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Tazelaar, G.H.P., Dekker, A.M., van Vugt, J.J.F.A. et al. (28 more authors) (2019) Association of NIPA1 repeat expansions with amyotrophic lateral sclerosis in a large international cohort. Neurobiology of Aging, 74. 234.e9-234.e15. ISSN 0197-4580

https://doi.org/10.1016/j.neurobiolaging.2018.09.012

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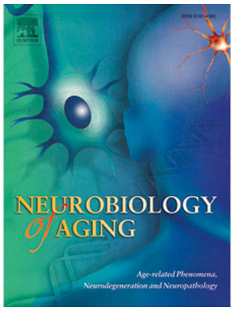

Association of *NIPA1* repeat expansions with amyotrophic lateral sclerosis in a large international cohort

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PII: S0197-4580(18)30336-1

DOI: 10.1016/j.neurobiolaging.2018.09.012

Reference: NBA 10374

To appear in: *Neurobiology of Aging*

Received Date: 11 May 2018

Revised Date: 8 August 2018

Accepted Date: 11 September 2018

Please cite this article as: Tazelaar, G.H., Dekker, A.M, van Vugt, J.J., van der Spek, R.A, Westeneng, H.-J., Kool, L.J., Kenna, K.P, van Rheenen, W., Pulit, S.L, McLaughlin, R.L, Sproviero, W., Iacoangeli, A., Hübers, A., Brenner, D., Morrison, K.E, Shaw, P.J, Shaw, C.E, Panadés, M.P., Mora Pardina, J.S, Glass, J.D, Hardiman, O., Al-Chalabi, A., van Damme, P., Robberecht, W., Landers, J.E, Ludolph, A.C, Weishaupt, J.H, van den Berg, L.H, Veldink, J.H, van Es, M.A, on behalf of the Project MinE ALS Sequencing Consortium, Association of *NIPA1* repeat expansions with amyotrophic lateral sclerosis in a large international cohort, *Neurobiology of Aging* (2018), doi: https://doi.org/10.1016/j.neurobiolaging.2018.09.012.

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Association of *NIPA1* repeat expansions with amyotrophic lateral sclerosis in a large international cohort.

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Abstract

NIPA1 (non-imprinted in Prader-Willi/Angelman syndrome 1) mutations are known to cause Hereditary Spastic Paraplegia type 6, a neurodegenerative disease that phenotypically overlaps to some extent with Amyotrophic Lateral Sclerosis. Previously, a genome-wide screen for copy number variants found an association with rare deletions in *NIPA1* and ALS, and subsequent genetic analyses revealed that long (or expanded) polyalanine repeats in *NIPA1* convey increased ALS susceptibility. We set out to perform a large-scale replication study to further investigate the role of *NIPA1* polyalanine expansions with ALS, in which we characterized *NIPA1* repeat size in an independent international cohort of 3,955 ALS patients and 2,276 unaffected controls and combined our results with previous reports. Meta-analysis on a total of 6,245 ALS patients and 5,051 controls showed an overall increased risk of ALS in those with expanded (>8) GCG-repeat length (odds ratio = 1.50, $P = 3.8\times10^{-5}$). Together with previous reports, these findings provide evidence for an association of an expanded polyalanine repeat in *NIPA1* and ALS.

Keywords

Amyotrophic lateral sclerosis; NIPA1; Repeat expansion

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disorder characterized by the loss of both upper and lower motor neurons leading to progressive weakness, spasticity and ultimately respiratory failure (Hardiman et al., 2011; van Es et al., 2017). The complex genetic architecture of ALS is characterized by 5-15% of patients with a positive family history, where it is assumed that there is a single causal mutation (Andersen and Al-Chalabi, 2011). However, even in the majority of seemingly sporadic patients a large genetic contribution is expected and causal mutations have been reported despite a negative family history (Al-Chalabi et al., 2016; McLaughlin et al., 2015). To date, mutations in more than 20 different genes have been implicated in ALS, one of the most prominent being an intronic repeat expansion in *C9orf72* (Al-Chalabi et al., 2016).

