL, Franssen H, Jacobs BC, Notermans NC. Clinical phenotype of patients with neuropathy associated with monoclonal gammopathy: a comparative study and a review of the literature. J Neurol. 2014;261(7):1398-404.

39. Halstead SK, Jackson M, Bianchi E, Rupp S, Granger N, Menchetti M, et al. Serum anti-GM2 and anti-GalNAc-GD1a ganglioside IgG antibodies are biomarkers for immune-mediated polyneuropathies in cats. J Peripher Nerv Syst. 2023;28(1):32-40.

40. Jacobs BC, van Doorn PA, Groeneveld JH, Tio-Gillen AP, van der Meche FG. Cytomegalovirus infections and anti-GM2 antibodies in Guillain-Barre syndrome. J Neurol Neurosurg Psychiatry. 1997;62(6):641-3.

41. Maeda H, Ishii R, Kusunoki S, Chiyonobu T. Childhood-onset multifocal motor neuropathy with IgM antibodies to GM2 and GalNac-GD1a. Brain Dev. 2020;42(1):88-92.

42. Ishigaki H, Hiraide T, Miyagi Y, Hayashi T, Matsubayashi T, Shimoda A, et al. Childhood-Onset Multifocal Motor Neuropathy With Immunoglobulin M Antibodies to Gangliosides GM1 and GM2: A Case Report and Review of the Literature. Pediatr Neurol. 2016;62:51-7.

43. Lambrecq V, Krim E, Rouanet-Larriviere M, Lagueny A. Sensory loss in multifocal motor neuropathy: a clinical and electrophysiological study. Muscle Nerve. 2009;39(2):131-6.

44. Cavanna B, Carpo M, Pedotti R, Scarpini E, Meucci N, Allaria S, et al. Anti-GM2 IgM antibodies: clinical correlates and reactivity with a human neuroblastoma cell line. J Neuroimmunol. 1999;94(1-2):157-64.

45. Vedeler C, Ulvestad E, Bjorge L, Conti G, Williams K, Mork S, Matre R. The expression of CD59 in normal human nervous tissue. Immunology. 1994;82(4):542-7.

46. Koski CL, Estep AE, Sawant-Mane S, Shin ML, Highbarger L, Hansch GM. Complement regulatory molecules on human myelin and glial cells: differential expression affects the deposition of activated complement proteins. J Neurochem. 1996;66(1):303-12.

47. Karbian N, Eshed-Eisenbach Y, Zeibak M, Tabib A, Sukhanov N, Vainshtein A, et al. Complement-membrane regulatory proteins are absent from the nodes of Ranvier in the peripheral nervous system. J Neuroinflammation. 2023;20(1):245.

48. Davoust N, Jones J, Stahel PF, Ames RS, Barnum SR. Receptor for the C3a anaphylatoxin is expressed by neurons and glial cells. Glia. 1999;26(3):201-11.

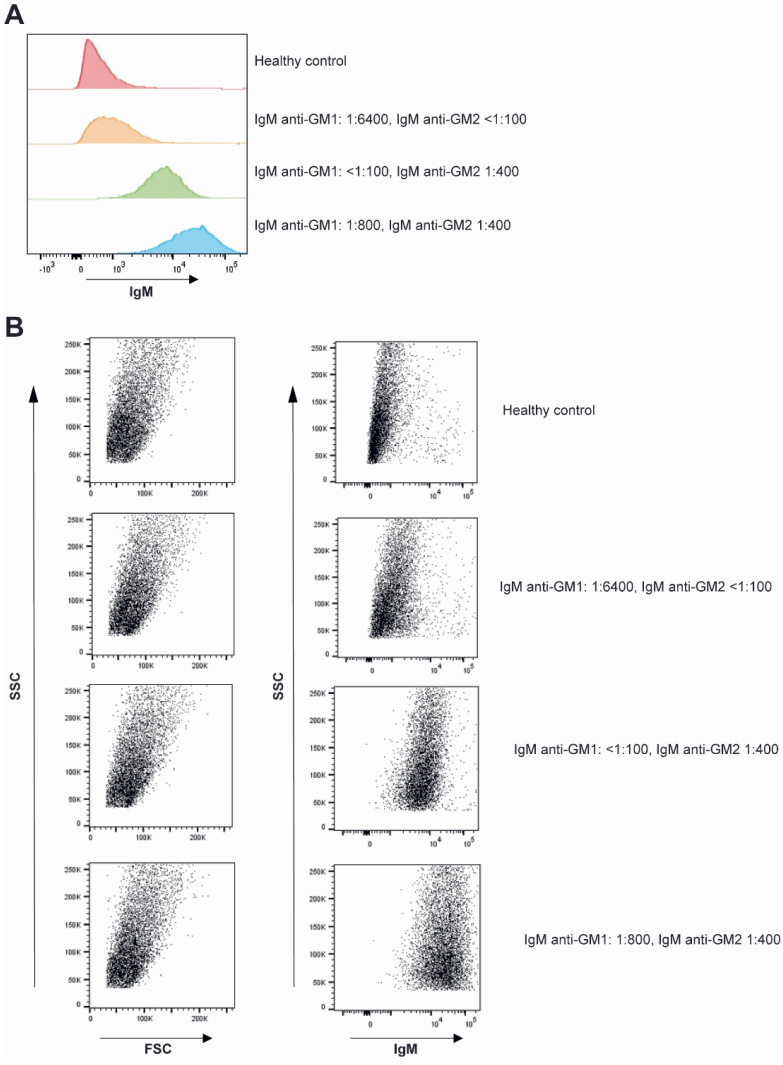
49. Meyer zu Horste G, Hu W, Hartung HP, Lehmann HC, Kieseier BC. The immunocompetence of Schwann cells. Muscle Nerve. 2008;37(1):3-13.

50. Ydens E, Lornet G, Smits V, Goethals S, Timmerman V, Janssens S. The neuroinflammatory role of Schwann cells in disease. Neurobiol Dis. 2013;55:95-103.

51. Van Rhijn I, Van den Berg LH, Bosboom WM, Otten HG, Logtenberg T. Expression of accessory molecules for T-cell activation in peripheral nerve of patients with CIDP and vasculitic neuropathy. Brain. 2000;123 (Pt 10):2020-9.

52. Rodella U, Negro S, Scorzeto M, Bergamin E, Jalink K, Montecucco C, et al. Schwann cells are activated by ATP released from neurons in an in vitro cellular model of Miller Fisher syndrome. Dis Model Mech. 2017;10(5):597-603.

Supplementary material



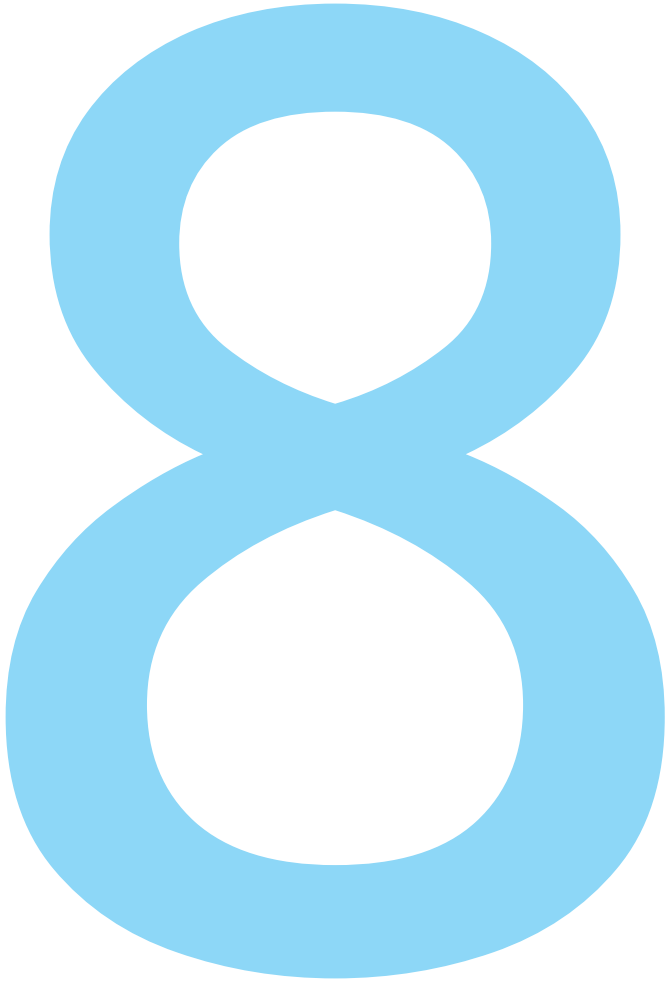
Supplemental Figure 1: Flow cytometry based analysis of IgM antibody binding to Schwann cells

A Schwann cells were incubated with healthy control or MMN patient sera. Respective overview of IgM binding intensities after opsonization with healthy control serum (red), MMN serum positive for IgM anti-GM1 and negative for IgM anti-GM2 (orange), MMN serum negative for IgM anti-GM1 and positive for IgM anti-GM2 (green), and positive for both IgM anti-GM1 and anti-GM2 (blue). **B** Forward scatter (FSC)/sideward scatter (SSC) and IgM/SSC plots of data depicted in **A**.

Supplemental Table 1. Anti-ganglioside antibody titer of MMN patients used in complement activation assays

Patient	IgM anti-GM1 titer	IgM anti-GM2 titer
MMN-004	200	200
MMN-005	800	800
MMN-007	200	200
MMN-010	<100	200
MMN-015	<100	400
MMN-022	400	200
MMN-024	6400	200
MMN-026	<100	400
MMN-048	100	1600
MMN-076	800	400
MMN-002	12800	<100
MMN-021	6400	<100
MMN-028	<100	<100
MMN-042	1600	<100
MMN-045	1600	<100
MMN-053	400	<100
MMN-068	1600	<100
MMN-070	200	<100
MMN-094	400	<100
MMN-106	100	<100

CHAPTER



Multifocal motor neuropathy is associated with an altered composition of gut microbiota

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In preparation

Abstract

Objective To determine the association between the composition of gut microbiota and susceptibility to multifocal motor neuropathy (MMN), its disease course and the presence of anti-GM1 IgM antibodies and MMN disease course.

Methods We collected fecal samples of 74 patients with MMN and 64 controls. Using 16S rRNA gene sequencing, we compared gut microbiota alpha and beta diversity, and differential abundances of bacterial genera between patients and controls. In addition, putative correlations with clinical parameters of patients with MMN including the presence of anti-GM1 IgM antibodies were examined.

Results Patients with MMN and controls had similar alpha and beta diversity. However, patients with MMN had significantly lower mean relative abundances of an uncultured genus of the Christensenellaceae family (MMN 0.022% vs. controls 0.058%; p=0.018) and *Eubacterium siraeum* (MMN 0.199% vs. controls 0.318%; p=0.020). Gut microbiota diversity and relative abundances were not associated with age at onset, nor with sex or IVIg treatment. Patients with anti-GM1 IgM antibodies had a significantly lower mean relative abundance of *Butyricicoccus* (anti-GM1 IgM^+ 0.29% vs anti-GM1 IgM^- 0.54%; p=0.049) and an increased abundance of *Mogibacterium* (anti-GM1 IgM^+ 0.088% vs. controls 0.019%; p=0.034).

Conclusion Multifocal motor neuropathy is associated with an altered abundance of minor genera of gut microbiota. Specifically, anti-GM1 IgM antibodies are associated with an increased abundance of *Mogibacterium*. The results of this study provide novel and important insights in the immunopathological processes underlying anti-GM1 IgM antibodies and MMN.

Introduction

Multifocal motor neuropathy (MMN) is a rare and chronic immune-mediated polyneuropathy that mostly affects young- to middle-aged men and causes progressive and ultimately severe asymmetrical weakness and disability of the hands and lower legs. MMN is associated with the presence of conduction block, i.e. the inability to propagate action potentials along axons of peripheral nerves, and the presence of IgM antibodies against the ganglioside GM1, a constituent of peripheral nerves.[1-5] Although reported prevalence figures of these antibodies vary, the use of standardized ELISA and glycoarray techniques has consistently shown that serum samples from approximately half of the patients with MMN contain anti-GM1 antibodies.[6-8] However, the true prevalence figure is probably higher, as shown in studies that used human motor neuron models rather than solid-phase assays.[9] The pathogenic potential of anti-GM1 IgM includes complement-activating properties that are associated with severity of muscle weakness, through the contribution of accumulating permanent axonal damage, and changes in the anatomy and function of the (peri)nodal region and possibly the neuromuscular junction.[7,9,10-12] Though short-lasting and partial, the efficacy of subcutaneously or intravenously administered immunoglobulins (IVIg), the standard of care for MMN treatment, is probably best explained by its interference with IgM-complement interaction.[13,14,15]

Anti-GM1 IgM antibodies are not unique to MMN. They are part of the repertoire of natural antibodies, which are produced without T-cell help and provide first-line protection against bacteria through their specificity for common epitopes including glycans expressed by bacterial glycoproteins and glycolipids. Anti-GM1 IgM antibodies appear in serum during the first months of life in parallel with other anti-bacterial anti-glycan IgM antibodies in response to the development of the respiratory and intestinal tract microbiome by bacteria colonizing the respiratory tract and gut.[16] The mechanisms that cause the unique increased titers and pathogenic properties of anti-GM1 IgM antibodies in inflammatory neuropathies such as MMN are unknown.[3,16,17] However, epidemiological and immunological studies of the Guillain-Barré syndrome (GBS) have shown that gut microbiota are a driving force behind the production of anti-GM1 antibodies, mostly as a result of preceding infections with strains of *Campylobacter jejuni* harboring GM1-like epitopes.[18,19] Similar associations of *C.jejuni* infections with MMN have not been found.[20] However, in a GBS mouse model, *C. Jejuni*-associated anti-ganglioside antibody production depended on gut microbiota composition.[21]

The aim of this study was therefore to study gut microbiota in relation to MMN susceptibility, anti-GM1 IgM antibody production and MMN disease course. To this end, we collected fecal samples of patients with MMN and controls, compared gut microbiota diversity and relative abundance of bacterial genera between groups, and their association with clinical parameters.

Methods

Study population

All patients were followed at the neuromuscular outpatient clinic of the University Medical Center Utrecht (UMCU), a national referral center for MMN. We previously performed population-based studies to determine natural history of MMN and all collected data were

stored in our national MMN database.[4,22] We contacted patients by telephone and asked whether they were willing to participate in this study. All patients had been diagnosed with MMN and fulfilled the 2010 diagnostic consensus criteria that distinguish the categories definite, probable and possible MMN, based on a typical MMN phenotype, combined with the finding of conduction block during nerve conduction studies and/or other findings on ancillary testing, including abnormal imaging of the brachial plexus, the presence of anti-GM1 IgM antibodies, moderately increased levels of protein in the cerebrospinal fluid (CSF) (< 1g/L), or a response to IVIg treatment.[23] Patients who agreed to participate were asked to include one or more control subjects in their vicinity. All subjects were included between May 2013 and February 2014.

Clinical data

We asked all participants to fill out a questionnaire that included questions regarding sex, age, date of sample production, weight, length, tobacco use, alcohol consumption and use of medication (including antacids and antibiotics). We used additional clinical data from our database, complemented with data from the UMCU patient files when necessary. We recorded age at onset, age at diagnosis, disease duration at the time of sample production, presence of anti-GM1 IgM antibodies and information on IVIg treatment. Age at onset was defined as the patient-reported onset of muscle weakness and date of diagnosis as the moment when a patient fulfilled diagnostic criteria for MMN. We examined the presence of anti-GM1 IgM antibodies by means of the standardized INCAT ELISA, as described previously.[6,7] We also documented whether patients were actively treated at the time of inclusion in this study, and if so, recorded the IVIg dosage per month.

Fecal sample collection and DNA isolation

Subjects participating in this study collected a fecal sample at home using the EasySampler kit (Alpha Laboratories) and sent it to the UMCU by mail. All samples were aliquoted in a laminar flow cabinet in 2ml Microtubes (Sarstedt, 72694006), which were stored at -80^{0}C until further analysis. Dates of sample production and storage at the UMCU were recorded. Only samples that were stored at the UMCU within two days after production were used in this study. DNA extraction was performed on 0.2g of feces using the PureLink Microbiome DNA purification kit (Invitrogen, Breda, The Netherlands). Extraction was performed according to manufacturer's instructions, with a modified bead beating step where two rounds of bead beating were applied at 3.5 m/s for 2 minutes, followed by two minutes incubation on ice using the FastPrep-24 (MP Biomedicals).

The 469 bp V3 and V4 hypervariable regions of the 16S rRNA gene were amplified and sequenced using the Illumina MiSeq instrument and Reagent Kit v3 (600-cycle) according to Fadrosh et al.[24] Negative controls, and mock communities (ZymoBIOMICS microbial community standard (D6300), ZymoBIOMICS microbial community DNA standard (D6305), ZymoBIOMICS Microbial Community Standard II (Log Distribution) (D6310), Zymo research, USA) were used from the beginning of DNA isolation up to the data analysis stage and matched with the distribution expected mock compositions.

