UNC13A in amyotrophic lateral sclerosis: from genetic association to therapeutic target

Sean W Willemse,1 Peter Harley,2 Ruben P A van Eijk,3,1,3 Koen C Demaegd,1 Pavol Zelina,4 R Jeroen Pasterkamp,4 Philip van Damme,5,6 Caroline Ingre,7 Wouter van Rheenen,1 Jan H Veldink,5,1 Matthew C Kiernan,8,9 Ammar Al-Chalabi1,10 Leonard H van den Berg,1 Pietro Fratta,2 Michael A van Es1

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder predominantly characterised by the loss of upper and lower motor neurons.1 Clinically, the loss of motor neurons results in weakness and spasticity, leading to progressive and severe disability over the course of months to years, and eventually death as a result of respiratory failure.2 The lifetime risk of developing ALS for women is 1:400 and is slightly higher for men at 1:350.3 With riluzole, Edaravone, Nuedexta and Albrioza, several drugs have now been approved for the treatment of ALS, but they have at best a limited effect on disease progression.4–6 As such, the prognosis of ALS remains poor, with a median survival of 3 to 4 years.

Over the past two decades, it has become evident that there is a broad clinical spectrum associated with ALS with multiple subtypes and extensive phenotypic heterogeneity.7 For instance, the average age of onset extends from the late teens to well over the age of 90. The initial presenting symptoms also vary greatly between patients and range from a foot drop to dysarthria. Cognitive and behavioural changes are present in up to 50% of patients and nearly 15% of individuals fulfil the criteria for frontotemporal dementia (FTD).1,8 Finally, survival is also highly variable and can be as short as a few months in some cases and well over 10 years in others.

This clinical heterogeneity may be in part attributed to the different subtypes of ALS. The disease may be genetic (familial ALS, FALS) or be (apparently) sporadic (SALS). FALS constitutes up to 10% of all cases and generally follows an autosomal dominant pattern of inheritance.9 Repeat expansions in the C9orf72 gene are the most common cause of FALS (±40%), followed by point mutations in SOD1, TARDBP and FUS. Mutations in these genes are also seen in about 10% of apparently sporadic cases. Classically, consensus was that the disease was caused by genetic risk factors in combination with environmental exposures, which together trigger disease.9,10 Despite large-scale epidemiological studies, robust environmental factors conferring a large risk of disease have not been identified. Putative risk factors include smoking, repeated head injury and physical activity.11 Currently, the classical distinction between familial and sporadic ALS seems artificial and genetic factors contribute considerably to SALS. Twin and family-based studies show that the genetic basis for the risk of developing SALS is estimated to be approximately 50%.12 Indeed, genomewide association studies (GWAS) in sporadic ALS have successfully identified multiple risk loci, including UNC13A.13

At a cellular level, the mislocalisation and cytoplasmatic aggregation of hyperphosphorylated and ubiquitinated TAR DNA-binding protein 43 (TDP-43) is a pathological hallmark of ALS that is seen in 98% of all cases.14 TDP-43 appears to

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with limited treatment options and an incompletely understood pathophysiology. Although genomewide association studies (GWAS) have advanced our understanding of the disease, the precise manner in which risk polymorphisms contribute to disease pathogenesis remains unclear. Of relevance, GWAS have shown that a polymorphism (rs12608932) in the UNC13A gene is associated with risk for both ALS and frontotemporal dementia (FTD). Homozygosity for the C-allele at rs12608932 modifies the ALS phenotype, as these patients are more likely to have bulbar-onset disease, cognitive impairment and FTD at baseline as well as shorter survival. UNC13A is expressed in neuronal tissue and is involved in maintaining synaptic active zones, by enabling the priming and docking of synaptic vesicles. In the absence of functional TDP-43, risk variants in UNC13A lead to the inclusion of a cryptic exon in UNC13A messenger RNA, subsequently leading to nonsense mediated decay, with loss of functional protein. Depletion of UNC13A leads to impaired neurotransmission. Recent discoveries have identified UNC13A as a potential target for therapy development in ALS, with a confirmatory trial with lithium carbonate in UNC13A cases now underway and future approaches with antisense oligonucleotides currently under consideration. Considering UNC13A is a potent phenotypic modifier, it may also impact clinical trial outcomes. This present review describes the path from the initial discovery of UNC13A as a risk gene in ALS to the current therapeutic options being explored and how knowledge of its distinct phenotype needs to be taken into account in future trials.
cause neurotoxicity through both dysregulation of nuclear RNA processing as well as cytoplasmic TDP-43 aggregation, which causes a series of aberrant processes including cellular stress, aberrant stress granule formation, mitochondrial dysfunction, altered translation, reduced autophagy and proteosomal dysfunction. More recently, the molecular mechanisms by which genetic variation in the UNC13A gene confers risk and modifies the ALS phenotype have been linked to TDP-43 pathology. This current review describes the path from the initial discovery of UNC13A as a risk gene and its effect on the ALS phenotype through to the understanding of its molecular action and yet-to-come elucidation of its pathophysiological impact and potential as a therapeutic target. The strategies and challenges associated with developing a targeted treatment for the UNC13A subgroup of ALS patients will likely also apply to other subgroups. As our understanding of the genetics of ALS grows, the lessons learnt here are likely to be more broadly applicable.