In addition to *C9orf72*, repeat expansions in other genes have been reported in ALS, including *ATXN2* and *NIPA1* (Blauw et al., 2012; Elden et al., 2010). *NIPA1* (non-imprinted in Prader-Willi/Angelman syndrome 1) mutations are known to cause Hereditary Spastic Paraplegia type 6, a neurodegenerative disease characterized by slowly progressive upper motor neuron signs (predominantly in the lower limbs) and is a condition that to some extent has phenotypic overlap with ALS (Rainier et al., 2003). Interestingly, a genome-wide screen for copy number variants found an association with rare deletions in *NIPA1* and ALS and subsequent genetic analyses revealed that long (or expanded) polyalanine repeats in *NIPA1* confer increased disease susceptibility (Blauw et al., 2012; 2010). In the majority of people (98%) the 5'-end of *NIPA1* (NCBI: NM_144599.4) encodes for a stretch of 12 or 13 alanine residues of which 7 or 8 are encoded by a $(GCG)_n$ trinucleotide repeat (TNR), although both shorter and longer GCG stretches have been reported in non-affected individuals (Chai et al., 2003). In this previous study, an analysis of an international cohort of 2,292 ALS patients and 2,777 controls showed that “long” repeats (>8) in *NIPA1* were enriched in ALS cases compared to controls (5.5% vs. 3.6%; OR 1.71; $P = 1.6 \times 10^{-4}$) (Blauw et al., 2012).

Although interesting and potentially relevant, only a small fraction of initially positive results from candidate gene studies (such as that performed on *NIPA1*) replicated consistently (Hirschhorn et al., 2002). Therefore, additional steps, such as replication of the findings and imposing a proper significance threshold (such as exome or genome-wide significance), are required to make any claims of causality (MacArthur et al., 2014).

We therefore set out to perform a large-scale replication study to further investigate the role of *NIPA1* polyalanine expansions with ALS, in which we characterized *NIPA1* repeat size in a large international cohort of ALS patients and unaffected controls and then meta-analyze our results with previous reports.

2. Material and Methods

2.1 Subjects

All participants gave written informed consent and approval was obtained from the local, relevant ethical committees for medical research. Genotyping experiments were performed on 6,231 samples comprising 3,955 ALS patients and 2,276 healthy controls from 6 populations. All patients were diagnosed according to the revised El Escorial criteria. Control subjects were from ongoing population-based studies on risk factors in ALS. All related individuals were excluded from further analysis. Baseline characteristics for available samples are provided in **Supplementary Table 1**.

2.2 PCR, sequencing and genotyping

Dutch samples obtained from 753 ALS and 603 unaffected individuals were analyzed using PCR according to protocols described previously and results were analyzed in a blinded and automated fashion with a call rate of 96.6% (Blauw, et al., 2012). Samples that failed genotyping, were additionally analyzed with Sanger sequencing to assess possible bias. An additional cohort of 767 unaffected controls and 764 ALS samples were genotyped using Sanger sequencing and automatically genotyped with a call rate of 99.1%. Primers: 5’-GCCCCTCTTCCTGCTCCT-3’ (forward) and 5’-CGATGCCCTTCTTCTGTAGC -3’ (reverse).

A total of 847 samples were analyzed using both methods (PCR and Sanger), with manual review of discordant genotypes (n = 35, 4.1%).

We analyzed *NIPA1* repeat size in whole-genome sequencing (WGS) data of 3,344 samples (2,438 cases and 906 controls) from the HiSeq X Sequencing platform, available to us through Project MinE (Project MinE ALS Sequencing Consortium, 2018), using the Illumina ExpansionHunter tool (Dolzhenko et al., 2017). There was a 691 sample overlap genotyped using both ExpansionHunter and Sanger sequencing, showing a 99% concordance (n = 684). Considering this 99% concordance between ExpansionHunter and Sanger results in the Dutch dataset, we did not perform additional validation experiments on the WGS samples and proceeded with the ExpansionHunter calls. *C9orf72* status had been determined for 3,907 ALS samples from the PCR, Sanger and ExpansionHunter cohorts. Additionally, the presence of rare non-synonymous and loss-of-function variants in the established ALS-associated genes *SOD1*, *FUS* and *TARDBP* was known for 5,030 cases and controls from all cohorts as described previously (Dekker et al., 2016; Project MinE ALS Sequencing Consortium, 2018).

2.3 Statistical analysis

All statistical procedures were carried out in R 3.3.0 (http://www.r-project.org). For association analyses we applied a logistic regression analysis to all subgroups, the effect of the expanded (>8) versus non-expanded polyalanine repeat length on the disease status, adjusting for sex at birth, method of genotyping and country of origin. Samples with missing sex at birth status (n = 108, 1%) were imputed using multivariate multiple imputation with the ‘mice’ 2.46.0 package.