Bioinformatics

The QIIME2 microbial community analysis pipeline (version 2021.4) was used with DADA2 for sequence variant detection (with default settings, except for --p-trunc-len-f 255 --p-trunc-len-r 240), and SILVA as 16S rRNA reference gene database (SILVA 138).[25-27] Sequencing data have

been made available on the European Nucleotide Archive. Samples with less than 5000 sequence reads were removed from further analyses.

Statistical analysis

We used R version 2022.02.2 to perform statistical analyses. We compared continuous variables using a Student's t-test or Mann Whitney U test and categorical variables using a Chi-squared or Fisher's test, as appropriate. Correlations between continuous variables were determined by calculating the Pearson correlation coefficient, or Spearman's rho, as appropriate. We used Bonferroni's method to correct for multiple testing.

All analyses of microbiome data were performed on the genus level. To assess alpha diversity, a measure for ecological diversity and evenness within a sample, we used the *vegan* package to calculate Chao1, Inverse Simpson's and Shannon's diversity scores, and compared scores between groups using the Mann-Whitney U test. A Bonferroni method was used to correct p-values for multiple testing. For beta diversity, a measure for overall community structure, we used the *phyloseq package* to perform principal coordinate analysis (PCoA) using Bray-Curtis ecological distances. Beta diversity was compared between groups by PERMANOVA using the *vegan* package. We analyzed differential abundance of bacteria using the ANCOMBC method using the *ANCOMBC* package, a function that compares relative abundance between groups, whilst controlling for compositionality of data.[28] P-values were corrected for multiple testing using the Benjamini-Hochberg method, and corrected p-values <0.05 were considered statistically significant.

Standard protocol approvals, registrations, and patient consents

The locally appointed medical ethical committee of the UMC Utrecht approved this study under protocol number METC-13-019/C. All included subjects gave written informed consent prior to inclusion in this study.

Data availability statement

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

Results

Study population

Between May 2013 and February 2014, we identified a total of 126 patients with MMN eligible for participation in this study. Of this group, 100 subjects could be contacted (79%), 88 of whom (88%) agreed to participate in this study. We received 83 fecal samples at our lab (94%), of which 75 (89%) could be stored within two days after sample production. DNA isolation and 16S rRNA sequencing were successfully performed in 74 samples (99%). We included an additional 78 controls and received a fecal sample of 71 (91%), 67 of which could be stored within two days. DNA isolation and 16S rRNA sequencing were successfully performed in 64 samples (96%).

Baseline characteristics of patients with MMN (n=74) and control subjects (n=64) included in our analyses are shown in Table 1.

Table 1. Baseline characteristics of controls and patients with MMN. All data represent data at the time of inclusion in this study, unless specified otherwise.

	Controls (N=64)	**MMN (N=74)**	**p-value**
Male sex (n (%))	13 (20)	56 (76)	<0.001*
Age at inclusion in years§	52 (17)	55 (16)	0.120
Body Mass Index (kg/m^2)	24.1 (5.8)	25.4 (4.5)	0.198
Tobacco use (n (%))	12 (19)	17 (24)	0.630
Alcohol use (n (%))	52 (81)	63 (88)	0.442
Antacid use (n (%))	11 (17)	10 (14)	0.796
Comorbidities (n (%))	4 (7)	3 (4)	0.704
Diabetes	3 (5)	2 (3)	0.669
Ankylosing spondylitis	0 (0)	1 (1)	-
Gout	0 (0)	1 (1)	-
Inflammatory bowel disease	1 (1)	1 (1)	1.000
Household controls (n (%))	55 (86)	-	-
Family controls (n/N (%))	11/53 (21)	-	-
Non-household controls (n (%))	9 (14)	-	-
Family controls (n/N (%))	5/9 (56)	-	-
Age at onset in years§	-	41 (11)	-
Age at diagnosis in years§	-	46 (12)	-
Disease duration at inclusion (months)§	-	173 (170)	-
EFNS 2010 diagnosis (n/N (%))			
Definite MMN	-	53/72 (74)	-
Probable MMN	-	15/72 (21)	-
Possible MMN	-	4/72 (6)	-
Anti-GM1 IgM antibodies (n/N (%))	-	35/61 (57)	-
IVIg treatment-naïve (n/N (%))	-	11/72 (15)	-
IVIg loading dose only	-	3/57 (5)	
IVIg maintenance therapy (n/N (%))	-	54/57 (95)	-
IVIg maintenance dose (grams/month)§	-	58 (48)	-
Cumulative IVIg dose (3 months) §	-	155 (159)	-
Cumulative IVIg dose (6 months) §	-	310 (330)	-

* Statistically significant

§ Value depicted as median (IQR)

EFNS = European Federation of Neurological Societies, IVIg = Intravenous immunoglobulins, MMN = Multifocal motor neuropathy,

Compared to controls, patients with MMN were significantly more often male (MMN vs. controls; 76% vs. 20%; X^2 test p<0.001), probably because most controls were partners of MMN patients. IVIg data were known in 72/74 (97%) of patients with MMN. 11/72 had never been treated with IVIg before participation in this study. Of 61 patients known to be treated with IVIg at or before inclusion in this study, detailed information on maintenance therapy was known in 57/61 (93%), of whom the majority was on maintenance IVIg therapy (54/57 (95%)). Patients and controls were well-matched for other variables, including age, BMI, tobacco and alcohol use and comorbidities.

Alpha diversity and community structure

The top 10 most abundant bacterial genera found in both patients with MMN and controls were *Blautia*, *Faecalibacterium*, *Bifidobacterium*, *Agathobacter*, *Bacteroides*, *Subdoligranulum*, *Collinsella*, *Fusicatenibacter*, *Prevotella* and *Coprococcus* (Fig. 1A). We compared various alpha diversity scores, measures reflecting ecological diversity and evenness within a sample, between patients with MMN and controls (Fig. 1B). None of the alpha diversity scores differed between groups, indicating that samples of patients and controls had a comparable richness and distribution of bacterial genera (all uncorrected alpha diversity scores p>0.18). In addition, no differences were found regarding beta diversity, indicating comparable community structures between patients with MMN and controls (p=0.65) (Fig. 1C).

Differential abundance of bacterial genera

To further determine differences in the composition of gut microbiota between patients with MMN and controls, we performed a supervised analysis (ANCOMBC) to explore bacterial differential abundance between patients and controls. Thirteen genera were found to be differentially abundant (uncorrected p-values <0.05), and after correction for multiple testing, two genera remained. Compared to controls, patients with MMN had a significantly lower relative abundance of an uncultured genus belonging to the Christensenellaceae family (mean relative abundance MMN 0.022% vs. controls 0.058%; p=0.0001, corrected p=0.018). Also, patients with MMN had a significantly lower relative abundance of *Eubacterium siraeum*, a genus of the Ruminococcaceae family (mean relative abundance (%) in MMN 0.199% vs. controls 0.318%; p=0.0002; corrected p=0.020). An overview of all bacterial genera that were differentially abundant between patients and controls is provided in Table 2.

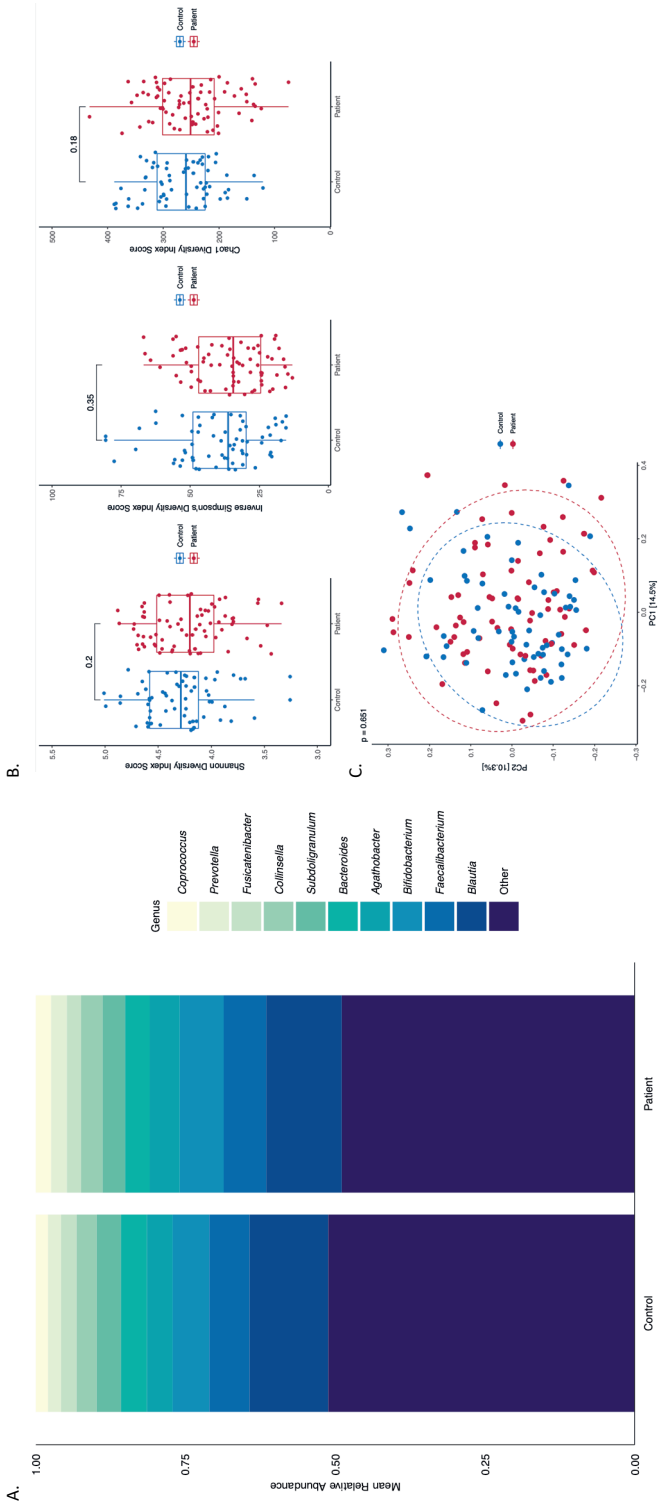

Figure 1

A. *Genus-level composition of patients with MMN and controls. Mean relative abundance of the top 10 most abundant bacterial genera are shown.*

B. *Microbial richness and diversity in patients with MMN and controls. Alpha diversity measures from left to right: Shannon, Inverse Simpson's, and Chao1 diversity score. Patients and controls showed comparable alpha diversity scores (all uncorrected p-values >0.18).*

C. *Principal coordinate analysis (PCoA) of 16S rRNA data from patients and controls. Beta diversity was compared by PERMANOVA using Bray-Curtis ecological distances. Ellipses indicate 95% confidence intervals. Patients and controls had comparable beta diversity (PERMANOVA p=0.651).*

Table 2. Differentially abundant bacteria as compared between patients with MMN (n=74) and controls (n=64). All bacteria, depicted by their family and genus name, with a ANCOMBC p-value < 0,05 are shown. The percentage of stool samples positive for a bacterium (Chi-squared test, uncorrected p-value) is shown. Mean relative abundances (% (SD)), along with the ANCOMBC uncorrected p-value and its Benjamini-Hochberg adjusted p-value, are depicted.

		Positive samples (%)			**Mean relative abundance (SD)**			
Family	Genus	Controls	MMN	p	Controls	MMN	p	Adjusted p
Christensenellaceae	Uncultured	87,5	63,5	0,002*	0,058 (0,119)	0,022 (0,026)	0,0001	0,0184*
Ruminococcaceae	*Eubacterium Siraeum*	85,9	67,6	0,020	0,318 (0,465)	0,199 (0,462)	0,0002	0,0202*
Erysipelatoclostridiaceae	*Catenibacterium*	4,7	16,2	0,058	0,040 (0,250)	0,282 (0,756)	0,0027	0,1656
Atopobiaceae	*Atopobium*	17,2	25,7	0,318	0,001 (0,004)	0,004 (0,008)	0,0053	0,2418
Anaerovoracaceae	*Mogibacterium*	23,4	35,1	0,189	0,020 (0,055)	0,056 (0,130)	0,0089	0,3186
Ruminococcaceae	*Caproiciproducens*	12,5	17,6	0,556	0,002 (0,008)	0,008 (0,030)	0,0104	0,3186
Lachnospiraceae	*Howardella*	31,3	41,9	0,265	0,025 (0,050)	0,041 (0,059)	0,0131	0,3429
Lachnospiraceae	*GCA-900066575*	76,6	59,5	0,051	0,037 (0,038)	0,024 (0,029)	0,0200	0,4582
Rikenellaceae	*RC9*	20,3	31,1	0,214	0,277 (1,999)	0,150 (0,629)	0,0374	0,6459
Prevotellaceae	*Paraprevotella*	45,3	55,4	0,312	0,133 (0,253)	0,239 (0,361)	0,0388	0,6459
Oscillospiraceae	*UCG-005*	96,9	87,8	0,101	0,794 (0,814)	0,612 (0,622)	0,0360	0,6459
Anaerovoracaceae	*Family XIII UCG-001*	89,1	81,1	0,287	0,069 (0,066)	0,046 (0,042)	0,0467	0,6571
Ruminococcaceae	*Ruminococcus*	96,9	87,8	0,101	2,473 (2,974)	1,929 (2,677)	0,0467	0,6571

* Statistically significant after p-value adjustment

The composition of gut microbiota is independent of sex

Since patients with MMN were significantly more often of the male sex (MMN 76% vs. controls 20%; p<0.0001), we investigated whether alpha and beta diversity differed between male and female control subjects. All alpha diversity scores did not differ between groups (p-values all >0.29, data not shown), nor did beta diversity (p 0.44, data not shown). We found no bacterial genera to be significantly differentially abundant between male and female subjects (all corrected p-values >0.27, data not shown).

Treatment with immunoglobulins does not affect gut microbiota composition

Treatment with IVIg is common among patients with MMN and could potentially alter gut microbiota composition and hence influence analyses. To determine the effect of IVIg treatment on gut microbiota composition, we compared patients that were treatment-naïve to patients that received IVIg treatment at the time of inclusion in this study (n=11 vs. n=61, unknown n=2, Table 1). No differences were found regarding alpha diversity (all p-values >0.78, data not shown), beta diversity (p=0.82, data not shown), and differential abundance of bacterial genera (all corrected p-values >0.11, data not shown).

Next, we dichotomized patients that were on IVIg maintenance therapy at the time of inclusion in this study (n=54) based on the median IVIg dosage per month (n=31 vs. n=23). We found no differences regarding alpha diversity scores (all p-values >0.23, data not shown), beta diversity (p=0.44, data not shown), nor differential abundance of bacterial genera (all corrected p-values >0.89, data not shown), suggesting that IVIg treatment, nor IVIg dosage, significantly affect the composition of gut microbiota.

Correlation to clinical parameters in patients with MMN

Next, we determined whether alterations in the composition of gut microbiota were associated with clinical parameters of patients with MMN. To this end, we correlated sex, age at onset of muscle weakness, and anti-GM1 IgM antibody status to alpha diversity scores, beta diversity and differential abundance of bacterial genera.

First, we compared male and female patients with MMN and found no differences regarding alpha diversity (all uncorrected p-values >0.09), beta diversity (p=0.15) and differential abundance of bacterial genera (all corrected p-values >0.26). Next, we compared male patients to male controls, and female patients to female controls. In all analyses, we found no significant differences, except for an increased relative abundance of *Izemoplasmatales* (genus in the family of Izemoplasmatales; mean relative abundance in male controls 0.00% vs. male patients with MMN 0.0061%, corrected p=0.0027) and *Coriobacteraceae UCG-003* (genus in the family of Atopobiaceae; mean relative abundance in male controls 0.00% vs. male patients with MMN 0.069%, corrected p=0.022) in male patients with MMN as compared to male controls. Since the male control group consisted of a small number of subjects (n=13) and since these genera were of either very low relative abundance or relatively high p-value, we considered these differences to be of uncertain relevance.

We dichotomized patients by median age at onset and compared groups (Table 1; n=34 vs. n=40). The median Chao1 and Shannon alpha diversity index scores were higher in patients with an above median age at onset (p=0.014 and p=0.069, respectively). When analyzing age at onset as a continuous variable, we also found a positive correlation between age at onset and increased alpha diversity scores (Chao1 diversity index score: Spearman's rho 0.30, p=0.010; Shannon diversity index score: Spearman's rho 0.24, p=0.038). We found no differences regarding beta diversity (p=0.16) and differential abundance (all p-values >0.48).

The composition of the gut microbiota is altered in patients with anti-GM1 IgM antibodies
We next stratified patients by anti-GM1 IgM antibody status and associated the presence of these antibodies with the gut microbiota composition.
In all comparisons, the presence or absence of anti-GM1 IgM antibodies was not associated with changes in alpha or beta diversity (p-values >0.22; data not shown). Compared to patients without anti-GM1 IgM antibodies, patients with anti-GM1 IgM antibodies had a lower relative abundance of *Butyricicoccus* (mean relative abundance (% (SD)) in anti-GM1 IgM^+ vs. anti-GM1 IgM^- patients with MMN; 0.29% (0.23) vs. 0.54% (0.32); p=0.0003; corrected p=0.049). *Butyricicoccus* was identified in 97% and 100% of the samples, respectively. When separately comparing patients with or without antibodies to controls, we reconfirmed the association between a lower relative abundance of a genus of the Christensenellaceae family and *Eubacterium siraeum* with MMN. In addition, we found that patients with MMN that have anti-GM1 IgM antibodies had an increased relative abundance of *Mogibacterium* (mean relative abundance (% (SD)) in anti-GM1 IgM^+ patients with MMN vs. controls; 0.088% (0.17) vs. 0.019% (0.055): p=0.0006; corrected p=0.034).