GENETIC ASSOCIATION STUDIES IN ALS

Genetic research in ALS, as well as in other fields, was initially focused on the identification of pathogenic mutations through classical linkage in ALS pedigrees in which there was a clear Mendelian pattern of inheritance. Using this approach, the first FALS gene (superoxidase dismutase 1, SOD1) was discovered in 1993, which led the subsequent discovery of major ALS genes like TARDBP and Fused In Sarcoma (FUS)/TLS. Given the large genetic contribution to SALS, researchers also started to look for genetic risk factors in sporadic cases through candidate gene approaches which were based on existing knowledge of gene/protein function and pathophysiology. Although state-of-the-art at the time, candidate gene approaches were inherently vulnerable to bias and did not significantly further our understanding of ALS. It was not until the introduction of large-scale, high-throughput genotyping techniques that ALS genetics progressed.

Initially, this was through GWAS that allow the screening of common genetic variation in large populations. GWAS makes use of high throughput genotyping and linkage disequilibrium (LD) to screen the genome for common variants (single-nucleotide polymorphisms, SNPs) associated with the trait of interest. GWAS were first applied in ALS in 2007 with initially varying success to identify new risk loci. Over time, GWAS methodology improved, and increasing sample size allowed the interrogation of both common (>1%) and rare (<1%) variants, which led to the identification of robust and replicable genetic risk factors. Importantly, GWAS identified the chromosome 9p21.2 locus that was already shown to be linked to familial ALS and 2 years later found to harbour the C9orf72 repeat expansion.

The latest and largest GWAS, to date, with 29,612 cases and 122,636 controls, identified 15 risk loci, 7 of which had been previously described. Through the use of novel sequencing techniques, over 60 different genes have been identified for ALS and combined have demonstrated a monogenic basis in ±70% of FALS cases and in 10% of SALS. Genetic mutations which are known to be monogenic are also detected though GWAS. The depths of the human genome combined with the aforementioned developments in the genetic research of ALS lead to believe that more different genetic risk factors and more certainty about their role in the pathophysiology will be discovered in the future. Despite the contribution of GWAS to our knowledge of ALS, translating these ALS risk SNPs into disease models has been challenging. SNPs serve as markers to highlight a genetic locus, leaving the true causal mutation unknown. The SNPs found by GWAS typically convey a limited effect on disease risk compared with classical Mendelian genes, which is reflected by small ORs (typically <1.5). The SNP at rs12608932 in UNC13A was one of the first and most robust ALS-associated loci to be identified through GWAS (figure 1A,B). In the latest GWAS, it has an OR of 1.12 and modestly increases the risk for ALS from 1 in 350 to 1.12 in 350. Interestingly, early studies found strong effects of this SNP on survival, which prompted further interrogation of UNC13A as a phenotypic modifier.

UNC13A-ALS: A DISTINCT PHENOTYPE

The association between rs12608932 (located in UNC13A) and ALS was first described in 2009. Since then, this association has been replicated repeatedly in larger GWAS cohorts of patients with ALS of European ancestries. Another SNP in UNC13A, rs12973192, was found to be associated with ALS in 2018. This SNP is in high LD with rs12608932 only in European populations and seems to have an additive pathophysiological effect. This finding might explain the less robust association for both SNPs with ALS in South-East Asian populations.