Subgroup effects were meta-analyzed using both fixed and random effects modelling using the ‘metafor’ 2.0 package. For the joint analysis on individual data, we used a generalized linear model (GLM) with fixed-effects covariates: sex, method of genotyping and country of origin. We additionally applied generalized linear mixed model (GLMM) on non-imputed data to account for possible random effects.

The survival after onset and age at onset analyses were performed using multivariate Cox regression with sex at birth, site of onset, age at onset (for survival only) and *C9orf72* status as covariates.

To assess whether the observed frequency of co-occurring genetic risk variants for ALS was in excess of what would be expected on the basis of chance, we used a method described previously by (Dekker et al., 2016). The expected frequency of co-occurring variants was calculated using the following formula: (the observed number of patients carrying a variant / the total number of patients) * (the observed number of controls carrying a variant / the total number of controls). This formula was used in order to take into account the higher frequency of just one variant in ALS patients (= frequency of variants in patients), multiplied by the chance probability of a second variant (= frequency of variants in controls). Then, a binomial test was performed to compare the observed frequency of co-occurring variants in ALS patients with the calculated expected frequency.

We specified a formal null model for an increase in repeat expansion with consideration of repeat confounding variables such as the genomic frequency and repeat size. Previous studies have shown that there are a total of 878 genes in the genome that contain a coding trinucleotide repeat (TNR) with a repeat size of 6 repeats or greater, 90 of which contain a polyalanine tract (Kozlowski et al., 2010). We therefore set two thresholds for significance in this study; 1) a relatively loose threshold, in which we correct for the number of genes that contain a polyalanine tract of 6 or larger resulting in $P = 0.05/90 = 5.6\times10^{-4}$ and 2) a more conservative threshold, in which we correct for the total number of genes in the genome that contain a coding TNR with a size of 6 or larger which gives $P = 0.05/878 = 5.7\times10^{-5}$.

3. Results

3.1 Replication

We first tried to replicate the initial findings in an independent Dutch cohort comprising 1,517 ALS cases and 1,370 unaffected controls by genotyping the GCG repeat length in

NIPA1 using repeat PCR and/or Sanger sequencing. As was reported previously, we found the most frequent alleles to consist of either 7 or 8 $(GCG)_n$ repeats (25% and 72% respectively) (**Figure 1**). Our analysis showed a similar allele frequency difference of expanded or “long” alleles (repeat length of 9 or longer) between ALS (n = 85, 2.80%) and controls (n = 51, 1.86%). Both ALS and control subgroups had only one single case with a homozygous expansion, indicating a dominant model for further analysis. This resulted in 84 individuals with ALS (5.54%) and 50 unaffected individuals (3.65%) as carriers of an expanded *NIPA1* polyalanine repeat length. Logistic regression analysis, corrected for sex at birth and method of genotyping (PCR or Sanger), revealed an effect of expanded *NIPA1* repeat length on disease susceptibility (OR = 1.54, *P* = 0.018).

3.2 Project MinE

To further increase sample size and investigate cohorts other than the Dutch population, we then analyzed *NIPA1* repeat expansion genotypes that were called using the Illumina ExpansionHunter tool in 2,438 independent ALS cases and 906 controls whole-genome sequenced (WGS) as part of the Project MinE ALS Sequencing Consortium (Project MinE ALS Sequencing Consortium, 2018). This multi-cohort WGS data showed a more equal distribution of expanded *NIPA1* carriers in ALS (114/2,438, 4.67%) and controls (40/906, 4.42%). A logistic regression analysis, corrected for country of origin and sex, showed no significant difference.