Combinations of genera associate with increased susceptibility to MMN
To further investigate the associations of *Eubacterium siraeum*, the uncultured genus of the Christensenellaceae family and *Mogibacterium* with MMN susceptibility, we combined the presence or absence of these genera into eight *MMN-associated genera combinations* (MAGCs), and compared these between patients and controls, and between patients with and without anti-GM1 IgM antibodies. As *Butyricicoccus* was found in almost all samples, this genus was not included in this specific analysis. The overall distribution of the MAGCs differed between patients and controls (Fisher's exact test, p=0.0085), driven by a major difference regarding the MAGC-2, consisting of the presence of *Eubacterium siraeum* and the uncultured genus of the Christensenellaceae family, in combination with the absence of *Mogibacterium* (MMN vs. controls; 29.7% vs. 62.5%; p=0.00023; Bonferroni corrected p=0.0018; OR 0.26 (95% CI 0.12-0.55)) (Table 3).
The presence of *Mogibacterium* was associated with anti-GM1 IgM antibodies (p=0.037; OR 3.88 (95% CI 1.09-16.2)). The aforementioned MAGC-2 was found significantly less frequently in patients with anti-GM1 IgM antibodies (p=0.030; OR 4.28 (95% CI 1.12-18.8)) (Table 4).
These analyses show that particular combinations of present or absent bacterial taxa may reveal synergistic associations between the microbial community composition and MMN susceptibility.

Table 3. Combined presence of three genera whose relative abundances are associated with MMN. The distribution of bacteria differed between controls and patients with MMN (Fisher's exact test, p 0.0085), the effect of which was carried by MAGC-2, comprising the combined presence of *Eubacterium Siraeum* and an uncultured genus of the Christensenellaceae family, in the absence of *Mogibacterium* (Chi-squared test, p 0.00023, OR MMN vs. controls 0.26 (0.12 – 0.55)).

MAGC	*E. siraeum*	Christensenellaceae	*Mogibacterium*	Controls (n (%))	MMN (n (%))	p	OR (95% CI)
1	+	+	+	10 (15,6)	14 (18,9)	0.78	-
2	+	+	-	40 (62,5)	22 (29,7)	0.00023*	0.26 (0.12 - 0.55))
3	+	-	+	2 (3,1)	5 (6,8)	0.14	-
4	+	-	-	3 (4,7)	9 (12,1)	0.11	-
5	-	-	-	2 (3,1)	8 (10,8)	0.50	-
6	-	-	+	1 (1,6)	5 (6,8)	0.22	-
7	-	+	-	6 (9,4)	9 (12,2)	0.79	-
8	-	+	+	0 (0)	2 (2,7)	0.45	-

* Statistically significant at p<0.0063 after Bonferroni p-value adjustment
MAGC = MMN-associated genera combination

Table 4. The presence of *Mogibacterium* and the absence of MAGC-2 are both associated with anti-GM1 IgM antibodies

	Anti-GM1 IgM^- (n=26)	Anti-GM1 IgM^+ (n=35)	p	OR (95% CI)
Mogibacterium -	21 (81)	18 (51)		
Mogibacterium +	5 (19)	17 (49)	0.037	3.88 (1.09 – 16.2)
MAGC-2				
Absent	15 (58)	30 (86)		
Present	11 (42)	5 (14)	0.030	4.28 (1.12 – 18.8)

* Statistically significant at p<0.05
MAGC-2 = MMN-associated genera combination 2 (see Table 3.)

Discussion

Bacteria that form part of the human gut microbiome form an incredible source of epitopes and metabolites and thus play an important role in the regulation of the human mucosal immune system. The relevance of the complex interplay of bacteria and the immune system at the gut mucosa level is illustrated by experimental studies that show the contribution of dysbiosis to susceptibility to various diseases.[29-32] The frequent presence of anti-GM1 antibodies in patients with MMN could be interpreted as an indication that alterations of microbiota underlie MMN susceptibility in a significant proportion of patients. Therefore, we set out to perform the first microbiome study in patients with MMN, using a sizeable cohort of well-characterized patients and controls, given the rarity of the disease. Through the design of this study, we created well-matched groups, minimizing potential effects of age, diet, and lifestyle on composition of gut microbiota. Overall, we did not observe major changes in microbiome composition, as we found no differences regarding alpha and beta diversity between patients and controls, nor did we find differences when looking at sex or IVIg treatment. However, patients with MMN had lower abundances of an uncultured genus of the Christensenellaceae family and *Eubacterium siraeum,* while the presence of anti-GM1 IgM antibodies as detected with ELISA appeared to be associated with a lower abundance of *Butyricicoccus* and a higher abundance of *Mogibacterium*. These associations were most pronounced when we examined the associations between *MMN-associated generatype combinations* (MAGCs) and MMN susceptibility. We therefore conclude that MMN is associated with changes in the composition of gut microbiota.

Dysbiosis of human gut microbiota alters immunological homeostasis at the gut mucosa level, shifting it from an IL-10 and regulatory T cell-mediated immunotolerant environment towards a more proinflammatory immunological site.[33] A previous study in neonates showed the direct relationship between production of anti-GM1 IgM antibodies and colonization of the gut that precedes constitution of the microbiome.[16] Moreover, acute motor axonal neuropathy (AMAN), a variant of the Guillain-Barré syndrome (GBS), an acute inflammatory polyneuropathy that not only shares the production of anti-ganglioside antibodies but also that of conduction block with MMN is often caused by preceding infections with *C. jejuni*. The presence of GM1-like epitopes on *C. Jejuni* lipo-oligosaccharides (LOS) provides the antigenic stimulus for anti-GM1 autoantibody production, while the infection itself may also trigger gut dysbiosis.[18,19] The relevance of gut dysbiosis in GBS was previously shown in a GBS mouse model, where antibiotic depletion of gut microbiota enhanced *C. Jejuni* colonization and invasion, increased T cell-mediated colitis and led to higher anti-ganglioside IgG antibody production.[21] There are, however, important differences in clinical characteristics between AMAN and MMN, particularly in onset (subacute versus insidious), disease course (monophasic versus chronic) and severity (loss of ambulation and respiratory muscle strength versus slowly progressive distal weakness). This may also indicate underlying differences in the nature and the impact of the gut microbiota composition and gut dysbiosis in AMAN and MMN. Despite these differences, the results of our study suggest that the composition of gut microbiota plays a role in the production of high titers of anti-GM1 IgM in patients with MMN.

How could we explain the association between MMN and changes in the relative abundance of a small number of low abundance bacterial genera, i.e. a lower abundance of an uncultured Christensenellaceae genus, *Eubacterium siraeum* and *Butyiricicoccus*, and a higher abundance

of *Mogibacterium*? Though seemingly counterintuitive, the presence of bacteria with a low abundance is associated with increased MHC class II expression by enterocytes and an upregulation of genes associated with T-cell cytokine production, indicating the effects that low abundance bacteria have on host immunity.[34] Indeed, a lower relative abundance of bacteria of the Christensenellaceae family has been found in metabolic syndrome and Crohn's disease, whereas higher abundances have been linked to multiple sclerosis and Parkinson's disease.[35-38] Variations in relative abundance of this bacterial family has been shown to depend on host genetics, as well as on fecal transit time, factors that might differ between patients with MMN and controls, but were not documented for the purpose of this study.[35,39] Increased abundance of *Eubacterium siraeum* has been associated with ankylosing spondylitis and lower abundance with gestational diabetes.[40,41] Finally, *Butyricicoccus* is found in lower abundances in patients with IBD.[42,43] A common denominator for the three aforementioned bacterial genera is their ability to produce short chain fatty acids (SCFA), such as acetate and butyrate, by fermentation of dietary fibers.[35,41,43,44] SCFAs promote an anti-inflammatory immunological environment by inducing regulatory T cell-mediated IL-10 production in the gut mucosa, and suppression of MHC class II upregulation on antigen-presenting cells.[44,45] The lower relative abundance of *Eubacterium siraeum* and *Butyiricicoccus* in patients with MMN could thus lead to proinflammatory settings at the gut mucosa.
The increased abundance of *Mogibacterium*, a strictly anaerobic Gram-positive genus, was associated with the presence of anti-GM1 IgM antibodies. *Mogibacterium* is a proinflammatory bacterium associated with periodontitis.[46-49] It is unlikely that *Mogibacterium* expresses GM1-like epitopes in a similar way to the Gram-negative bacterium *C. jejuni*, since it lacks lipooligosaccharide (LOS). Yet, GM1-like structures that bind cholera toxin have been shown to be expressed by bacteria other than *C. jejuni*, including Firmicutes, a Gram-positive phylum to which *Mogibacterium* belongs.[50] It is unknown whether *Mogibacterium* indeed expresses GM1-like structures via as of yet unknown mechanisms, but if so, this raises the possibility of molecular mimicry explaining the association with MMN. Alternatively, *Mogibacterium* may contribute to more proinflammatory stimuli at the mucosal interface, a scenario supported by the finding that the presence of a combination of the aforementioned genera with maximal anti-inflammatory properties, i.e. an uncultured Christensenellaceae genus, *Eubacterium siraeum* and *Butyricicoccus*, in the absence of *Mogibacterium,* was found significantly less common in patients with MMN.

The results of our study add to our understanding of the immunopathogenesis of MMN but acknowledge that longitudinal microbiome studies would corroborate and strengthen the associations that we report in this cross-sectional study. Moreover, the design of this study does not allow for answering the question of causality of the found associations, nor for determining the theoretical possibility of MMN-associated inflammation or anti-GM1 IgM antibodies on gut microbiota. Yet, it is important to point out that previous studies failed to show signs of systemic inflammation in patients with MMN, such as increased cytokine concentrations in serum.[51,52]

We show that multifocal motor neuropathy is associated with changes in the composition of the gut microbiome that predispose to a more proinflammatory mucosal status. Patients with MMN have lower abundance of bacterial genera with anti-inflammatory effects on the immune system. More specifically, patients with high levels of anti-GM1 IgM antibodies have a higher abundance of proinflammatory *Mogibacterium*, a genus that could be hypothesized

to express GM1-like structures via as of yet unknown mechanisms.[50] The results of our study provide important and novel insights regarding the unique immunopathological mechanisms that underlie MMN.

References

1. Pestronk A, Cornblath DR, Ilyas AA, et al. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. Ann Neurol 1988;24(1):73-78

2. Nobile-Orazio E, Cappellari A, Prior A. Multifocal motor neuropathy: current concepts and controversies. Muscle Nerve 2005;31(6):663-680

3. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

4. Herraets I, Van Rosmalen M, Bos J, et al. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. Neurology 2020;95(14):e1979-e1987

5. Herraets IJT, Bakers JNE, Van Eijk RPA, et al. Human immune globulin 10% with recombinant human hyaluronidase in multifocal motor neuropathy. J Neurol 2019;266(11):2734-2742

6. Kuijf ML, Van Doorn PA, Tio-Gilles AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci 2005;239(1):37-44

7. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010;75(22):1961-1967

8. Nobile-Orazio E, Giannotta C, Musset L, et al. Sensitivity and predictive value of anti-GM1/galactocerebroside IgM antibodies in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry 2014;85(7):754-758

9. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol 2016;80(1):71-88

10. Van Asseldonk JTH, Van den Berg LH, Van den Berg-Vos RM, et al. Demyelination and axonal loss in multifocal motor neuropathy: distribution and relation to weakness. Brain 2003;126(1):186-198

11. Susuki K, Rasband MN, Tohyama K, et al. Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. J Neurosci 2007;27(15):3956-3967

12. Fewou SN, Plomp JJ, Willison HJ. The pre-synaptic motor nerve terminal as a site for antibody-mediated neurotoxicity in autoimmune neuropathy and synaptopathies. J Anat 2014;224(1):36-44

13. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol 2010;229(1-2):256-262

14. Lutz HA, Stammler P, Bianchi V, et al. Intravenously applied IgG stimulates complement attenuation in a complement-dependent autoimmune disease at the amplifying C3 convertase level. Blood 2004;103(2):465-472

15. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2015;2(4):e119

16. Alaniz ME, Lardone RD, Yudowski SL, et al. Normally occurring human anti-GM1 immunoglobulin M antibodies and the immune response to bacteria. Infect Immun 2004;72(4):2148-2151

17. Yeh WZ, Dyck PJ, Van den Berg LH, et al. Multifocal motor neuropathy: controversies and priorities. J Neurol Neurosurg Psychiatry 2020;91(2):140-148

18. Yuki N, Yoshino H, Sato S, et al. Acute axonal polyneuropathy associated with anti-GM1 antibodies following Campylobacter enteritis. Neurology 1990;40(12):1900-1902

19. Jacobs BC, Van Doorn PA, Schmitz PI, et al. Campylobacter jejuni infections and anti-GM1 antibodies in Guillain-Barré syndrome. Ann Neurol 1996;40(2):181-187

20. Terenghi F, Allaria S, Scarlato G, et al. Multifocal motor neuropathy and Campylobacter Jejuni reactivity. Neurology 2002;59(2):282-284

21. Brooks PT, Bell JA, Bejcek CE, et al. An antibiotic depleted microbiome drives severe Campylobacter jejuni-mediated type 1/17 colitis, type 2 autoimmunity and neurologic sequelae in a mouse model. J Neuroimmunol 2019;337:577048

22. Cats EA, Van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology 2010;75(9):818-825

23. Van Schaik IN, Leger JM, Nobile-Orazio E, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society--first revision. J Peripheral Nerv Syst 2010;15(4):295-301

24. Fadrosh DW, Ma B, Gajer P, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2014;2(1):6

25. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensive microbiome data science using QIIME 2. Nat Biotechnol 2019;37(8):852-857

26. Callahan BJ, McMurdie PJ, Rosen MJ, et al. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13(7):581-583

27. Quast C, Pruesse E, Yilmax P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590-596

28. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. Nat Commun 2020;11(1):3514

29. Van der Houwen TB, Van Laar JAM, Kappen JH, et al. Behçet's disease under microbiotic surveillance? A combined analysis of two cohorts of Behçet's disease patients. Front Immunol 2020;11:1192

30. iMSMS Consortium. Gut microbiome of multiple sclerosis patients and paired household healthy controls reveal associations with disease risk and course. Cell 2022;185(19):3467-3486

31. Zhang L, Qing P, Yang H, et al. Gut microbiome and metabolites in systemic lupus erythematosus: link, mechanisms and intervention. Front Immunol 2021;12:686501

32. Clemente JC, Manasson J, Scher JU. The role of gut microbiome in systemic inflammatory disease. BMJ 2018;360:5145

33. Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. Immunity 2017;46(4):562-576

34. Han G, Luong H, Vaishnava S. Low abundance members of the gut microbiome exhibit high immunogenicity. Gut Microbes 2022;14(1):2104086

35. Waters JL, Ley RE. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. BMC Biol 2019;17(1):83

36. Galluzzo P, Capri FC, Vecchioni L, et al. Comparison of the intestinal microbiome of Italian patients with multiple sclerosis and their household relatives. Life (Basel);11(7):620

37. Shen T, Yue Y, He T, et al. The association between the gut microbiota and Parkinson's disease, a meta-analysis. Front Aging Neurosci 2021;13:636545

38. Li X, Li Z, He Y, et al. Regional distribution of Christensenellaceae and its associations with metabolic syndrome based on a population-level analysis. PeerJ 2020;8:e9591

39. Goodrich JK, Waters JL, Poole AC, et al. Human genetics shape the gut microbiome. Cell 2014;159(4):789-799

40. Zhou C, Zhao H, Xiao X, et al. Metagenomic profiling of the pro-inflammatory gut microbiota in ankylosing spondylitis. J Autoimm 2020;107:102360

41. Ye D, Huang J, Wu J, et al. Integrative metagenomic and metabolomic analyses reveal gut microbiota-derived multiple hits connected to development of gestational diabetes mellitus in humans. Gut Microbes 2023;15(1):2154552

42. Bajer L, Kverka M. Kostovcik M, et al. Distinct gut microbiota profiles in patients with primary sclerosing cholangitis and ulcerative colitis. World J Gastroenterol 2017;23(25):4548-4558

43. Eeckhaut V, Machiels K, Perrier C, et al. Butyricicoccus pullicaecorum in inflammatory bowel disease. Gut 2013;62(12):1745-1752

44. Kim CH. Control of lymphocyte functions by gut microbiota-derived short-chain fatty acids. Cell Mol Immunol 2021;18(5):1161-1171

45. Qiu J, Ma Y, Qiu J. Regulation of intestinal immunity by dietary fatty acids. Mucosal Immunol 2022;15(5):846-856

46. Nakazawa F, Sato M, Poco SE, et al. Description of Mogibacterium pumilum gen. nov., sp. nov. and Mogibacterium vescum gen. nov., sp. nov., and reclassification of Eubacterium timidum (Holdeman et al. 1980) as Mogibacterium timidum gen. nov., comb. nov. Int J Syst Evol Microbiol 2000;50(2):679-688