Homozygosity for the C-allele at rs12608932 was found to lead to a shorter survival when compared with homozygous (A/A) or heterozygous individuals (A/C), with a decrease in median survival reported ranging from 5 months to 1 year. One study finding a higher median age of onset (65.5 vs 63.5 for A/A homozygosity). Other research found no significant relation for UNC13A and age of onset. Patients homozygous for the C-allele at SNP rs12608932 were more likely to have a bulbar onset of symptoms (43.1% vs 30.6%) and a lower functional vital capacity at baseline (86.5% vs 90.1%). Furthermore, behavioural disinhibition (13.2% vs 7.1%) and cognitive deficits were more frequent and there was a marked thinning in several frontal and temporal cortical regions. Pathological examination of the middle frontal, temporal and motor cortices of patients with ALS homozygous for the C-allele at rs12608932 and age of onset are less robust, with one study finding a higher median age of onset (65.5 vs 63.5 for A/A homozygosity). Other research found no significant relation for UNC13A and age of onset.

Patients homozygous for the C-allele at SNP rs12608932 were more likely to have a bulbar onset of symptoms (43.1% vs 30.6%) and a lower functional vital capacity at baseline (86.5% vs 90.1%). Furthermore, behavioural disinhibition (13.2% vs 7.1%) and cognitive deficits were more frequent and there was a marked thinning in several frontal and temporal cortical regions. Pathological examination of the middle frontal, temporal and motor cortices of patients with ALS homozygous for the C-allele at SNP rs12608932 were found to have strongly increased burden of TDP-43 compared with AA-homozygous patients, indicating that these individuals were more severely affected. An overview of clinical characteristics of UNC13A C/C homozygosity is found in figure 3.

UNC13A AS A COMMON RISK FACTOR FOR ALS AND FTD WITH TDP-43 PATHOLOGY

ALS forms a disease spectrum with FTD, with clinical phenotypes displaying characteristics of both disease. FTD is a progressive dementia characterised by either behavioural disinhibition or several different types of aphasia and has a yearly incidence of 15 to 22 per 100,000. Pathologically, FTD is characterised by clustering of either TDP-43 or FUS pathology. The relation for ALS and FTD specifically concerns FTD with TDP-43 inclusions (FTD-TDP). Approximately 10%–15% of all patients with ALS meet criteria for FTD at baseline, and inversely, 15% of patients with FTD develop motor neuron symptoms over the course of the disease. Presence of FTD in patients with ALS is associated with a shorter survival reported ranging from 5 months to 1 year.
Neurodegeneration with a worse survival. 42 The C-allele at rs12608932 was found to convey risk for both ALS and FTD-TDP. 43 Later findings confirmed that UNC13A is part of a shared genetic basis for both diseases, among other genes such as C9orf72. 13 44 Patients with ALS homozygous for the C-allele at rs12608932 in UNC13A were found to have an increased risk of FTD at baseline, independent of age at onset.37 Inversely, rs12608932 in UNC13A was found to confer risk of TDP-43-associated FTD, both with and without motor neuron symptoms.41 44 A specific association was found for subtype B of FTD-TDP, and rs12973192 in UNC13A, which is in LD with rs12608932. 41 Type B FTD-TDP is pathologically characterised by round TDP-43 inclusions throughout the depths of cortical layers and is clinically associated with both behavioural FTD and FTD with motor neuron symptoms; patients with type B FTD-TDP have a markedly shorter survival than other subtypes.41

**STRUCTURE AND FUNCTION OF UNC13A**

UNC13 was first described in the worm species Caenorhabditis Elegans, named as such because mutant worms were grouped based on their movement phenotypes. Defects in this gene were associated with ‘uncoordinated’ movement and paralysis. 45 In 1995, the Mammalian UNC13 (Munc-13) protein group was discovered in the central nervous system of rats, of which Munc13-1 was most ubiquitous. 46 Munc13-1 is a large protein with a molecular weight around 200 kDa, containing several binding domains with different functions at its C-terminal and
Neurotransmitters are released into the synapse where they bind to the corresponding receptors and are eventually recycled. UNC13A limits the site where neurotransmission can take place by initiating and maintaining synaptic active zones. UNC13A inhibits axonal growth both in vitro and in vivo in mice and triggers axonal branching, suggesting expression of UNC13A is typical for fully grown neurons and makes it possible for connections with nearby cells to be formed.