3.3 Meta-analysis

Finally, we sought to perform an analysis of all available *NIPA1* polyalanine expansion data, combining our data with the original data published previously (Blauw et al., 2012). After exclusion of duplicate samples; individual level data was available for a total of 5,056 samples (2,290 cases and 2,775 controls) in the discovery dataset published by Blauw *et al.* (2012). Our replication cohort (including results from PCR, Sanger and Expansion Hunter) comprised 3,955 cases and 2,276 controls. The final dataset included 6,245 ALS patients and 5,051 controls, reaching a final number of 11,296 unique individuals. We combined this data in a fixed-effects meta-analysis and found an overall risk of expanded *NIPA1* repeat length on ALS (odds ratio (OR) = 1.50, $P = 3.8\text{x}10^{-5}$) (**Figure 2**). Since individual level data was available, we additionally performed a multivariate logistic regression analysis, using sex at birth, method of genotyping and country of origin as covariates in the pooled data, resulting in an equal effect and significance (OR = 1.48, $P = 6.2\text{x}10^{-5}$). Other association models that account for random effects, such as random effect meta-analysis and a generalised linear mixed model gave similar results (data not shown). Repeating the analysis excluding the 322 *C9orf72* repeat expansion carriers yielded a *P* value of $7.7\text{x}10^{-5}$ for the fixed-effects meta-analysis (OR = 1.49, 95% confidence interval (CI) = 1.22-1.81) and a *P* value of $1.0\text{x}10^{-4}$ for the multivariate logistic regression analysis (OR = 1.47, 95% CI = 1.21-1.78). Exclusion of an additional 171 samples (133 cases and 38 controls) carrying a non-synonymous or loss-of-function mutation in *SOD1*, *FUS* or *TARDBP* did not alter the results (fixed-effects meta-analysis *P* value = $7.5\ \text{x}10^{-5}$, OR = 1.49, 95% CI = 1.22-1.81) (**Supplementary Figure 1**).

3.4 Survival

Clinical data and survival data was available for 1,954 out of 3955 ALS patients from the combined replication cohorts (**Supplementary Table 2**). After correction for sex, age at onset, bulbar site of onset and *C9orf72* status, we used a Cox regression model in this mixed population to test if *NIPA1* conferred any risk for shorter survival time; we found no evidence for such an effect (Hazard ratio (HR) = 1.16; 95% CI = 0.94-1.45; *P* = 0.16) (**Supplementary Figure 2**). Also, there was no significant association between *NIPA1* repeat length and age at onset in this replication cohort with correction for sex, site of onset and the presence of a *C9orf72* expansion (**Supplementary Figure 3).**

3.4 Co-occurrence with C9orf72 repeat expansion

Since a significant number of *NIPA1* expansion carriers was reported in a subgroup of ALS patients that also carried a *C9orf72* repeat expansion (Dekker et al., 2016), we evaluated this co-occurrence in 4,619 participants genotyped for both loci in all cohorts (n = 712 for the discovery cohort; n = 3,907 for the combined replication cohorts).

Although we did observe a higher than expected frequency of co-occurrence of the repeat expansions, our data did not robustly replicate the previously published finding (0.37% observed vs 0.26% expected; $P = 0.06$) (**Supplementary Table 3**).

4. Discussion

In this study, we included a large international cohort and additionally meta-analyzed the *NIPA1* expansion genotypes in a total of 6,245 ALS patients and 5,051 controls. Given that we were able to replicate our previous results in an independent cohort and observed an increase in significance in the overall meta-analysis, our data adds to the evidence that expanded *NIPA1* repeats are a risk factor for sporadic ALS. Mutations in *NIPA1* were already known to cause Hereditary Spastic Paraplegia type 6, a neurodegenerative disease with motor-neuron involvement, whereas the 15q11.2 microdeletions are better known for low penetrant neurodevelopmental phenotypes, further adding to the complexity of the *NIPA1* locus (Butler, 2017; Rainier et al., 2003). Interestingly, genetic pleiotropy between HSP and ALS appears to be more widespread, as recently it has been shown that mutations in different domains in *KIF5A* either cause HSP or ALS (Brenner et al., 2018; Nicolas et al., 2018).

After *C9orf72* and *ATXN2*, *NIPA1* is the third reported expanded genomic repeat motif associated with an increased risk for ALS. Its initial discovery in ALS by identification of copy number variants in the chromosome 15q11.2 locus containing *NIPA1,* was followed by further genetic screening in a large international cohort consisting of Belgian, Dutch, and German subjects (Chai et al., 2003). This subsequent study in 2,292 ALS patients and 2,777 controls revealed that, although *NIPA1* deletions and missense mutations were identified in ALS patients, it actually was an increase of the $(GCG)_n$ repeat motif in the 5'-end of *NIPA1* that seemed to associate with ALS (OR = 1.71 with $P = 1.6\text{x}10^{-4}$). Knowing that positive results derived from candidate gene studies often fail to replicate, we sought to replicate the *NIPA1* finding in ALS, particularly given the complex genotypic and phenotypic architecture of the *NIPA1* locus (Messaed and Rouleau, 2009).