47. Casarin RCV, Saito D, Santos VR, et al. Detection of Mogibacterium timidum in subgingival biofilm of aggressive and non-diabetic and diabetic chronic periodontitis patients. Braz J Microbiol 2012;43(3):931-937

48. Nibali L, Sousa V, Davrandi M, et al. Differences in the periodontal microbiome of successfully treated and persistent aggressive periodontitis. J Clin Periodontol 2020;47(8):980-990

49. Li Q, Pu Y, Lu H, et al. Porphyromonas, Treponema, and Mogibacterium promote IL8/IFNγ/TNFα- based pro-inflammation in patients with medication-related osteonecrosis of the jaw. J Oral Microbiol 2020;13(1):1851112

50. Patry RT, Stahl M, Perez-Munoz ME, et al. Bacterial AB5 toxins inhibit the growth of gut bacteria by targeting ganglioside-like glycoconjugates. Nat Commun 2019;10(1):1390

51. Vlam L, Stam M, De Jager W, et al. Cytokine profiles in multifocal motor neuropathy and progressive muscular atrophy. J Neuroimmunol 2015;286:1-4

52. Bos JW, Groen EJW, Budding K, et al. Multifocal motor neuropathy is not associated with altered innate immune responses to endotoxin. J Neurol Sci 2023;451;120692

CHAPTER

9

General discussion

MMN from a neurological perspective

From the first descriptions by the group of Pestronk *et al.* in 1988, MMN has been recognized as a treatable disorder with a distinct clinical phenotype, consisting of markedly asymmetrical and mostly distal muscle weakness of mainly the hands, in the absence of sensory abnormalities.[1] The severity of MMN shows considerable variation, as exemplified by two case histories. The first is a man in his sixties, who experienced muscle weakness in his right hand from the age of 25 and was diagnosed with MMN 20 years later. He has been treated with immunoglobulins at 50 grams per month ever since. His muscle weakness of the right hand and left foot have not deteriorated and is completely independent. The other patient is man of comparable age, diagnosed with MMN at the age of 40. Despite treatment with increasing dosages of IVIg, with now 45 grams at weekly infusions and loading dosages of 180 grams every three months, his muscle strength steadily decreased. This has led to severe proximal (i.e., upper) arm weakness and minimal remaining hand function. He had to stop working and requires professional care to help him with his activities of daily living. Although he remains ambulant, he needs an electrical wheelchair for longer distances.

How can MMN affect patients this differently? The answer to this question is probably key to improving treatment of MMN. Previous cross-sectional studies showed that patients with anti-GM1 IgM antibodies have more severe weakness, and that muscle strength deteriorates over time.[2-5] In **Chapter 2** of this thesis, we report the results the MAIN study, a combined cross-sectional and longitudinal study in a cohort of 100 patients with MMN, 60 of whom underwent clinical measurements with an interval of 8 years. We show that deterioration of strength and increasing disability is the rule rather than the exception in MMN, but that there is considerable variation between patients. The presence of anti-GM1 IgM antibodies detectable with ELISA is associated with more severe muscle weakness. Patients with the most pronounced deterioration of muscle strength more often had more severe muscle weakness and loss of deep tendon reflexes at baseline. This gives us some indication of which patients may follow a more severe disease course, i.e., those who present with a more rapid progression of weakness. The data also show that awareness of MMN has improved, as shown by a decrease of diagnostic delay over time. The increase of the age at diagnosis probably indicates that MMN is increasingly recognized in people of higher age as well. Yet, the most important conclusion is that MMN remains a progressive disease despite maintenance treatment, with severe disability as a possible outcome.[4]

Despite shortening of reported diagnostic delays, we should aim to further improve diagnostic sensitivity for MMN, because an early start of treatment correlates with better outcome.[3] This may be particularly important for other presentations than asymmetric weakness of the hands, for example those with foot drop, upper arm muscle weakness, faster progression from start, and the presence of brisk as opposed to decreased deep tendon reflexes. Although the systematic diagnostic approach as described in the consensus diagnostic criteria is an important aid to ensure timely diagnosis, the execution and interpretation of specific nerve conduction study protocols remains an important challenge. The use of ultrasound of peripheral nerves of the upper arms and brachial plexus facilitates diagnosis of both common and uncommon phenotypes. Thickening of arm nerves and brachial plexus has very high diagnostic sensitivity for MMN and is reproducible across centers, which makes it a useful first

tier assessment tool. Importantly, ultrasound also can identify treatment-responsive patients with MMN who would have been missed with the current diagnostic criteria.[6]
The progressive disease course of MMN demonstrates the need for new treatment strategies. We aimed at improving insights in underlying disease mechanisms to facilitate the development of new therapies.

The immunopathological disease model of MMN

The currently available model of the pathology underlying MMN is based on three main processes[7,8]:

1. The production of anti-GM1 IgM autoantibodies by activated, possibly autoreactive B cells;
2. The binding of these antibodies to GM1 on the axolemma of motor neurons (or other nerve constituents) at and near the node of Ranvier, hindering saltatory conduction and disrupting normal axonal-Schwann cell interaction; and
3. The subsequent activation of the classical pathway of the complement cascade by these antibodies, leading to formation of the membrane attack complex (MAC) and causing structural motor neuron damage.

The main goal of this thesis was to gain a more detailed insight in the immunopathology of MMN by investigating genetic susceptibility, and by studying the presence, origin, and effects of antibodies in MMN.

Genetic susceptibility to MMN

MMN may be associated with autoimmune diseases. The prevalence of type 1 diabetes, Hashimoto's thyroid disease, and celiac disease was increased in first-degree relatives of patients with MMN as compared to controls in one study, suggesting possibly overlapping genetic susceptibility.[9] Genetic association studies have yielded mixed results. MMN is not associated with polymorphisms in genes involved in B cell signaling or activation (*PTPN22*, *BANK1*, *Blk*) or non-peptide antigen presentation (*CD1A* and *CD1E*), but the HLA-DRB1*15 allele group, a genetic variant within the *DRB1* gene of the Major Histocompatibility Complex (MHC) class II locus, was found more frequently in patients with MMN.[10,11] In this thesis, we further explored genetic associations.

In **Chapter 4**, we focused on copy number variation in the *SMN* locus in patients with motor neuron disorders. Normal levels of the Survival Motor Neuron (SMN) protein are essential for proper motor neuron function, and the homozygous deletion of one of the two human genes encoding SMN protein (*SMN1*) causes the severe genetic motor neuron disorder *hereditary proximal spinal muscular atrophy* (SMA). Severity of SMA correlates inversely with the copy number of the second, backup human SMN gene, *SMN2*. Although the exact mechanisms by which the ubiquitously expressed SMN protein is vital for motor neuron survival are unknown, as a subunit of larger protein complexes it has been shown to be involved in processes such as mRNA trafficking and splicing, axonal transport, and endocytosis.[12] A possibly unifying explanation may be found in the association between SMN protein and motor neuron-specific ribosomal functioning.[13]

Several studies have suggested that the *SMN* locus is more broadly associated with motor neuron vulnerability. A previous pilot study in 102 patients with MMN suggested that *SMN1* duplications associated with MMN, and other studies showed that *SMN1* duplications are associated with ALS. Combined, these studies suggest that high intracellular SMN protein levels may exert unknown neurotoxic effects.[14-17] Indeed, Van Alstyne *et al.* in 2021 showed that *over*expression of SMN protein causes neurotoxicity and in mice leads to a lower motor neuron-like disease.[18]

However, we found no association between *SMN1* or *SMN2* copy numbers and MMN susceptibility or disease course. In our study, *SMN1* duplications were associated with susceptibility to progressive muscular atrophy (PMA), a motor neuron disease that specifically affects lower motor neurons. Since we found no association between altered *SMN* copy number and primary lateral sclerosis (PLS), a motor neuron disease that affects upper motor neurons, and especially since a 2021 study by Moisse *et al.* showed that altered copy numbers in the *SMN* locus do not associate with ALS, our study suggests the relevance of SMN protein levels in the processes underlying the loss of lower motor neurons in PMA.[19]
Although motor neuron vulnerability and axonal loss observed in patients with MMN is not explained by genetic variation within the *SMN* locus, this study does call for further research on SMN-related neurotoxicity in lower motor neuron syndromes such as PMA. SMN overexpression as observed in AAV-9-treated mice has been shown to exert neurotoxic effects and this may be mimicked by SMN1 gene duplication. Since the total number of duplications in our PMA cohort was relatively low (n=18 in 150 patients with PMA), the found association needs validation in a larger cohort of patients. iPSC-derived motor neurons from patients with PMA with different *SMN1* but equal *SMN2* copy numbers could then be used to investigate how SMN overexpression exerts neurotoxicity.

In **Chapter 3**, we investigated a previously described association between MMN and the MHC class II locus in more detail.[11] In a cohort of patients larger than in the initial study, we showed that MMN is associated with specific HLA class II haplotypes. We corroborated the previously described association with the DRB1*15 allele and extended it to the DRB1*15:01-DQB1*06:02 haplotype. We found a second association within the HLA class II region in the form of the HLA-DRB1*12:01-DQB1*03:01 haplotype. We suggest three hypotheses that could explain the association between the HLA class II locus and MMN (Fig. 1).
The first explanation revolves around the HLA class II-molecule itself and its function in the presentation of peptides to $CD4^+$ T-cells. Since GM1 itself is a non-peptide, direct presentation by HLA class II molecules is unlikely, a hypothesis that seems to be supported by the lack of association between the abovementioned haplotypes and anti-GM1 IgM antibodies or their fine epitope specificity.[20,21] A different explanation would assume that through the process of linked antigen presentation, currently unidentified peptides associated with GM1 and presented through HLA class II molecules would ensure co-stimulation that would lead to proliferation of GM1-autoreactive B-cells. However, this T cell-dependent process fails to explain why IgM is the only antibody isotype found in practically all patients with MMN, since T-cell engagement generally leads to class switching the production of IgG.

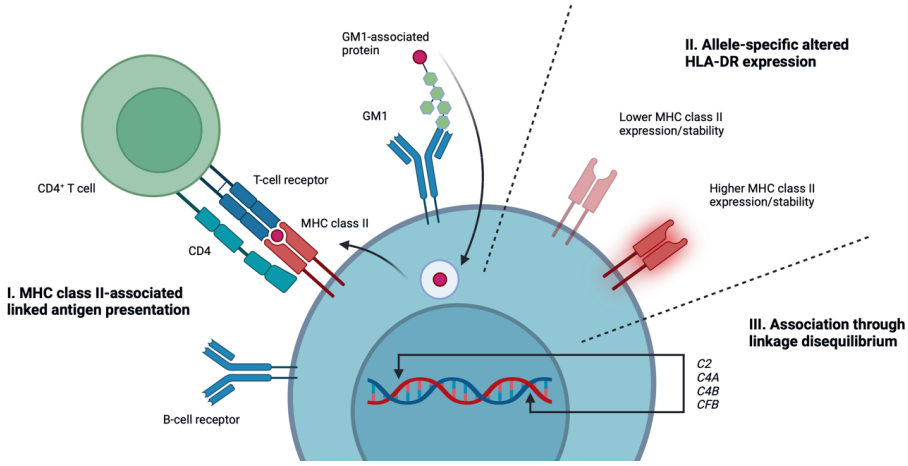

Figure 1. *Hypotheses on the explanation of MMN being associated with the HLA class II locus.*[86] *I) Certain HLA class II molecules may lead to stronger co-stimulation by CD4⁺ T-cells; II) Some HLA class II alleles lead to altered HLA class II expression, which is associated with autoimmunity; III) The association with the HLA class II region may be a flag for other genetic variants associated with MMN within the MHC III region, such as complement genes.*

A second explanation concerns the consequences of altered expression and stability that are intrinsic to certain HLA class II molecules.[22,23] For example, the association between HLA-DRB1*15:01 and multiple sclerosis (MS) is probably in part caused by hypomethylation of this specific allele, leading to increased HLA-DR expression on lymphocytes and monocytes.[24] Yet, such mechanisms inherent to hypomethylation would still fail to explain the presumed T-cell independent pathology in MMN. Alternatively, HLA class II alleles may be associated with lower expression or lower stability of HLA class II molecules.[22,23] Lower expression may be associated with autoimmunity by increasing the pool of autoreactive B-cells through defective negative selection.[25] Whether the MMN-associated haplotypes harbor epigenetic changes that have functional consequences is unknown. Future studies assessing methylation profiles and comparing HLA-DR and -DQ expression on lymphocytes and monocytes using flow cytometry (FACS) would address this issue.

Third and last, strong linkage disequilibrium within the MHC locus implies that any HLA association could serve as a tag for other disease-associated genes, for example those encoding components of the complement cascade in the MHC class III locus (Fig. 1 part III.). The MHC III locus contains the *C4A*, *C4B* and *C2* genes, encoding upstream classical complement pathway proteins, and the *CFB* gene that encodes factor B, a protein involved in formation of the alternative pathway's C3 convertase.[26,27] Complement activation is central in MMN immunopathogenesis (see below), and high innate activity of the classical pathway of complement is associated with MMN disease course.[28,29]

The results of chapters 3 and 5, discussed in more detail below, demonstrate that genetic variation underlies MMN susceptibility. To further our understanding of genetic susceptibility, it would be worthwhile to perform a genome-wide association study (GWAS), or immuno-targeted GWAS, in patients with MMN. Although the lack of statistical power intrinsic to studying rare diseases may complicate interpretation, it is important to note that the first-ever reported GWAS proved that sample sizes may give biologically meaningful insights.[30] Moreover, MMN is a relatively homogeneous disease both in clinical phenotype and antibody specificity. Yet, GWAS would not elucidate whether the HLA association with MMN is caused by linkage disequilibrium. Long-read sequencing that allows for genomic and epigenetic genotyping of long DNA segments simultaneously could be an alternative approach.[31,32]

Antibodies and their correlation with MMN phenotype

The presence of IgM antibodies against GM1 has been part of the first descriptions of MMN. GM1 is part of a family of molecules known as glycosphingolipids, consisting of a ceramide stalk with a variety of attached sugar groups. This variation causes antigenic variation that is reflected by the variety of anti-ganglioside antibodies, such as GM1, GM2, GD1a, GD1b and GQ1b (Fig. 2).[21]

The reported prevalence of anti-GM1 IgM antibodies in MMN initially varied strongly due to methodological differences in detection methods.[3,33-35] For example, anti-GM1 IgM antibodies are found in increased titers in about 50% of patients when using an ELISA-based method.[2] Their detection is increased to 67% using a glycoarray, and even to about 80% when looking for anti-GalC/GM1 IgM complex antibodies.[36] Yet, when using an *in vitro* iPSC-derived motor neuron model, basically all patients with MMN seem to harbor complement-activating anti-GM1 IgM antibodies, including those patients considered anti-GM1 IgM negative based on ELISA testing.[37] This shows that anti-GM1 IgM antibodies are present in more patients with MMN than previously reported, if not in all.

How anti-GM1 antibodies exert their effects on motor neurons has been studied in a rabbit model of acute motor axonal neuropathy (AMAN), a variant of the Guillain-Barré syndrome (GBS). This subacute pure-motor inflammatory neuropathy is associated with anti-GM1 antibodies of the IgG isotype. Susuki et al. showed that anti-GM1 IgG disrupts the normal clustering of voltage-gated sodium channels at the nodes of Ranvier, and in the paranodal region interferes in the interactions between the microvilli of Schwann cells and the motor neuron, both in a complement-dependent manner.[38] Indeed, both these nodal and paranodal changes have been implicated as the cause of conduction block in MMN.[39] Studies performed in an iPSC-derived motor neuron model have shown that patient-derived anti-GM1 IgM antibodies bind to the axolemma and upon binding activate the classical pathway of the complement cascade, ultimately leading to formation of the membrane attack complex (MAC) and causing structural motor neuron damage.[37] These experiments show that anti-GM1 antibodies are pathogenic in MMN, and that the activation of complement is crucial in this process.