Several domains of the UNC13A protein are involved in synaptic plasticity, the ability of neurons to change their connections based on activity either over short periods of time, which is involved in processes such as motor control, and longer periods, involved in learning and memory. Heterodimerisation of the C2A domain of UNC13A with RIM, which recruits Ca2+-channels in the active zones, is important for docking and priming. In order to effectuate short-term plasticity, SVs are recruited into readily releasable pools (RRPs), which are stand-by in the presynaptic terminal in order to effectuate rapidly fuse when needed, for which the DAG-binding C1 domain of UNC13A plays a crucial role. UNC13A and UNC18 interact with the SNARE complex in order to prevent NSF/SNAPs depinning which keeps the RRP fusion competent. The calmodulin binding domain is involved in activity-dependent RRP refilling which is attenuated to residual Ca2+, shaping short-term plasticity during sustained neurotransmission. Finally, the C2B-domain of UNC13A regulated the recovery from high frequency stimulation through interaction with voltage-gated calcium channels, further enabling short-term plasticity.

PATHOPSYCHOLOGICAL MECHANISMS IN UNC13A

Mutations in UNC13A have also been reported in other diseases. A homozygous nonsense loss of function mutation in the N-terminal of UNC13A was identified in a patient with severe hypotonia, cortical hyperexcitability and fatal myasthenia. In vitro analysis of cultured muscle cells of this patient showed end plate potentials which were 2% of normal, indicating a severe decrease in neurotransmission. These clinical signs were thought to be the result of a loss of functional syntxin-1B, which was left in a non-operational state due to the loss of UNC13A. A gain of function mutation in UNC13A was shown to have opposite effects; a Pro814Leu exchange was found to lead to a dyskinetic movement disorder caused by an increase in the probability of SV release. This gain of function mutation was induced in neuronal cultures and was shown to increase synaptic strength of hippocampal and striatal neurons of mouse models and cholinergic neurons in neuromuscular junctions due to a higher SV release rate.

Based on the known variation in UNC13A, genetic mutations leading to nonsense mutations or mutations leading to loss of function are much more rare than is to be expected based on chance. As such, UNC13A is a very constrained gene, which could lead to believe that mutations leading to an alteration or loss of its function is much more often than not inconvertible. Importantly, the UNC13A SNPs associated with ALS are intronic and do not lead to changes in translation of the main UNC13A transcripts. Indeed, although rs12608932 and rs12973192 are associated with ALS, they also occur at a high frequency in healthy individuals, and around 10% of the European population is homozygous without pathophysiological consequences. This makes their role in ALS pathogenesis more elusive and questioning whether they had a direct effect or were merely tagging other yet-to-be identified changes.
Cryptic exon inclusion secondary to TDP-43 mislocalisation

Cryptic exons have recently been identified as the basis of UNC13A ALS pathophysiology. The process of splicing is regulated by TDP-43, a widely expressed protein which plays a role in RNA processing and transport. Specifically, one of TDP-43’s functions is that of binding to intronic sequences and repressing the inclusion of certain segments into mature RNA. On TDP-43 depletion, this repression is lost and normally intronic sequences, called cryptic exons, are included in messenger RNA (mRNA). Cryptic exons often introduce premature stop codons or frameshift mutations, which lead to nonsense-mediated decay of mRNA. In ALS-FTD, TDP-43 cytosolic aggregates are found in up to 98% of sporadic ALS cases and in 60%–83% of FTD pathology, depending on the clinical phenotype and are often accompanied by a nuclear depletion of TDP-43, leading to the appearance of cryptic exons. The cryptic exon is detected specifically in tissues affected by TDP-43 pathology (motor cortex and spinal cord in ALS patients and frontal and temporal cortices in FTD patients) and is absent in the (although small) percentage of non-TDP-43-related ALS-FTD.

The two previously described non-SNPs (rs12608932(A>C) and rs12973192(C>G)) and a novel third mutation (rs56041637) are located either within the C2B domain; a point mutation induced to abolish Ca2+ binding in this domain showed a slower recovery rate of the RRP. The loss in synaptic transmission indicates that mutations in UNC13A mainly affect excitatory neurotransmission.