Our results showed a very similar effect of increased *NIPA1* polyalanine expansions on ALS-susceptibility in a new Dutch cohort of 1,517 ALS cases and 1,370 unaffected controls tested via PCR or Sanger sequencing. Given the high concordance between Sanger/PCR results and the calls from the bioinformatic tool ExpansionHunter on WGS data, we were able to further increase the sample size of our study by including data from Project MinE (Shinchuk et al., 2005). This allowed us to additionally evaluate the role of *NIPA1* repeat sizes in non-Dutch cohorts. The size of this cohort was similar in the number of cases compared to the original discovery cohort, but smaller in number of controls compared to that original cohort. Also, the Project MinE dataset is more heterogeneous compared to the original discovery cohort. This is a possible explanation as to why the overall *NIPA1* signal was not replicated in the WGS data. However, we did find a similar direction and effect size in 4 out of the 6 WGS cohorts (Ireland, Spain, the United States of America and the United Kingdom).

While empirical thresholds for genome-wide and exome-wide significance have been derived for studies assessing associations between phenotypes and single nucleotide variants, these thresholds are likely to be too stringent in the context of screening for coding repeat expansions, as the genome contains only ~900 genes with a coding TNR tract with a length of 6 or more, 90 of which code for a polyalanine tract (Messaed and Rouleau, 2009). We therefore set the significance threshold for associations with TNRs to be approximately $P = 5.6\text{x}10^{-4}$, correcting for polyalanine only, or (more conservative) $P = 5.7\text{x}10^{-5}$, correcting for all TNRs with a length of 6 repeats or more. The meta-analysis results are significant regardless of the threshold applied. Furthermore, exclusion of samples carrying a mutation in established ALS genes (*C9orf72, SOD1*, *TARDBP* and *FUS*) yielded somewhat lower *P* values (due to loss of power corresponding to lower number of included samples) with similar magnitude of effect, further supporting the role of *NIPA1* as independent risk factor for developing ALS.

Although we did see a higher than expected number of ALS cases carrying both *NIPA1* and *C9orf72* repeat expansions in this study (n = 17, *P* = 0.06), we did not robustly reproduce the co-occurrence of *C9orf72* expansion carriers in the *NIPA1* expanded cases described by Dekker *et al.* (2016). This might be attributed to the relatively small sample size in the original study (755 ALS patients), resulting in broad confidence intervals that overlap with our results (frequency = 0.004 [0.002-0.006] in the current study; frequency = 0.009 [0.004-0.019] in Dekker *et al.* (2016). Alternatively, the co-occurrence might be relevant in some, but not all included populations. Additionally, we were unable to replicate the effect of *NIPA1* expansions on ALS survival and age at onset (Blauw et al., 2012). These findings again reemphasize the necessity for replication and the importance of tracking clinical characteristics in large genetic databases. Currently, we were able to perform a survival analysis on just 50% of our replication set and further evaluation in a larger and complete dataset is therefore recommended.

Interestingly, the increase in the *NIPA1* repeat size seems to be limited to the addition of mostly two GCG repeats. However, this seemingly small addition might well have protein conformational effects as has been shown in-vitro; polyalanine stretches between 7 to 15 alanines transition from a monomeric alpha helix to a predominant macromolecular beta sheet, which in turn may lead to stronger protein–protein interactions and aggregation (Blauw et al., 2010). Additionally, a patient with a mutation in *NIPA1* suffering from a progressive motor neuron phenotype was shown to have TDP-43 inclusions, very similar to effects seen in ALS and ALS-FTD cases(Martinez-Lage et al., 2012). These findings might explain how alterations in *NIPA1* could increase ALS risk.

In conclusion, our data adds to the evidence for an association of *NIPA1* expansions and ALS. Future investigations may provide further insights in the role of *NIPA1* and polyalanine stretches in the development and possibly treatment of motor neuron disease.