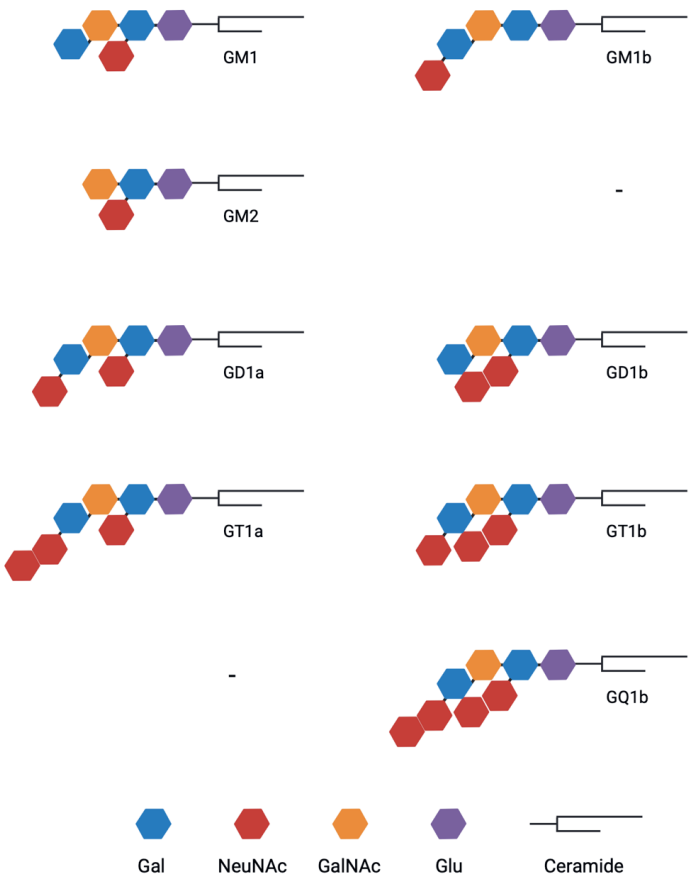

Fig. 2 *Molecular composition of gangliosides.*[21,86]
Gal = galactose; NeuNAc = N-acetylneuraminic acid; GalNAc = N-acetylgalactosamine; Glu = glucose

The relevance of anti-GM1 antibodies in the pathogenesis of MMN is further suggested by the correlation of more severe muscle weakness and axonal damage with increasing titers.[3,28,29,33] Whether other anti-ganglioside antibodies than those specific for GM1, such as anti-GD1b IgM, found in about 9% of patients and associated with vibration sense abnormalities, and anti-GM2 IgM antibodies, present in 6%-10% of patients with MMN, contribute to the immunopathogenesis of MMN was unclear.[2,40,41]

In **Chapter 7**, we show that anti-GM2 IgM antibodies in sera of patients with MMN bind to Schwann cells (SCs) and have the capacity to activate complement. High SC membrane expression of the complement regulating protein CD59 probably prevented complement-mediated SC lysis. Our data amend the disease model of MMN. First, by identifying a subgroup

of patients with a different and relevant antibody that is associated with an earlier onset of muscle weakness. Second, since patients with anti-GM2 IgM antibodies, apart from an earlier onset, show a similar disease course as patients without, we believe that the results of this study suggest that developing MMN may be a threshold-driven process. In this process, the nerve damage that leads to MMN is the result of a net effect of complement activation and inhibition that is skewed towards activation, which is likely most strongly driven by anti-GM1 IgM antibodies. Factors that further push this balance towards complement activation are higher anti-GM1 IgM titers, increased innate activity of the complement system, impaired complement inhibition (discussed below), and the presence of other complement-activating antibodies such as anti-GM2 IgM.[2,28,29] Third, the data support involvement of SCs in the disease model of MMN. SCs are immune-competent cells that express MHC class II molecules, can present glycolipids via CD1b, produce pro-inflammatory cytokines and express complement receptors.[42-44] Anti-GM2 IgM antibodies may therefore, through activation of SCs, amplify local inflammatory processes.

What explains the presence of anti-GM1 antibodies in MMN?

Anti-GM1 IgM antibodies are absent in umbilical cord blood, but appear during the first months of life, in concordance with other anti-bacterial anti-glycan IgM antibodies. This suggests that anti-GM1 IgM antibodies are probably formed as an innate immune response to bacteria colonizing the respiratory tract or gut.[45] The presence of anti-GM1 IgM antibodies by itself is therefore not abnormal. They form part of the repertoire of *natural antibodies*, an innate-like pool of antigen-independent IgM antibodies that bind to microbial epitopes and self-antigens with low specificity and low affinity.[35,46-48] The mechanisms that lead to the ongoing production of anti-GM1 IgM antibodies in higher titers are probably central to the pathogenesis of MMN.
The production of anti-GM1 IgM antibodies is probably the result of a single-cell event, as about 10% of patients with MMN have an IgM paraproteinemia and the large majority of MMN-associated anti-GM1 IgM antibodies are at least oligoclonal.[49,50] Yet, in contrast to, for example, anti-MAG polyneuropathy, the absence of clinical evidence of an increased risk of hematological malignancies suggests a non-malignant proliferation of a small group of B-cells autoreactive to GM1.[49-52] Currently, the mechanisms that lead to the activation, proliferation, and possibly ultrastructural B-cell receptor changes of GM1-reactive B-cells are unknown.

In the Guillain-Barré syndrome, anti-ganglioside antibodies are associated with preceding infections with *Campylobacter jejuni* and *Haemophilus influenzae*, both bacteria that can express GM1-like epitopes on the outer cell membrane.[53-55] Therefore, a molecular mimicry-like process underlying the production of anti-GM1 IgM antibodies in MMN is one of the possible mechanisms, although evidence for an association with exposure to GM1-presenting micro-organisms such as *C. jejuni* is lacking.[7,8,56] Although about 5% of patients with MMN have anti-GM1 IgA antibodies, molecular mimicry arising as a result from infection would fail to explain the lack of isotype switching to IgG in MMN.[2,35]

In addition to the antigenic drive provided by molecular mimicry, host factors may determine a person's likelihood of developing AMAN. Increased innate, i.e. antigen-independent, immune responses are an example. Huizinga *et al.* showed that dendritic cells, the major

antigen-presenting cell, of patients with GBS have increased innate immune responses to *C. jejuni*-derived LOS, as indicated by increased upregulation of CD38 and CD40, and increased production of type I interferons. Innate immune responses, mediated through Toll-like receptor 4 (TLR4), could fuel an exaggerated immune response.[57]
To further explore whether patients with MMN have increased innate immune responses, we stimulated blood of patients with MMN with lipopolysaccharide (LPS), and measured levels of various cytokines before and after stimulation (**Chapter 6)**. Patients with MMN did not seem to have altered TLR4-mediated innate immune responses to endotoxin. We corroborated data from another cytokine study in patients with MMN that showed a striking lack of systemic inflammation in patients with MMN.[58] The reported elevated cytokines concentrations in another study are likely the result of treatment with IVIg, rather than a consequence of the immunopathology underlying MMN.[59] Future studies could focus on the production of B cell stimulating cytokines, which emerge after longer periods of stimulation. MMN susceptibility and the production of anti-GM1 IgM antibodies do not seem associated with altered TLR4-mediated innate immune responses.

Since the formation of the gut microbiome in newborns precedes production of anti-GM1 IgM, and since B cell receptors of GM1-reactive B cells in patients with MMN use somatically mutated variable genes (V genes), we hypothesized that we should focus our attention to the mucosal interface between the immune system and the gut microbiome.[45,60] We performed the first gut microbiome study in patients with MMN and showed that patients with MMN, specifically those with anti-GM1 IgM antibodies, have an altered composition of gut bacteria (**Chapter 8**). *Eubacterium siraeum* and a genus of the Christensenellaceae family had a lower abundance in patients with MMN, whilst lower abundance of *Butyricicoccus* and a higher abundance of *Mogibacterium* were specifically associated with the presence of anti-GM1 IgM antibodies. Interestingly, all bacteria with lower abundances in MMN produce short-chain fatty acids (SCFAs), which have an anti-inflammatory effect on the host immune system.[61-64] *Mogibacterium* is regarded pro-inflammatory and is associated with chronic periodontitis.[65-68] Combinations of these associated genera have a synergistic effect on MMN susceptibility, since the theoretically most anti-inflammatory combination was less frequently found in patients with MMN (Fig. 3).

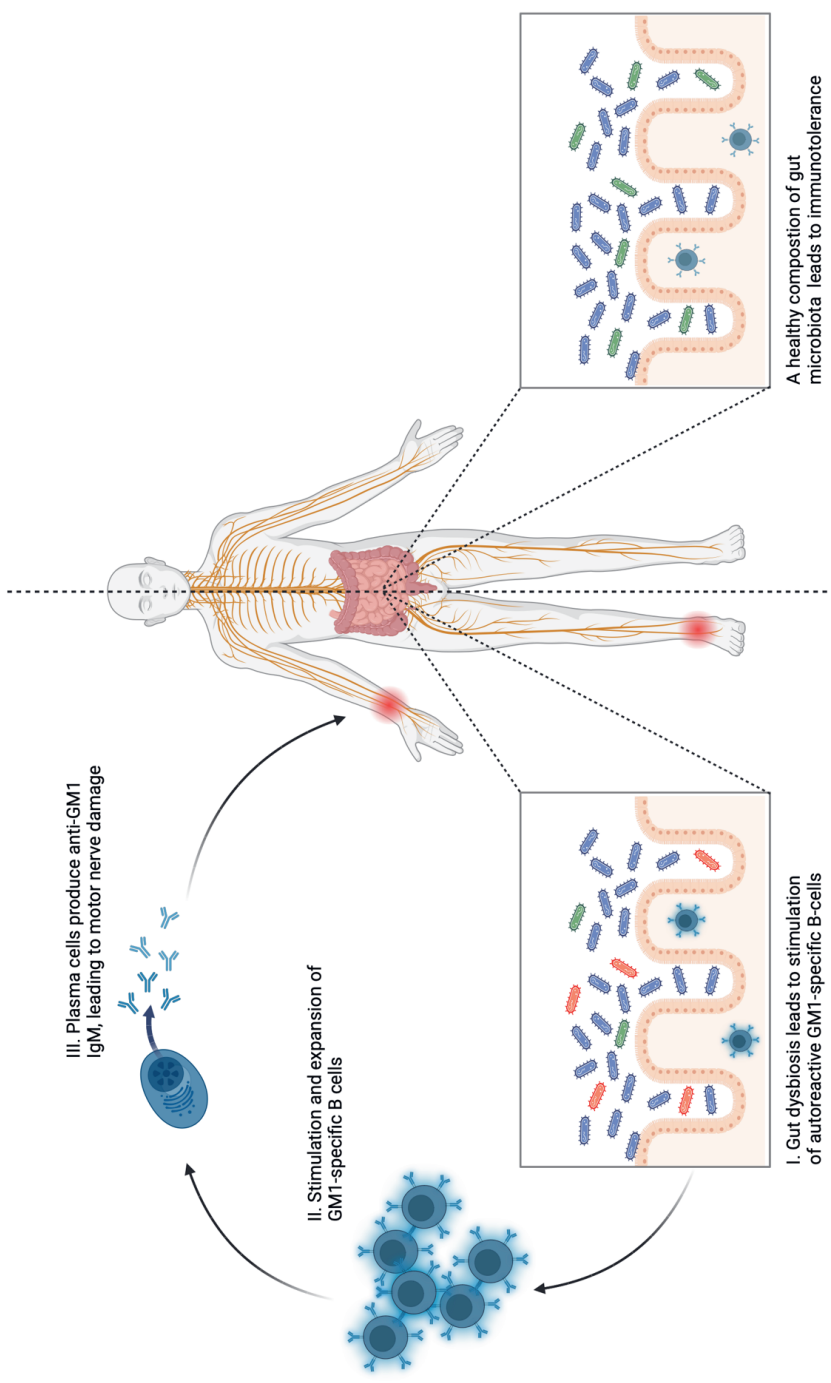

Fig. 3 *MMN is associated with an altered composition of bacteria constituting the gut microbiome. A lower abundance of bacteria with anti-inflammatory properties, combined with a higher abundance of the pro-inflammatory Mogibacterium contribute to MMN susceptibility.*[86]

This study, for the first time, links MMN susceptibility and anti-GM1 IgM antibodies to the composition of the gut microbiome, but we need to further elucidate underlying mechanisms. The association with genera with a lower abundance in patients could be biologically relevant if patients with MMN are indeed shown to have lower concentrations of short-chain fatty acids in blood or fecal water.[64] The finding of an association of anti-GM1 IgM antibodies with an increased abundance of *Mogibacterium* is surprising, since as a Gram-positive bacterium, it probably does not express GM1-like epitopes.[65] Yet, in a study assessing the binding potential of GM1-binding cholera toxin on gut bacteria of chicken, it was shown that GM1 can be found on bacteria other than *C. jejuni*, including bacteria of the Firmicutes family, to which *Mogibacterium* belongs.[69] *Mogibacterium* may via currently unknown mechanisms and despite being Gram-positive, express GM1-like epitopes. Experiments that demonstrate the binding of cholera toxin, a molecule known to bind to GM1 specifically, or patient-derived IgM or IgA to *Mogibacterium* would further support a more direct biological link between this bacterium and MMN-associated antibodies.[37] Finally, we do not yet know whether associations between MMN susceptibility and bacteria are limited to the gut, and the results of our study warrant further research on other microbiomes, such as the respiratory tract.

MMN pathogenesis – The complement system

Their potential to bind to motor neurons and activate complement probably explains the pathogenicity of antibodies in MMN. Anti-GM1 IgM antibodies activate the classical complement pathway, and *in vitro* studies showed that a high complement-activating potential of anti-GM1 IgM antibodies, and a higher innate complement activity are both associated with more severe muscle weakness in patients with MMN.[28,29] Moreover, the efficacy of immunoglobulin treatment seems to relate to inhibition of complement activation.[28,37] Various mechanisms have been proposed, including scavenging of complement components (C1, C3 and C4) and C3 convertases, and the effect of IVIg in MMN seems to correlate to the IgG increase after infusion.[28,70-72] Since complement activation may cause undesired tissue damage, cells innately express molecules that ensure complement inhibition via membrane-bound complement regulators such as CD46, CD55 and CD59.[73-75]

In **Chapter 5**, we investigated genetic variation of these complement regulating molecules and found that a 21-bp deletion polymorphism in the promotor region of *CD55* is associated with MMN susceptibility. Moreover, patients carrying this polymorphism probably have a better outcome after initiation of treatment with immunoglobulins. CD55, also known as decay-accelerating factor (DAF), inhibits the complement cascade at the C3 level by preventing the formation of C3 convertases, thereby inhibiting the formation of C3b and attenuating further complement activation through the C3 amplification loop (Fig. 4).[76] It thereby mimics the effect of IVIg, which by binding to C3b also inhibits the complement cascade at the C3 level.[71,77] By specifically acting on an impaired mechanism associated with MMN susceptibility, IVIg may have a stronger beneficial effect in patients carrying the 21bp deletion in the promotor region of *CD55*.

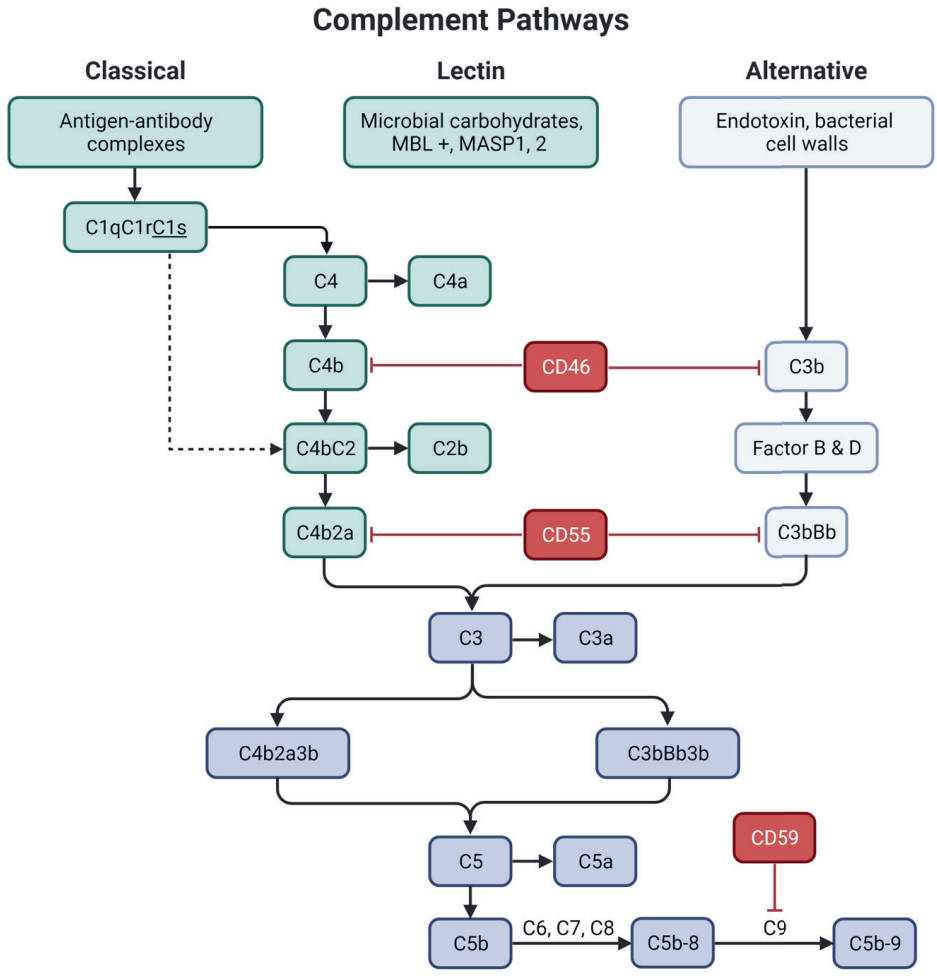

Fig. 4 *The complement system, consisting of the classical, lectin and alternative pathway. Anti-GM1 IgM antibodies activate the classical pathway, resulting in formation of C5b-9, also known as the membrane attack complex (MAC). The membrane-bound complement regulatory proteins CD46 and C55 inhibit the cascade proximally (i.e., at the pre-C5 level), and CD59 distally.*

We hypothesize that therapies targeting the proximal complement cascade may benefit all patients with MMN. Eculizumab, a monoclonal antibody inhibiting the distal complement cascade at the C5 level, has proven its benefit in other complement-mediated diseases such as neuromyelitis optica spectrum disorder (NMOSD), paroxysmal nocturnal hemoglobinuria (PNH), CHAPLE disease, and possibly in myasthenia gravis.[78-82] Yet, a clinical trial with eculizumab in patients with MMN failed to show a clear additional effect to treatment with IVIg, a finding consistent with the results of our study.[83] Studies assessing the expression of membrane-bound complement regulators CD46, CD55 and CD59 using peripheral nerve biopsies, biopsy-derived Schwann cell cultures and iPSC-derived motor neurons all show a moderate expression of CD55 and CD46 on neurons, and a high expression of CD59 on both neurons and Schwann cells.[75,84,85] This high innate CD59 expression probably explains why eculizumab, basically having a CD59-like mechanism of action, and genetic variation in the *CD59* promotor region do not affect MMN disease course after initiation of IVIg treatment. The same study assessed the therapeutic potential of an anti-C2 monoclonal antibody, which *in vitro* led to complete abrogation of the classical complement pathway at the C2 level.[75] At this moment, we know that formation of the membrane-attack complex (MAC) causes structural motor neuron damage *in vitro* and that inhibiting this process with immunoglobulin treatment partly alleviates this damage.[37] Yet, we currently do not know if and how products of the proximal complement cascade affect motor neurons. Future studies using iPSC-derived motor neuron models might give us more insight in these processes, especially since peripheral motor neurons express receptors for the proximal complement components C3a (C3aR) and C3b/C4b (CD35).[75] Increased pro-inflammatory cytokine production, a downstream effect of said receptors, may promote (local) inflammation. Interestingly, although complement is crucial for the pathogenicity of anti-GM1 IgM antibodies in MMN, complement-independent effects of these antibodies on motor neurons may be relevant as well.[37]

Extending the MMN disease model

The MMN disease model as described prior to this thesis explained the pathogenesis of MMN via three main processes. These encompass the production of anti-GM1 IgM antibodies by autoreactive B-cells, their binding to motor neurons, and the subsequent activation of complement. The research presented in this thesis has significantly increased our knowledge of the pathogenesis of MMN.