Loss in synaptic transmission

As can be expected according to the main function of UNC13A, loss of UNC13A leads to impairment of neurotransmission (figure 4B). Loss of UNC13A leads to less efficient or total absence of evoked neurotransmission, with cells lacking UNC13A releasing 5Vs at a nearly four times slower rate, mainly due to a dramatic decrease in the RRP and a lack in bridging. UNC13A was shown to mediate SV release through activity-dependent Ca2+-phospholipid binding in the C₂B domain; a point mutation induced to abolish Ca2+ binding in this domain showed a slower recovery rate of the RRP. The decrease in RRP recovery increased the latency between stimulus and recovery, leading to believe that Ca2+ binding to UNC13A is instrumental in fidelity of neurotransmission during maintained firing of action potentials.

In cultivated hippocampal cells, loss of UNC13A was shown to mainly affect glutamatergic neurotransmission. GABAergic neurons seem to have an inherently lower threshold for vesicular fusion and are more affected by the loss of all UNC13 proteins, instead of UNC13A specifically. This might indicate that mutations in UNC13A mainly affect excitatory neurotransmission.

Maintaining neuronal health and function

Apart from its role in neurotransmission, loss of UNC13A affects neuronal structure and health. Absence of UNC13A decreased the release of dense core vesicles up to 60%. Dense core vesicles contain neuropeptides, neurotrophic factors and other regulatory substances which develop neurons in the adult brain and are important in the formation of synaptic active zones. A downstream effect of the loss in UNC13A is a simultaneous loss of syntaxin-1, which is instrumental in both vesicle docking and maintaining neuronal health, as it leads to increased rates of degradation in both developing and adult neuronal cells. Expression levels of both syntaxin-1B, an isoform, and UNC13A were lowered in the transcriptome of ventral horns of ALS patients. Furthermore, there was reduced expression of circulating microRNA related to either UNC13A or UNC18 in ALS pathology,75 depending on the clinical phenotype and are often accompanied by a nuclear depletion of TDP-43, leading to the appearance of cryptic exons.15,72 The cryptic exon is detected specifically in tissues affected by TDP-43 pathology (motor cortex and spinal cord in ALS patients and frontal and temporal cortices in FTD patients) and is absent in the (although small) percentage of non-TDP-43-related ALS-FTD.

The above described mechanism linking TDP-43 pathology with the presence of these variants, their presence was shown to alter the affinity of TDP-43 binding and make the inclusion of cryptic exon in mRNA more likely. Therefore, the loss of TDP-43, combined with the presence of these risk variants, leads to a drastic reduction in viable UNC13A mRNA and proteins, as can be seen in figure 4A. The above described mechanism linking TDP-43 pathology with UNC13A loss explains how risk SNPs can occur frequently in the general population, as they exert their action only when the TDP-43 pathology has started and might also explain in part the effect of the SNPs on disease progression.

Furthermore, TDP-43 pathology seems to affect neurons in a cell-specific manner, indicating that downstream processes, such as UNC13A pathology, are also selective to these groups of cells. Finally, TDP-43 pathology has been found to be associated with impaired synaptic transmission and synaptic loss, but it is as of yet unclear if, or in what manner, this is dependent on loss of UNC13A.

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ALS, implicating a loss of neuronal transmission in part in the pathophysiology of ALS.83

POTENTIAL TREATMENTS TARGETING UNC13A
Lithium carbonate

The application of lithium carbonate in treating ALS has been researched extensively. Initially, the results of a phase two trial showed a very promising effect on survival in patients with ALS when compared with treatment with riluzole alone.86 After this initial publication, five randomised controlled trials were conducted with none finding a benefit of lithium carbonate compared with control.87-89 A meta-analysis confirmed that there is no benefit of lithium carbonate to the ALS population as a whole.90 In post-hoc analyses, it was interrogated whether genetic subgroups (C9ORF72 and UNC13A) responded differently to treatment with lithium carbonate.90 Strikingly, patients homozygous for the C/C genotype of rs12608932 allocated to lithium carbonate exhibited a significantly higher 12-month survival probability compared with those in controls arms (69.7% compared with 40.1%, respectively).90 Although these data suggested that lithium carbonate might be effective in this genetic subgroup, these represent results will need to be replicated in a confirmatory trial. Therefore, an international, multicentre, double-blind, placebo-controlled study enrolling patients with ALS with homozygous for the risk SNP rs12608932 has been initiated. The study is being undertaken in 15 ALS centres originating from seven countries in Europe and Australia (EudraCT 2020-000579-19). A total of 171 patients homozygous for the C-allele at rs12608932 will be included. A protocol for this study has recently been published.96 99

While the mechanism by which lithium carbonate could influence disease progression in UNC13A-ALS remains unclear, preclinical studies show that lithium induces sprouting of pyramidal neurons and induces synaptogenesis, which might counter-effect the previously described loss in synaptic transmission resulting from UNC13A depletions.92 93 Other proposed mechanisms relate to regulation of intracellular calcium homeostasis and an increase in autophagy, a process through which cells are able to degrade intracellular components, which was shown to be activated by lithium.93 94 Furthermore, it may also be that the effect of lithium can be detected in the trial context in faster-progressing individuals, which are more likely to be homozygous for the risk SNP.