Disclosure statement

L.H. van den Berg serves on scientific advisory boards for the Prinses Beatrix Spierfonds, Thierry Latran Foundation, Biogen and Cytokinetics; and serves on the editorial board of Amyotrophic Lateral Sclerosis And Frontotemporal Degeneration and The Journal of Neurology, Neurosurgery, and Psychiatry. O. Hardiman has received speaking honoraria from Novarits, Biogen Idec, Sanofi Aventis and Merck-Serono, has been a member of advisory panels for Biogen Idec, Allergen, Ono Pharmaceuticals, Novartis, Cytokinetics and Sanofi Aventis and serves as Editor-in-Chief of Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. A. Al-Chalabi has consulted for OrionPharma, Biogen Idec, Cytokinetics Inc, Treeway Inc, and Chronos Therapeutics. J.H. Veldink reports that his institute received consultancy fees from Vertex Pharmaceuticals outside the submitted work. M.A. van Es received grants from the Netherlands Organization for Health Research and Development (Veni scheme), The Thierry Latran foundation, The Netherlands ALS foundation (Stichting ALS Nederland) and the Joint Program Neurodegeneration (JPND). He has received travel grants from Baxalta and serves on the biomedical research advisory panel of the motor neurone disease association (MNDA). Other authors have no reported conflicts of interest.

Acknowledgements

This study was supported by the ALS Foundation Netherlands, the Belgian ALS Liga and National Lottery, and Agency for Innovation by Science and Technology (IWT), and the MND Association (UK) (Project MinE, www.projectmine.com). Research leading to these results has received funding from the European Community's Health Seventh Framework Programme (FP7/2007-2013). This study was supported by ZonMW under the frame of E-Rare-2, the ERA Net for Research on Rare Diseases (PYRAMID). This is an EU Joint Programme–Neurodegenerative Disease Research (JPND) project (STRENGTH, MEND, SOPHIA, ALS-CarE). The project is supported through the following funding organizations under the aegis of JPND: UK, Medical Research Council (MR/L501529/1; MR/R024804/1) and Economic and Social Research Council (ES/L008238/1); Ireland, Health Research Board; Netherlands, ZonMw; Belgium, FWO-Vlaanderen. Samples used in this research were in part obtained from the UK National DNA Bank for MND Research, funded by the MND Association and the Wellcome Trust. This project was supported by supported by the MND Association of England, Wales and Northern Ireland and the Netherlands Organisation for Health Research and Development (Vici scheme to L.H. van den Berg and veni scheme to M.A. van Es). NDAL cordially thanks Suna and Inan Kirac Foundation for their generous support. M.A. van Es is supported by the Thierry Latran Foundation, the Dutch ALS foundation and the Rudolf Magnus Brain Center Talent Fellowship. C.E. Shaw and A Al-Chalabi receive salary support from the National Institute for Health Research (NIHR) Dementia Biomedical Research Unit and Biomedical Research Centre in Mental Health at South London and Maudsley NHS Foundation Trust and King’s College London. O. Hardiman is funded by the Health Research Board Clinician Scientist Programme and Science Foundation Ireland. J.E. Landers is supported by the US National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS) (R01NS073873) and the American ALS Association. R.L. McLaughlin is supported by the Thierry Latran Foundation (ALSIBD) and the ALS Association (2284). P. Van Damme holds a senior clinical investigatorship from FWO-Vlaanderen.

Figure 1. *NIPA1* polyalanine repeat length distribution.

Proportion of total alleles grouped per *NIPA1* polyalanine repeat size. Alleles displayed were observed multiple times in the Dutch replication cohort of 1517 individuals affected with ALS (blue) and 1370 unaffected controls (orange).

Figure 2. *NIPA1* polyalanine repeat expansion meta-analysis.

Forest plot for the fixed-effect meta-analysis and joint analysis on individual level data of the effect of expanded *NIPA1* polyalanine (>8 GCG repeats) on ALS risk with the initial discovery reports (Blauw, et al., 2012) and current replication using PCR, Sanger or whole genome sequencing (WGS) grouped per cohort/country of origin. Weights depending on number of participants. CI, confidence interval.