First, we have reconfirmed and extended the concept of MMN being an antibody-mediated disease. In Chapter 2, we reconfirmed the association of increased titers of anti-GM1 IgM antibodies with MMN severity, and in Chapter 7, we presented the novel finding of the biological and clinical relevance of anti-GM2 IgM antibodies in MMN. We found that whereas anti-GM1 IgM antibodies bind to neurites, anti-GM2 IgM antibodies bind to Schwann cells, and both subsequently activate complement. Patients with anti-GM2 IgM antibodies had a younger age at disease onset, but otherwise followed a similar disease course as patients with anti-GM2 IgM antibodies. This thesis adds anti-GM2 IgM to the repertoire of antibodies associated with MMN and suggests a possible role for Schwann cells in the local inflammatory processes that cause MMN.

Next, we focused on the elusive question regarding the origin of antibodies and, as described in Chapter 8, found that MMN is associated with a dysbiosis of gut microbiota. Patients with MMN lack a certain combination of bacteria that theoretically have a synergistic anti-

inflammatory effect on the host immune system. Especially since *Mogibacterium* was found to be associated with the presence of anti-GM1 IgM antibodies, this thesis extends the current disease model of MMN with microbial dysbiosis, be it of the gut or of other microbiomes, as a possible driving force behind the production of antibodies associated with MMN.
Lastly, we finetuned our knowledge of the complement system in MMN by incorporating complement regulation. In Chapter 5, we found that MMN is associated with a *CD55* promotor polymorphism, which was associated with MMN disease course. Next to corroborating the idea of genetic susceptibility underlying MMN (see also Chapter 3), this thesis indicates that despite MAC formation being the final product of the complement activation which *in vitro* leads to motor neuron damage, the proximal (i.e. pre-C5) complement cascade may serve as a target for novel therapies.

Concluding remarks and future directions

This thesis yielded new insights in the relevance of antibody specificity, the origin of antibodies, and genetic contributions to MMN susceptibility. The results of the studies converge on the interaction of antibodies and complement and provide clues for improved treatment. I hope follow-up studies will follow soon (Fig. 5). The first study assessing the long-term safety and efficacy of an anti-C2 monoclonal antibody in patients with MMN has started in 2022 (trial number NCT05225675); results will be available in 2024. If successful, the approach to dissect the immunopathology of MMN may eventually benefit the broader group of patients with inflammatory neuropathies mediated by anti-ganglioside antibodies.

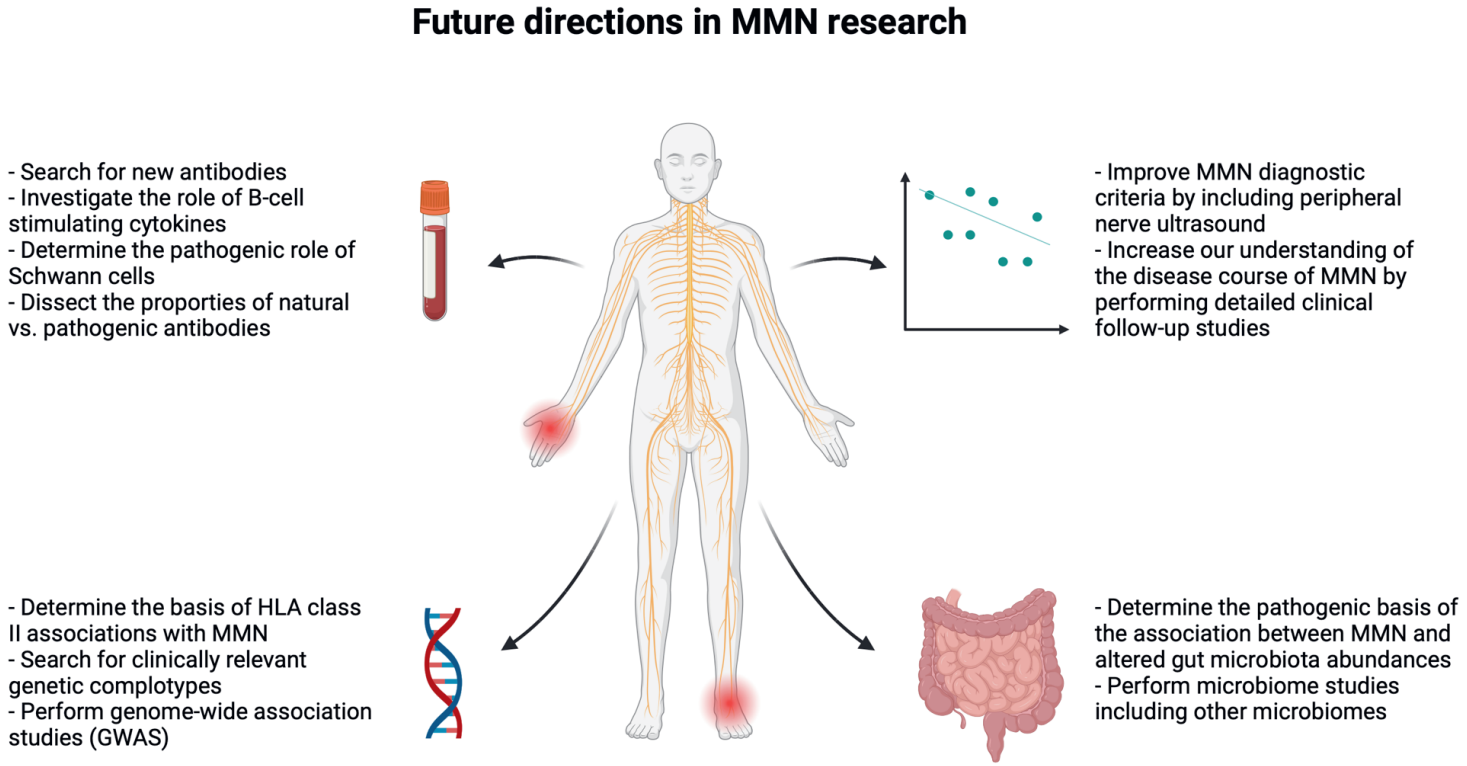

Fig. 5 Suggestions for further research in the field of MMN.

References

1. Pestronk A, Cornblath DR, Ilyas AA, et al. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. Ann Neurol 1988;24(1):73-78

2. Cats EA, Jacobs BC, Yuki N, et al. Multifocal motor neuropathy: association of anti-GM1 IgM antibodies with clinical features. Neurology 2010;75(22):1961-1967

3. Cats EA, van der Pol WL, Piepers S, et al. Correlates of outcome and response to IVIg in 88 patients with multifocal motor neuropathy. Neurology. 2010;75:818-825

4. Lange DJ, Weimer LH, Trojaborg W, et al. Multifocal motor neuropathy with conduction block: slow but not benign. Arch Neurol. 2006;63:1778-1781

5. Loscher WN, Oberreiter EM, Erdler M, et al. Multifocal motor neuropathy in Austria: a nationwide survey of clinical features and response to treatment. J Neurol. 2018;265:28342840

6. Herraets IJT, Goedee HS, Telleman JA, et al. Nerve ultrasound improves detection of treatment-responsive chronic inflammatory neuropathies. Neurology 2020;94(14):e1470-e1479

7. Vlam L, van der Pol WL, Cats EA, et al. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. Nat Rev Neurol 2011;8(1):48-58

8. Harschnitz O, Jongbloed BA, Franssen H, et al. MMN: from immunological cross-talk to conduction block. J Clin Immunol 2014;34(Suppl 1):S112-119

9. Cats EA, Bertens ASB, Veldink JH, et al. Associated autoimmune diseases in patients with multifocal motor neuropathy and their family members. J Neurol 2012;259(6):1137-1141

10. Vlam L, Cats EA, Seelen M, et al. Multifocal motor neuropathy is not associated with genetic variation in PTPN22, BANK1, Blk, FCGR2B, CD1A/E, and TAG-1 genes. J Peripher Nerv Syst 2011;16(3):175-179

11. Sutedja NA, Otten HG, Cats EA, et al. Increased frequency of HLA-DRB1*15 in patients with multifocal motor neuropathy. Neurology 2010;74(10):828-83

12. Singh RN, Howell MD, Ottesen EW, et al. Diverse role of survival motor neuron protein. Biochim Biophys Acta Gene Regul Mech 2017;1860(3):299-315

13. Lauria F, Bernabò P, Tebaldi T, et al. SMN-primed ribosomes modulate the translation of transcripts related to spinal muscular atrophy. Nat Cell Biol 2020;22(10):1239-1251

14. Blauw, HM, Barnes, CP, Van Vught, PWJ, et al. SMN1 gene duplications are associated with sporadic ALS. Neurology 2012;78(11):776-780

15. Veldink, JH, Kalmijn, A, Van der Hout, AH, et al. SMN genotypes producing less SMN protein increase susceptibility to and severity of sporadic ALS. Neurology 2005;65(6):820-825

16. Wang, X, Cui, N, Gao, J, Qiu, X., Zheng, F. SMN1 duplications contribute to sporadic amyotrophic lateral sclerosis susceptibility: evidence from a meta-analysis. J Neurol Sci 2014;340(1-2):63-68

17. Vlam L. (2015). Multifocal motor neuropathy and progressive muscular atrophy. Chapter 7. Ph. D. Thesis. Utrecht University

18. Van Alstyne M, Tattoli I, Delestrée N, et al. Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit. Nat Neurosci 2021;24(7):930-940.

19. Moisse M, Zwamborn RAJ, Van Vugt J, et al. The effect of SMN gene dosage on ALS risk and disease severity. Ann Neurol 2021;89(4):686-697

20. Bos JW, Otten HG, Herraets IJT, et al. High-resolution mapping identifies HLA class II associations with multifocal motor neuropathy. Neurobiol Aging 2021;101:79-84

21. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002;125:2591-2625

22. Dendrou CA, Petersen J, Rossjohn J, et al. HLA variation and disease. Nat Rev Immunol 2018;18(5):325-339

23. Miyadera H, Ohashi J, Lernmark Å, et al. Cell-surface MHC density profiling reveals instability of autoimmunity-associated HLA. J Clin Invest 2015;125(1):275-291

24. Kular L, Liu Y, Ruhrmann S, et al. DNA methylation as a mediator of HLA -DRB1*15:01 and a protective variant in multiple sclerosis. Nat Commun 2018;9(1):2397

25. Chen JW, Schickel J-N, Tsakiris N, et al. Positive and negative selection shape the human naïve B cell repertoire. J Clin Invest 2022;132(2):e150985

26. Matzaraki V, Kumar V, Wijmenga C, et al. The MHC locus and genetic susceptibility to autoimmune and infectious diseases. Genome Biol 2017;18(1):76

27. Gruen JR, Weissman SM. Human MHC class III and IV genes and disease associations. Front Biosci 2001;6;960-972

28. Piepers S, Jansen MD, Cats EA, et al. IVIg inhibits classical pathway activity and anti-GM1 IgM-mediated complement deposition in MMN. J Neuroimmunol 2010;229(1-2):256-262

29. Vlam L, Cats EA, Harschnitz O, et al. Complement activity is associated with disease severity in multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2015;2(4):e119

30. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. Science 2005;308(5720):385-389

31. Nurk S, Koren S, Rhie A, et al. The complete sequence of a human genome. Science 2022;376(6588):44-53

32. Vollger MR, Guitart X, Dishuck PC, et al. Segmental duplications and their variation in a complete human genome. Science 2022;376(6588):eabj6965

33. Herraets I, Van Rosmalen M, Bos J, et al. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. Neurology 2020;95(14):1979-1987

34. Van Schaik IN, Bossuyt PM, Brand A, et al. Diagnostic value of GM1 antibodies in motor neuron disorders and neuropathies: a meta-analysis. Neurology 1995;45(8):1570-1577

35. Kuijf ML, Van Doorn PA, Tio-Gilles AP, et al. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. J Neurol Sci 2005;239(1):37-44

36. Galban-Horcajo F, Vlam L, Delmont E, et al. The diagnostic utility of determining anti-GM1:GalC complex antibodies in multifocal motor neuropathy: a validation study. J Neuromuscul Dis 2015;2(2):157-165

37. Harschnitz O, van den Berg LH, Johansen LE, et al. Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. Ann Neurol 2016;80(1):71-88

38. Susuki K, Rasband MN, Tohyama K, et al. Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. J Neurosci 2007;27(15):3956-3967

39. Franssen H. The node of Ranvier in multifocal motor neuropathy. J Clin Immunol 2014;34(Suppl 1):S105-111

40. Nobile-Orazio E, Gallia F, Terenghi F, et al. How useful are anti-neural IgM antibodies in the diagnosis of chronic immune-mediated neuropathies? J Neurol Sci 2008;266(1-2):156-163

41. Nobile-Orazio E, Giannotta C, Musset L, et al. Sensitivity and predictive value of anti-GM1/galactocerebroside IgM antibodies in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry 2014;85(7):754-758

42. Meyer zu Hörste G, Hu W, Hartung HP, et al. The immunocompetence of Schwann cells. Muscle Nerve 2008;37(1):3-13

43. Ydens E, Lornet G, Smits V, et al. The neuroinflammatory role of Schwann cells in disease. Neurobiol Dis 2013;55:95-103

44. Van Rhijn I, Van den Berg LH, Bosboom WM, et al. Expression of accessory molecules for T-cell activation in peripheral nerve of patients with CIDP and vasculitic neuropathy. Brain 2000;123(10):2020-2029

45. Alaniz ME, Lardone RD, Yudowski SL, et al. Normally occurring human anti-GM1 immunoglobulin M antibodies and the immune response to bacteria. Infect Immun 2004;72(4):2148-2151

46. García Guijo C, García-Merino A, Rubio G. Presence and isotype of anti-ganglioside antibodies in healthy persons, motor neuron disease, peripheral neuropathy, and other diseases of the nervous system. J Neuroimmunol 1995;56(1):27-33

47. Holdick NE, Rodríguez-Zhurbenko N, María Hernández A. Defining natural antibodies. Front Immunol 2017;8;872

48. Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. J Immunol 2015;194(1):13-20

49. Vlam L, Piepers S, Sutedja NA, et al. Association of IgM monoclonal gammopathy with progressive muscular atrophy and multifocal motor neuropathy: a case-control study. J Neurol 2015;262(3):666-673

50. Cats EA, Van der Pol WL, Tio-Gillen AP, et al. Clonality of anti-GM1 IgM antibodies in multifocal motor neuropathy and the Guillain-Barré syndrome. J Neurol Neurosurg Psychiatry 2015;86(5):502-504

51. Steck AJ. Anti-MAG neuropathy: from biology to clinical management. J Neuroimmunol 2021;361:577725

52. Van de Mortel JPM, D'Sa S, Vrancken AFJE, et al. Polyneuropathy associated with IgM monoclonal gammopathy: advances in genetics and treatment, focusing on anti-MAG antibodies. Hemato 2022;3(4):663-688