Other potential therapeutic strategies

Drugs with a similar structure to DNA and RNA, called nucleic acid therapeutics, can be used to specifically target mRNA in order to regulate protein expression.95 Single-stranded synthetic antisense oligonucleotides (ASOs) are able to bind the junctions between introns and exons in mRNA in order to regulate splicing and induce exon skipping.96 This could be used to inhibit the inclusion of the cryptic exon in UNC13A induced by TDP-43 pathology, leading to a rescue of functional protein levels. Splicing modulating therapies are already being used to inhibit the inclusion of the cryptic exon in UNC13A induced by TDP-43 pathology, leading to a rescue of functional protein levels, in turn likely affecting neurotransmissions, synaptic plasticity and neuronal health. Finally, we have described possible treatment strategies, in particular, those targeting UNC13A sub-populations, such as lithium carbonate and exon skipping ASOs. Clinical, genetic and biological findings all demonstrate that UNC13A is a potent phenotypic modifier in ALS, but with a much smaller effect on disease risk. This finding warrants further research on genetic modifiers of ALS phenotypes as further knowledge of these modifiers could guide trial design and therapy development.

CONCLUSION

Here, we have reviewed the association of UNC13A with ALS, its effect on survival and the distinct clinical phenotype associated with it. We described how TDP-43 pathology, the hallmark of ALS, leads to the inclusion of a cryptic exon in UNC13A and subsequent nonsense-mediated decay lowering functional protein levels, in turn likely affecting neurotransmissions, synaptic plasticity and neuronal health. Finally, we have described possible treatment strategies, in particular, those targeting UNC13A sub-populations, such as lithium carbonate and exon skipping ASOs. Clinical, genetic and biological findings all demonstrate that UNC13A is a potent phenotypic modifier in ALS, but with a much smaller effect on disease risk. This finding warrants further research on genetic modifiers of ALS phenotypes as further knowledge of these modifiers could guide trial design and therapy development.

Author affiliations

1Department of Neurology, UMC Utrecht Brain Center Rudolf Magnus, Utrecht, The Netherlands
2UCU Queen Square Motor Neuron Disease Centre, Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology, London, UK
3Biostatistics & Research Support, Julius Center for Health Sciences and Primary Care, UMC Utrecht, Utrecht, The Netherlands
4Department of Translational Neuroscience, UMC Utrecht Brain Center Rudolf Magnus, Utrecht, The Netherlands
5Department of Neurology, KU Leuven Hospital, Leuven, Belgium
6Department of Neurology, KU Leuven Hospital, Leuven, Belgium
7Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden
8Bushell Chair of Neurology, Brain and Mind Centre, University of Sydney, Sydney, New South Wales, Australia
9Neurology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia
10Department of Neurology, King’s College Hospital, London, UK

Twitter Matthew C Kiernan @jnnp_bmj and Ammar Al-Chalabi @AmmarAlChalabi

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ORCID iDs

Sean W Willemsen http://orcid.org/0000-0002-0198-5740
Ruben P.A.van Eijk http://orcid.org/0000-0002-7132-5967
Philip van Damme http://orcid.org/0000-0002-4010-2357
Jan H Veldink http://orcid.org/0000-0001-5752-9657
Matthew C Kieman http://orcid.org/0000-0001-9054-026X
Ammar Al-Chalabi http://orcid.org/0000-0002-4924-7712

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Supplementary Figure 1: Overall survival for rs12608932 polymorphism under an additive model.

The p-value indicates the difference in cumulative risk between homozygous C-allele carriers vs heterozygotes vs homozygous A-allele carriers.
Supplementary figure 2: Survival probability adjusted for sex, age at onset and site of onset for rs12608932 polymorphism under a recessive model.
Supplementary figure 3: Survival probability adjusted for sex, age at onset and site of onset for rs12608932 polymorphism under a recessive model.