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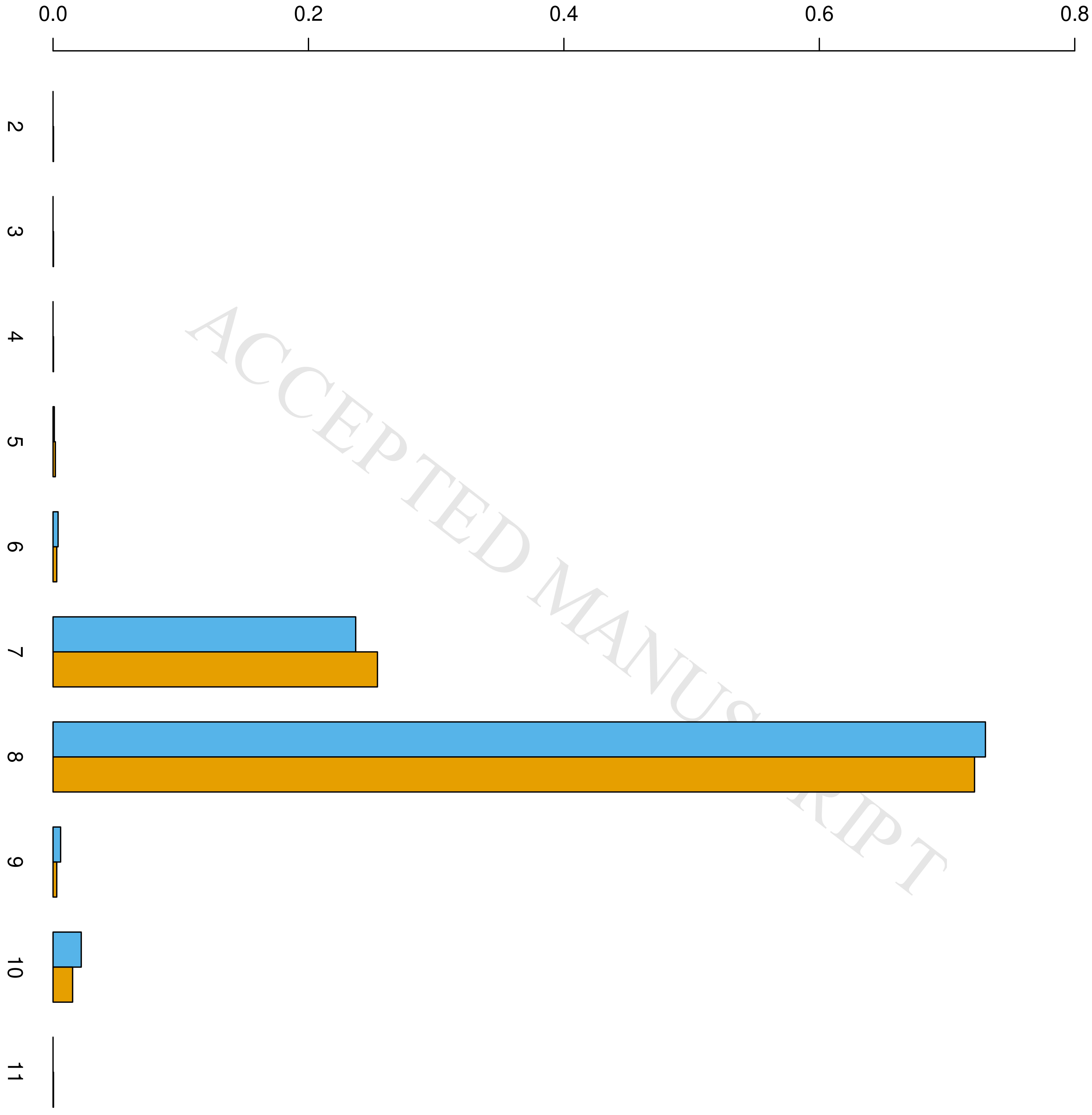
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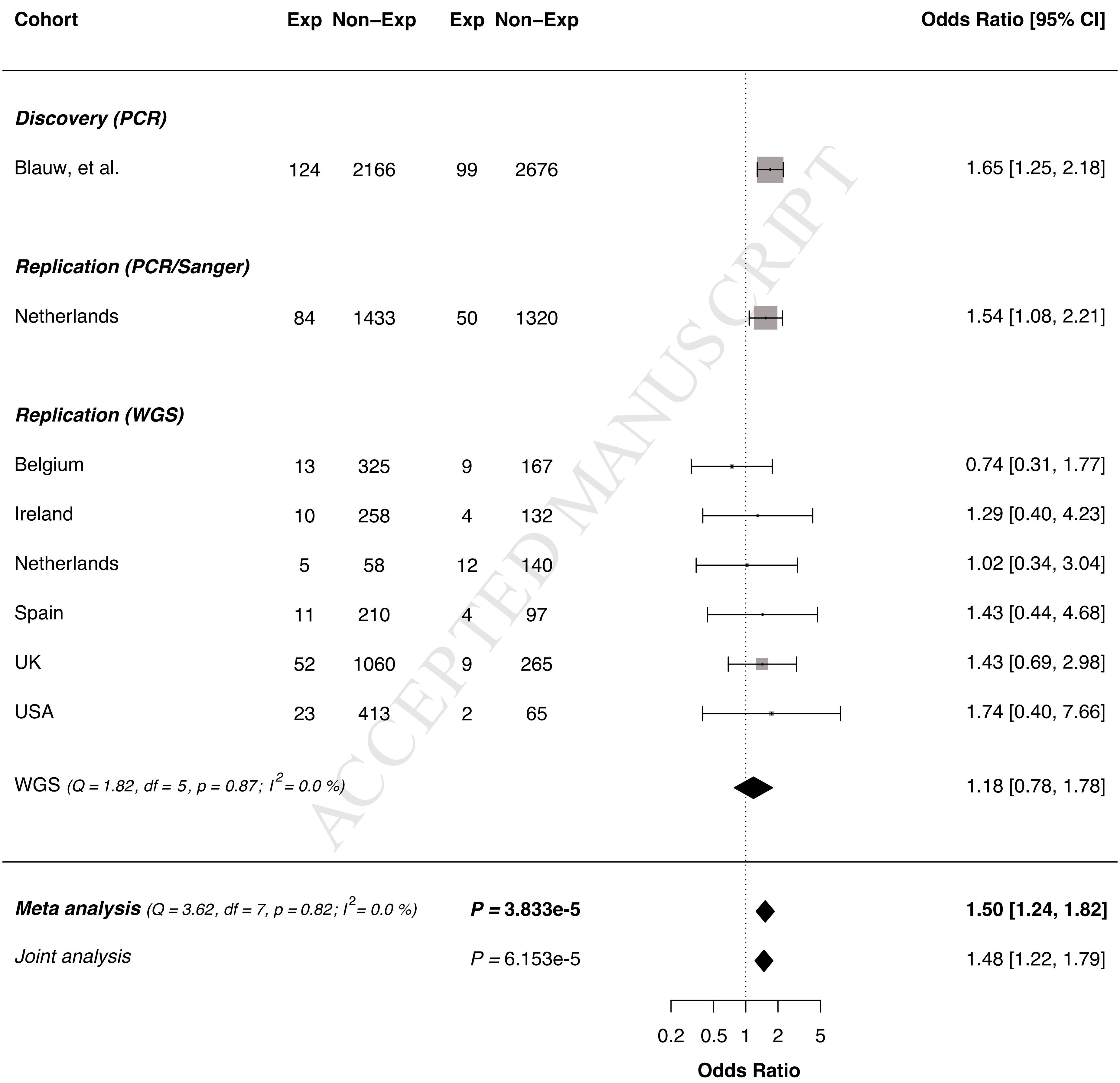