53. Yuki N, Yoshino H, Sato S, et al. Acute axonal polyneuropathy associated with anti-GM1 antibodies following Campylobacter enteritis. Neurology 1990;40(12):1900-1902

54. Jacobs BC, Van Doorn PA, Schmitz PI, et al. Campylobacter jejuni infections and anti-GM1 antibodies in Guillain-Barré syndrome. Ann Neurol 1996;40(2):181-187

55. Mori M, Kuwabara S, Miyake M, et al. Haemophilus influenzae infection and Guillain-Barré syndrome. Brain 2000;123(10):2171-2178

56. Terenghi F, Allaria S, Scarlato G, et al. Multifocal motor neuropathy and Campylobacter Jejuni reactivity. Neurology 2002;59(2):282-284

57. Huizinga R, Van den Berg B, Van Rijs W, et al. Innate immunity to campylobacter jejuni in Guillain-Barré syndrome. Ann Neurol 2015;78(3):343-354

58. Vlam L, Stam M, De Jager W, et al. Cytokine profiles in multifocal motor neuropathy and progressive muscular atrophy. J Neuroimmunol 2015;286:1-4

59. Furukawa T, Matsui N, Fujita K, et al. Increased proinflammatory cytokines in sera of patients with multifocal motor neuropathy. J Neurol Sci 2014;346(1-2):75-79

60. Paterson G, Wilson G, Kennedy PG, et al. Analyse of anti-GM1 ganglioside IgM antibodies cloned from motor neuropathy patients demonstrates diverse V region gene usage with extensive somatic hypermutation. J Immunol 1995;155(6):3049-3059

61. Waters JL, Ley RE. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. BMC Biol 2019;17(1):83

62. Ye D, Huang J, Wu J, et al. Integrative metagenomic and metabolomic analyses reveal gut microbiota-derived multiple hits connected to development of gestational diabetes mellitus in humans. Gut Microbes 2023;15(1):2154552

63. Eeckhaut V, Machiels K, Perrier C, et al. Butyricicoccus pullicaecorum in inflammatory bowel disease. Gut 2013;62(12):1745-1752

64. Kim CH. Control of lymphocyte functions by gut microbiota-derived short-chain fatty acids. Cell Mol Immunol 2021;18(5):1161-1171

65. Nakazawa F, Sato M, Poco SE, et al. Description of Mogibacterium pumilum gen. nov., sp. nov. and Mogibacterium vescum gen. nov., sp. nov., and reclassification of Eubacterium timidum (Holdeman et al. 1980) as Mogibacterium timidum gen. nov., comb. nov. Int J Syst Evol Microbiol 2000;50(2):679-688

66. Casarin RCV, Saito D, Santos VR, et al. Detection of Mogibacterium timidum in subgingival biofilm of aggressive and non-diabetic and diabetic chronic periodontitis patients. Braz J Microbiol 2012;43(3):931-937

67. Nibali L, Sousa V, Davrandi M, et al. Differences in the periodontal microbiome of successfully treated and persistent aggressive periodontitis. J Clin Periodontol 2020;47(8):980-990

68. Li Q, Pu Y, Lu H, et al. Porphyromonas, Treponema, and Mogibacterium promote IL8/IFNγ/TNFα- based pro-inflammation in patients with medication-related osteonecrosis of the jaw. J Oral Microbiol 2020;13(1):1851112

69. Patry RT, Stahl M, Perez-Munoz ME, et al. Bacterial AB5 toxins inhibit the growth of gut bacteria by targeting ganglioside-like glycoconjugates. Nat Commun 2019;10(1):1390

70. Liu X, Cao W, Li T. High-dose intravenous immunoglobulins in the treatment of severe acute viral pneumonia: the known mechanisms and clinical effects. Front Immunol 2020;11:1660

71. Lutz HA, Stammler P, Bianchi V, et al. Intravenously applied IgG stimulates complement attenuation in a complement-dependent autoimmune disease at the amplifying C3 convertase level. Blood 2004;103(2):465-472

72. Vlam L, Cats EA, Willemse E, et al. Pharmacokinetics of intravenous immunoglobulin in multifocal motor neuropathy. J Neurol Neurosurg Psychiatry 2014;85(10):1145-1148

73. Noris M, Remuzzi G. Overview of complement activation and regulation. Semin Nephrol. 2013;33(6):479-492

74. Mathern DR, Heeger PS. Moleculues great and small: the complement system. Clin J Am Soc Nephrol. 2015;10(9):1636-1650

75. Budding K, Johansen LE, Van de Walle I, et al. Anti-C2 antibody ARGX-117 inhibits complement in a disease model for multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2021;9(1):e1107

76. Lukacik P, Roversi P, White J, et al. Complement regulation at the molecular level: the structure of decay-accelerating factor. PNAS 2004;101(5):1279-1284

77. Lutz HU, Stammler P, Jelezarova E, et al. High dosis of immunoglobulin G attenuate immune aggregate-mediated complement activation by enhancing physiologic cleavage of C3b in C3bn-IgG complexes. Blood 1996;88(1):184-193

78. Versmold K, Alashkar F, Raiser C, et al. Long-term outcomes of patients with paroxysmal nocturnal hemoglobulinuria treated with eculizumab in a real-world setting. Eur J Haematol 2023;111(1):84-95

79. Pittock SJ, Fujihara K, Palace J, et al. Eculizumab monotherapy for NMOSD: data from PREVENT and its open-label extension. Mult Scler 2022;28(3):480-486

80. Ozen A, Kasap N, Vujkovic-Cvijin I, et al. Broadly effective metabolic and immune recovery with C5 inhibition in CHAPLE disease. Nat Immunol 2021;22(2):128-139

81. Howard Jr JF, Utsugisawa K, Benatar M, et al. Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study. Lancet Neurol 2017;16(12):976-986

82. Howard Jr JF, Barohn RJ, Cutter GR, et al. A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis. Muscle Nerve 2013;48(1):76-84

83. Fitzpatrick AM, Mann CA, Barry S, et al. An open label clinical trial of complement inhibition in multifocal motor neuropathy. J Peripher Nerv Syst 2011;16(2):84-91

84. Vedeler C, Ulvestad E, Bjørge L, et al. The expression of CD59 in normal human nervous tissue. Immunology 1994;82(4):542-547

85. Koski CL, Estep AE, Sawant-Mane S, et al. Complement regulatory molecules on human myelin and glial cells: differential expression affects the deposition of activated complement proteins. J Neurochem 1996;66(1):303-312

86. Created with BioRender.com

Appendices

Samenvatting in het Nederlands

Voor mensen met MMN, hun naasten, en de leek

In het proefschrift dat voor u ligt, vindt u verschillende onderzoeken die ik tijdens mijn promotietraject heb mogen uitvoeren. Alle onderzoeken richtten zich op de aandoening multifocale motorische neuropathie (MMN), een heel zeldzame ziekte van zenuwen in armen en benen. Ze zijn echter geschreven op een manier die doorgaans minder toegankelijk is voor de leek; ze zijn geschreven in het Engels, gaan over ingewikkelde biologische processen, en maken gebruik van vaktaal. Ik wil de gelegenheid van mijn proefschrift aangrijpen om mijn onderzoek op een begrijpelijke manier uit te leggen. Enerzijds voor mensen om mij heen die geen medische achtergrond hebben, maar in het bijzonder voor mensen met MMN en hun naasten, die door hun grote bereidwilligheid om deel te nemen aan wetenschappelijk onderzoek nieuwe kennis over MMN mogelijk hebben gemaakt.

Multifocale motorische neuropathie

MMN is een heel zeldzame neurologische aandoening, die voor het eerst werd beschreven in de jaren '80. De diagnose wordt in Nederland zo'n 5-6 keer per jaar gesteld en in totaal zijn er zo'n 225-250 Nederlanders met MMN. Zeker als u deze getallen afzet tegen die van andere aandoeningen die als zeldzaam worden beschouwd, zoals MS of ALS met ieder zo'n 500 nieuwe diagnoses per jaar, heeft u een idee van de zeldzaamheid van MMN.

De aandoening treft vooral mannen - zo'n 70% is man - en leidt bij zowel mannen als vrouwen tot symptomen vanaf de leeftijd van 35-45 jaar, met uitschieters richting de 20 en 70 jaar. De exacte oorzaak van MMN is niet geheel bekend, maar we weten dat het een ontstekingsziekte is van de zenuwen in armen en benen, en dat het nooit het ruggenmerg of de hersenen aantast. Het geeft hoofdzakelijk een probleem van de zenuwen die de spieren moeten aansturen, wat leidt tot spierzwakte. Die is in de regel opvallend asymmetrisch en treft vooral spieren van de handen en de voeten, en soms van de bovenarmen of -benen. De gemiddelde persoon met MMN heeft daardoor voornamelijk last van een verminderde handvaardigheid, wat zich kan uiten in moeite met schrijven of een verminderde fijne motoriek, of van een probleem met het lopen door een klapvoet. Vrijwel iedereen met MMN heeft beperkende klachten, maar bij zo'n 20% is sprake van een ernstige beperking. Hierbij moet u denken aan mensen bij wie MMN beide handen dusdanig heeft aangetast dat ze geen wezenlijke functie meer hebben, of bij wie de spierzwakte in de benen dermate ernstig is dat een rolstoel noodzakelijk is. Doordat de ademhalings- en hartspier in de regel niet worden aangetast gaat MMN niet gepaard met een beperkte levensverwachting.

Gelukkig is er voor mensen met MMN een behandeling met een middel dat immunoglobuline wordt genoemd. Dit medicijn, dat bestaat uit samengevoegde antistoffen verkregen uit het bloed van mensen die hun bloed doneren, wordt elke zoveel weken via een infuus direct in de bloedbaan (intraveneus) toegediend en wordt daarom IVIg genoemd (**I**ntra**V**eneus **I**mmuno**g**lobuline). Hoe dit middel exact werkt is niet geheel bekend, maar het leidt in de regel tot een duidelijke en langdurige toename van de spierkracht. Toch zijn er mensen met MMN die vanwege bijwerkingen dit medicijn niet kunnen krijgen, of bij wie ondanks behandeling met IVIg de spierkracht achteruitgaat. Voor hen is er op dit moment helaas nog geen alternatief.

Het afweersysteem, antistoffen, en MMN
Het wetenschappelijk onderzoek in het UMC Utrecht richt zich hoofdzakelijk op de vraag hoe MMN zich ontwikkelt, omdat wij geloven dat een beter inzicht in deze processen kan leiden tot nieuwe, betere behandelingen. Voordat de onderzoeken in dit proefschrift werden uitgevoerd was er al een duidelijk idee over de ziektemechanismen die leiden tot MMN. Centraal hierin staat een antistof, *anti-GM1 IgM* genaamd, die bij minstens 50% van de mensen met MMN wordt gevonden.

Ons afweersysteem helpt ons om gezond te blijven, onder andere door ons te beschermen tegen bacteriën en virussen. Dat doet het bijvoorbeeld door antistoffen te maken, eiwitten die de vorm van een i-grec (Y) hebben. Met de bovenste twee armen van de Y-vorm binden ze aan een specifiek molecuul dat bijvoorbeeld op zo'n virus of bacterie zit, en met het onderste deel kunnen ze vervolgens delen van het immuunsysteem activeren, waardoor een bacterie of virus wordt opgeruimd. Welke delen van het immuunsysteem geactiveerd worden is afhankelijk van het type antistof. Antistoffen helpen ons dus om gezond te blijven, maar soms maakt ons afweersysteem een vergissing. Het produceert dan bijvoorbeeld antistoffen die niet (alleen) aan bacteriën of virussen binden, maar (ook) aan onze eigen cellen. Wat dan volgt is een ziekte als gevolg van een immuunreactie van ons eigen immuunsysteem tegen onze eigen cellen. Dat noemen we een auto-immuunziekte.
In het geval van de auto-immuunziekte MMN binden anti-GM1 antistoffen van het type IgM aan het molecuul GM1 dat zich op zenuwcellen bevindt. Hoe meer van deze antistof iemand heeft, hoe ernstiger de spierzwakte is. De antistoffen activeren het complementsysteem, een onderdeel van het immuunsysteem dat bestaat uit een groep van negen eiwitten (C1 t/m C9 genaamd), die elkaar één voor één activeren. Gaandeweg dit proces, dat we de complementcascade noemen, raken meer en meer moleculen geactiveerd - een soort sneeuwbaleffect. Uiteindelijk vormen meerdere C9-eiwitten een cirkel op het celmembraan van de zenuwcel, het *membrane attack complex* (MAC) genaamd. Dit MAC prikt een gaatje in de zenuwcel, wat leidt tot schade. We weten dat IVIg de activatie van complementeiwitten, en daarmee schade aan zenuwcellen, gedeeltelijk stopt.

Samenvatting van het proefschrift

Er was dus een goed begrip over de ontstaanswijze en de ziekteprocessen van MMN. Toch bleven er nog veel vragen onbeantwoord. Hoe is bijvoorbeeld het beloop van MMN op de lange termijn, en zijn er factoren die het verloop voorspellen? Er is een bepaalde genetische gevoeligheid voor het ontwikkelen van MMN? Zijn er naast anti-GM1 IgM antistoffen nog andere antistoffen die bijdragen aan het ontwikkelen van MMN? En hoe komt iemand eigenlijk aan anti-GM1 IgM antistoffen?
Deze vragen vormden de basis voor mijn proefschrift. In onderstaande alinea's neem ik de hoofdstukken van mijn proefschrift met u door en leg ik uit wat voor onderzoek we gedaan hebben en waarom de resultaten relevant zijn voor een beter begrip van MMN

In **Hoofdstuk 2** beschrijven we de resultaten van een onderzoek naar het langdurige beloop van MMN. Zowel in 2007 als in 2015 zijn in een groep mensen met MMN o.a. metingen van de spierkracht in armen en benen verricht. Tussen 2007 en 2015 zagen we dat achteruitgang van de spierkracht eerder regel dan uitzondering was, ondanks behandeling met IVIg. Mensen

met MMN die in 2007 meer spierzwakte hadden, hadden dat in 2015 nog steeds, en de aanwezigheid van anti-GM1 IgM antistoffen correleerde met ernstigere spierzwakte. Deze resultaten geven ons enig idee van welke mensen met MMN een ernstiger beloop kunnen hebben, maar bovenal laat het zien dat MMN, ondanks behandeling met IVIg, een progressieve ziekte is. Dat sterkt het idee dat er nieuwe, betere behandelingen nodig zijn. Hiervoor is een betere kennis over de ontstaanswijze van MMN noodzakelijk.

In Hoofdstuk 3, 4 en 5 verkenden we de mogelijkheid van genetische risicofactoren voor MMN. MMN is geen erfelijke ziekte; mensen met MMN krijgen geen kinderen die ook MMN krijgen, en MMN komt niet vaker voor binnen families. Toch zou het mogelijk kunnen zijn dat mensen met MMN een bepaalde genetische opmaak hebben die een groter risico geeft op het ontwikkelen van MMN. Zulke risicofactoren kunnen ons dan soms ook iets leren over relevante ziektemechanismen.

In **Hoofdstuk 3** onderzochten we bepaalde genen in ons erfelijk materiaal (DNA), die betrokken zijn bij het activeren van ons immuunsysteem na een infectie. Wanneer een bacterie ons lichaam binnendringt worden kleine delen van zo'n bacterie door bepaalde immuuncellen aan ons immuunsysteem getoond, zodat er een afweerreactie op gang kan komen. De 'dienbladen' die deze cellen hiervoor gebruiken, worden HLA-eiwitten genoemd en er bestaat een enorme variatie in hoe de 'dienbladen' eruitzien. Wij hebben gekeken naar varianten in de genen voor de eiwitten met de namen HLA-DRB1, HLA-DQA1 en HLA-DQB1. Verschillende HLA-eiwitten kunnen op verschillende manieren hun deeltjes presenteren en dus een verschillende mate van activatie van het immuunsysteem veroorzaken. Dit is dan ook de reden dat genetische varianten in deze HLA-eiwitten gelinkt zijn aan allerlei soorten auto-immuunziekten.
Wij vonden twee varianten in het HLA-DRB1 gen vaker bij mensen met MMN dan bij mensen zonder MMN. Deze varianten heten HLA-DRB1*15:01 en HLA-DRB1*12:01. Om na te gaan of een grotere gevoeligheid om MMN te ontwikkelen een direct gevolg kon zijn van deze genetische link, gingen we na of er een relatie was tussen deze genetische varianten en de aanwezigheid van anti-GM1 antistoffen, de hoogte hiervan, of de ernst van het ziektebeloop. Dit bleek niet het geval. Daarom concluderen we dat de gevoeligheid om MMN te ontwikkelen weliswaar gelinkt is aan deze genetische varianten van het HLA-DRB1-eiwit, maar dat dat minder waarschijnlijk komt door een effect van het HLA-eiwit zelf. Vrijwel direct naast het HLA-DRB1 gen liggen genen voor bepaalde eiwitten van het complementsysteem. Mogelijk is het signaal dat we opgepikt hebben een marker voor het werkelijke signaal dat in een van deze naburige genen ligt.

Motorische zenuwcellen hebben een bepaald eiwit nodig om goed te functioneren, het SMN-eiwit genaamd. De totale hoeveelheid van dit eiwit wordt met name bepaald door het aantal kopieën van het SMN1-gen, en in mindere mate van het SMN2-gen. Normaliter hebben we van ieder gen twee kopieën; één van moeder, en één van vader. De genen SMN1 en SMN2 hebben hierin echter een grote variatie, tot wel vijf kopieën van ieder. Als iemand geen SMN1 kopieën heeft en dus heel weinig SMN-eiwit, ontwikkelt hij of zij de spierziekte SMA. Er zijn echter ook onderzoeken gedaan naar ALS, die suggereerden dat juist een tevéél aan SMN1-kopieën, en dus waarschijnlijk een teveel aan SMN-eiwit, eveneens schadelijk kan zijn voor motorische zenuwcellen.
Er zijn verschillende aandoeningen waarbij er specifiek een probleem is van motorische zenuwcellen. MMN is hier een voorbeeld van, net als bepaalde varianten van ALS, PSMA en

PLS genaamd. In **Hoofdstuk 4** onderzochten we het aantal SMN1- en SMN2-kopieën in deze drie aandoeningen. Het aantal kopieën van zowel SMN1 als SMN2 verschilde niet tussen mensen met en zonder MMN. De specifieke gevoeligheid voor schade van motorische zenuwen bij MMN wordt dus niet verklaard door verschillen in het aantal kopieën in deze genen.

In hoofdstuk 5 keken we naar enkele andere interessante genen, maar voor de loop van het verhaal neem ik u eerst mee naar **Hoofdstuk 6** van mijn proefschrift. Ons afweersysteem bestaat grofweg uit twee delen. Enerzijds het verworven afweersysteem, dat heel specifiek reageert op één micro-organisme. Activatie hiervan leidt tot de vorming van antistoffen en tot immunologisch geheugen. Aangezien mensen met MMN bepaalde antistoffen produceren, is dit deel van het immuunsysteem betrokken bij MMN. Anderzijds is er het aangeboren afweersysteem, dat juist níet specifiek is; het raakt actief bij haast alle infecties met virussen of bacteriën. Cellen van het aangeboren afweersysteem produceren na activatie signaalmoleculen, cytokinen genaamd, die een effect hebben op cellen van het verworven afweersysteem. Cytokinen vormen dus een brug tussen beide afweersystemen.
Wij wilden onderzoeken of mensen met MMN een overmatig actief aangeboren afweersysteem hebben. Zo ja, dan zou dit een oorzaak kunnen zijn van een overactief verworven immuunsysteem, dat op zijn beurt doorslaat naar auto-immuniteit. We hebben vers bloed (met dus nog levende cellen daarin) van mensen met en zonder MMN blootgesteld aan een bepaald bacterieel molecuul, LPS genaamd. Het aangeboren immuunsysteem reageerde op de aanwezigheid van dit molecuul door cytokinen te produceren, en na vier uur blootstelling hebben we de concentraties van een aantal van deze cytokinen gemeten. We vonden geen grote verschillen tussen mensen met en zonder MMN. Wel zagen we na vier uur blootstelling een hogere concentratie van het cytokine IL-21 bij mensen met MMN die anti-GM1 IgM antistoffen hebben. Mogelijk is er een rol weggelegd voor IL-21 in de productie van anti-GM1 IgM antistoffen, maar onze conclusie luidt dat er geen sprake lijkt van een algeheel actiever aangeboren afweersysteem bij mensen met MMN.

Zoals hiervoor besproken staat de antistof anti-GM1 IgM centraal in het ziektemechanisme dat leidt tot MMN. In **Hoofdstuk 7** laten we echter zien dat zo'n 10% van de mensen met MMN (ook) een andere antistof heeft, anti-GM2 IgM. We weten dat anti-GM1 IgM antistoffen aan de zenuw zelf binden en daar leiden tot activatie van complement. Wij zagen onder de microscoop dat anti-GM2 IgM antistoffen binden aan een ander deel van de zenuwcel, een Schwanncel genaamd, en daarna eveneens het complementsysteem activeren. Schwanncellen zijn cellen die om zenuwen heen gerold zijn en myeline maken, de isolatielaag van zenuwcellen. Hoewel deze antistoffen niet gelinkt zijn aan een andere mate van achteruitgang van spierkracht over de jaren, zijn mensen met MMN die anti-GM2 IgM antistoffen hebben gemiddeld maar liefst 12 jaar jonger op het moment dat de spierzwakte van MMN startte.
Met dit onderzoek hebben we laten zien dat niet alleen anti-GM1 IgM antistoffen, maar ook anti-GM2 IgM antistoffen relevant zijn in de ziektemechanismen van MMN. Waar we eerst dachten dat alleen de zenuwcel aangedaan raakte in het ziekteproces, hebben we nu laten zien dat in een deel van de mensen met MMN waarschijnlijk ook de Schwanncel betrokken is. Dit breidt onze kennis over de ziektemechanismen van MMN significant uit.

Een belangrijke vraag bleef nog onbeantwoord: hoe komt iemand aan anti-GM1 IgM antistoffen? Een hint werd ons gegeven door een studie uit Argentinië uit 2004, waarin in het

bloed van pasgeborenen gekeken is naar de aanwezigheid van anti-GM1 IgM antistoffen. Ze zagen dat kinderen geboren worden zonder anti-GM1 IgM antistoffen, maar dat zij deze in de loop van de eerste weken na de geboorte ontwikkelden. Op basis van enkele andere data concluderen de onderzoekers dat de productie van anti-GM1 IgM antistoffen te linken is aan de vorming van het microbioom, de samenstelling van bacteriën in de dikke darm. Deze link wordt verklaard doordat sommige bacteriën eiwitten produceren die heel erg kunnen lijken op GM1. Als het immuunsysteem met deze bacteriën in aanraking komt, kan het gebeuren dat het antistoffen maakt tegen deze op GM1 gelijkende eiwitten. Die antistoffen vinden vervolgens echter ook hun weg naar het GM1 dat op zenuwcellen zit. Op die manier kan een afweerrespons tegen bacteriën in de darm uiteindelijk leiden tot een ontsteking van zenuwen.
In **Hoofdstuk 8** hebben we het microbioom van mensen met en zonder MMN onderzocht. In het verleden zijn ontlastingsmonsters verzameld van mensen met MMN en hun naasten, veelal hun partners. Hieruit is bacterieel DNA geïsoleerd en op basis van de opbouw van dit DNA kon worden herleid welke bacteriën in het ontlastingsmonster aanwezig waren, en in welke hoeveelheid.
We zagen dat de opbouw van het microbioom in grote lijnen gelijk was tussen mensen met en zonder MMN. Toch zagen we ook belangrijke verschillen. Twee bacteriesoorten, *Eubacterium siraeum* en een *Christensenellaceae*-soort, kwamen significant in mindere mate voor bij mensen met MMN. Daarnaast zagen we dat mensen met MMN die anti-GM1 IgM antistoffen hadden meer *Mogibacterium* in hun darm hadden. De samenstelling van het microbioom bleek een risicofactor voor het ontwikkelen van MMN; microbiomen waarin *Eubacterium siraeum* en de *Christensenellaceae*-soort onbraken, en *Mogibacterium* juist aanwezig was, werden bijna vier keer vaker gevonden bij mensen met MMN dan bij mensen zonder MMN. Daarmee is de samenstelling van het microbioom op dit moment de grootste risicofactor voor het ontwikkelen van MMN, specifiek voor MMN met aanwezigheid van anti-GM1 IgM antistoffen.
Mogelijk kan een dysbalans in het microbioom leiden tot overactivatie van ons afweersysteem. Specifiek zou het interessant zijn om na te gaan of de bacteriesoort *Mogibacterium* aan de buitenkant structuren heeft die lijken op GM1; dit zou een directe link tussen de overgroei aan deze bacteriesoort en anti-GM1 IgM antistoffen bij mensen met MMN kunnen verklaren.

We weten dat anti-GM1 IgM antistoffen leiden tot schade aan zenuwcellen doordat ze het complementsysteem activeren. Dit is een proces waarbij negen complementeiwitten, C1 t/m C9 genaamd, elkaar één voor één activeren. Het lichaam beschermt zichzelf tegen overactiviteit middels complementregulatoren, eiwitten die complementactivatie remmen. Om beter te weten welke delen van het complementsysteem relevant zijn voor MMN hebben we tot slot in **Hoofdstuk 5** gekeken naar genen die coderen voor de complementregulatoren die CD46, CD55 en CD59 heten.
We zagen dat mensen met MMN bijna 2.5 keer vaker een genetische variant in het gen voor CD55 hebben, wat ertoe zou kunnen leiden dat zij minder van dit eiwit maken. CD55 remt complement vrij vroeg in het proces, rondom het eiwit C3. Wanneer er minder complementregulatie is, neemt de rem op het complementsysteem af en volgt er dus een overactiviteit. Dit zou kunnen verklaren waarom deze genetische variant een risicofactor vormt voor MMN; antistoffen die aan zenuwen binden leiden tot een hogere complementactivatie en daarmee schade. We vonden ook dat de variant in CD55 te linken was aan een milder beloop van MMN, mogelijk doordat mensen die deze variant bij zich dragen om nog onbekende redenen gevoeliger zijn voor IVIg.

De resultaten van dit onderzoek suggereren dat de eerste stappen van de complementcascade interessante targets kunnen zijn voor nieuwe medicijnen die MMN moeten remmen.

Conclusie

Alle onderzoeken in dit proefschrift tezamen hebben onze kennis over MMN, en specifiek over de ziektemechanismen van MMN, significant verbeterd. We toonden aan dat MMN ondanks behandeling met IVIg een progressieve aandoening is, wat de noodzaak voor nieuwe en betere behandelingen onderstreept. Hoewel MMN geen erfelijke ziekte is, hebben we laten zien dat bepaalde genetische varianten in genen die betrokken zijn bij activatie van het afweersysteem een risicofactor vormen voor MMN. Waar we eerst dachten dat alleen anti-GM1 IgM antistoffen betrokken zijn bij MMN, hebben we in dit proefschrift laten zien dat ook anti-GM2 IgM antistoffen relevant zijn, wat ons idee over MMN als ziekte veroorzaakt door antistoffen uitbreidt. We hebben voor het eerst onderzoek gedaan naar de herkomst van anti-GM1 IgM antistoffen bij MMN, en toonden aan dat de aanwezigheid van deze antistoffen gelinkt kan worden aan een afwijkende samenstelling van het microbioom van de dikke darm.

Het onderzoek binnen het UMCU naar de betrokkenheid van het complementsysteem bij MMN heeft geleid tot de ontwikkeling van een nieuw medicijn voor MMN dat momenteel enkel nog in onderzoeksverband wordt getest (de ARDA-trial). Hopelijk mogen de resultaten van mijn proefschrift een opmaat zijn voor meer van dergelijke ontwikkelingen, die op hun beurt van belang kunnen zijn voor de grotere groep van mensen met een ontstekingsziekte van de zenuwen.

Dankwoord

Het proces waarin ik MMN tot in detail heb mogen leren kennen en wat heeft geresulteerd in het proefschrift dat voor u ligt, heeft zich uitgestrekt over een periode van zo'n tien jaren. Dit soort getallen stemt mij nostalgisch, en wanneer ik een moment neem om over mijn schouder te kijken, zie ik een grote groep mensen staan zonder wie het simpelweg niet was gelukt. Bij dezen maak ik graag van de gelegenheid gebruik om iedereen te bedanken die direct of indirect heeft bijgedragen aan dit proefschrift, mijn opleiding tot neuroloog, of, niet onbelangrijk, mijn levensgeluk.

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Curriculum Vitae

Jeroen Wessel Bos (November 12th 1991, Wageningen, The Netherlands) grew up in the city of Veenendaal, where he graduated secondary school with honors at the Christelijk Lyceum Veenendaal (CLV) in 2009. Torn between his love for both music and medicine, he decided to be educated both. In 2013, he graduated with honors at the Conservatory of Utrecht as a classical pianist. In 2016, he obtained his medical degree with honors at Utrecht University.

From the beginning of his medical training, the field of Neurology has fascinated him. This led to his first steps in scientific research in 2014, where he explored imaging modalities in motor neuron diseases under the supervision of Bas Jongbloed, Leonard van den Berg and Ludo van der Pol. This internship resulted in a PhD project on multifocal motor neuropathy under the supervision of dr. Ewout Groen, prof. Leonard van den Berg and prof. Ludo van der Pol, that focused on clinical, genetic, and immunological aspects of this disorder. At the 2023 Peripheral Nerve Society conference, he was awarded the Arthur K. Asbury prize for best presentation in the category of inflammatory neuropathies for his talk on complement inhibition in multifocal motor neuropathy. The work performed during his PhD project resulted in the thesis that is before you.

In 2017, Jeroen also started the Neurology Residency program at the University Medical Center Utrecht, trained by late prof. J.H.J. Wokke, dr. Janneke van den Bergen, prof. G.J. Biessels and prof. T. Seute. Through the experiences obtained during his residency and his PhD project, his interest in rare, and especially neuroinflammatory disorders, grew. He therefore followed an internship focusing on rheumatological and systemic autoimmune diseases at the Clinical Immunology department of the UMCU, under supervision of dr. Helen Leavis and dr. Maarten Limper, and on neuroinflammatory disorders at the Erasmus MC Rotterdam, under supervision of dr. Beatrijs Wokke and dr. Maarten Titulaer. He will complete his residency in 2027.

List of publications

Jongbloed BA, Haakma W, Goedee HS, **Bos JW**, Bos C, Hendrikse J, Van den Berg LH, Van der Pol WL. Comparative study of peripheral nerve MRI and ultrasound in multifocal motor neuropathy and amyotrophic lateral sclerosis. Muscle Nerve 2016;54(6):1133-1135

Jongbloed BA, **Bos JW**, Rutgers D, Van der Pol WL, Van den Berg LH. Brachial plexus magnetic resonance imaging differentiates between inflammatory neuropathies and does not predict disease course. Brain Behav 2017;7(5):e00632

Terhal PA, Vlaar JM, Middelkamp S, Nievelstein RAJ, Nikkels PGJ, Ross J, Creton M, **Bos JW**, Voskuil-Kerkhof ESM, Cuppen E, Knoers N, Van Gassen KLI. Biallelic variants in POLR3GL cause endosteal hyperostosis and oligodontia. Eur J Hum Genet 2020;28(1):31-39

Herraets I, Van Rosmalen M, **Bos JW**, Van Eijk R, Cats E, Jongbloed B, Vlam L, Piepers S, Van Asseldonk JT, Goedee HS, Van den Berg L, Van der Pol WL. Clinical outcomes in multifocal motor neuropathy: a combined cross-sectional and follow-up study. Neurology 2020;95(14):e1979-e1987

Budding K, Johansen LE, Van de Walle I, Dijkxhoorn K, De Zeeuw E, Bloemenkamp LM, **Bos JW**, Jansen MD, Curial CAD, Silence K, De Haard H, Blanchetot C, Van de Ven L, Leusen JHW, Pasterkamp RJ, Van den Berg LH, Hack CE, Boross P, Van der Pol WL. Anti-C2 antibody ARGX-117 inhibits complement in a disease model for multifocal motor neuropathy. Neurol Neuroimmunol Neuroinflamm 2021;9(1):e1107

Bos JW, Otten HG, Herraets IJT, Goedee HS, Cats EA, De Hoop T, Verduijn W, Van der Pol WL, van den Berg LH. High-resolution mapping identifies HLA class II associations with multifocal motor neuropathy. Neurobiol Aging 2021;101:79-84

Bos JW, Groen EJN, Wadman RI, Curial CAD, Molleman NN, Zegers M, Van Vught PWJ, Snetselaar R, Vijzelaar R, Van der Pol WL, Van den Berg LH. *SMN1* duplications are associated with progressive muscular atrophy, but not with multifocal motor neuropathy and primary lateral sclerosis. Neurol Genet 2021;7(4):e598

Bos JW, Groen EJN, Budding K, Delemarre EM, Goedee HS, Knol EF, Van den Berg LH, Van der Pol WL. Multifocal motor neuropathy is not associated with altered innate immune responses to endotoxin. J Neurol Sci 2023;451:120692

Bos JW, Groen EJN, Otten HG, Budding K, Van Eijk RPA, Curial C, Kardol-Hoefnagel T, Goedee HS, Van den Berg LH, Van der Pol WL. A 21-bp deletion in de complement regulator CD55 promotor region is associated with multifocal motor neuropathy and its disease course. J Peripher Nerv Syst 2024

Budding K*, **Bos JW***, Dijkxhoorn K, De Zeeuw E, Bloemenkamp LM, Zekveld EM, Groen EJN, Jacobs BC, Huizinga R, Goedee HS, Cats EA, Leusen JHW, Van den Berg LH, Hack CE, Van der Pol WL. IgM anti-GM2 antibodies in patients with multifocal motor neuropathy target Schwann cells and are associated with early onset. J Neuroinflammation 2024;21(1):100

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