Cohort	Case Exp	Case Non−Exp	Control Exp	Control Non−Exp	Odds Ratio [95% CI]
Discovery (PCR)					
Blauw, et al.	124	2166	99	2676	1.65 [1.25, 2.18]
Replication (PCR/Sanger)					
Netherlands	84	1433	50	1320	1.54 [1.08, 2.21]
Replication (WGS)					
Belgium	13	325	9	167	0.74 [0.31, 1.77]
Ireland	10	258	4	132	1.29 [0.40, 4.23]
Netherlands	5	58	12	140	1.02 [0.34, 3.04]
Spain	11	210	4	97	1.43 [0.44, 4.68]
UK	52	1060	9	265	1.43 [0.69, 2.98]
USA	23	413	2	65	1.74 [0.40, 7.66]
WGS *($Q = 1.82$, $df = 5$, $p = 0.87$; $I^2 = 0.0$ %)*					1.18 [0.78, 1.78]
Meta analysis *($Q = 3.62$, $df = 7$, $p = 0.82$; $I^2 = 0.0$ %)*			***P* = 3.833e-5**		**1.50 [1.24, 1.82]**
Joint analysis			*P* = 6.153e-5		1.48 [1.22, 1.79]

Highlights

- We replicated the association of long PolyA-repeats in *NIPA1* with ALS in an independent Dutch cohort.
- A subsequent meta-analysis on 6,245 cases and 5,051 controls yielded $P = 3.8\text{x}10^{-5}$ with OR = 1.50.
- *NIPA1* repeat expansions are not associated with ALS age of onset or survival.
- *NIPA1* repeat expansions (>8 GCG-repeats) are a risk factor for sporadic ALS.