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Julian, T.H. [orcid.org/0000-0002-5488-5620](https://orcid.org/0000-0002-5488-5620), Boddy, S., Islam, M. [orcid.org/0000-0002-2296-0120](https://orcid.org/0000-0002-2296-0120) et al. (9 more authors) (2022) A review of Mendelian randomization in amyotrophic lateral sclerosis. *Brain*, 145 (3). pp. 832-842. ISSN 0006-8950

<https://doi.org/10.1093/brain/awab420>

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This is a pre-copyedited, author-produced version of an article accepted for publication in *Brain* following peer review. The version of record, Thomas H Julian, Sarah Boddy, Mahjabin Islam, Julian Kurz, Katherine J Whittaker, Tobias Moll, Calum Harvey, Sai Zhang, Michael P Snyder, Christopher McDermott, Johnathan Cooper-Knock, Pamela J Shaw, A review of Mendelian randomization in amyotrophic lateral sclerosis, *Brain*, Volume 145, Issue 3, March 2022, Pages 832–842, is available online at: <https://doi.org/10.1093/brain/awab420>

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# **A review of Mendelian randomization in amyotrophic lateral sclerosis**

Thomas H Julian<sup>1</sup>, Sarah Boddy<sup>1</sup>, Mahjabin Islam<sup>1</sup>, Julian Kurz<sup>1</sup>, Katherine J Whittaker<sup>1</sup>, Tobias Moll<sup>1</sup>, Calum Harvey<sup>1</sup>, Sai Zhang<sup>2,3</sup>, Michael P Snyder<sup>2,3</sup>, Christopher McDermott<sup>1</sup>, Johnathan Cooper-Knock<sup>1\*</sup>, Pamela J Shaw<sup>1\*</sup>

<sup>1</sup>Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, UK

<sup>2</sup>Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA

<sup>3</sup>Center for Genomics and Personalized Medicine, Stanford University School of Medicine, Stanford, CA, USA

\*These authors contributed equally

Correspondence should be addressed to PJS ([pamela.shaw@sheffield.ac.uk](mailto:pamela.shaw@sheffield.ac.uk)) and JCK ([j.cooper-knock@sheffield.ac.uk](mailto:j.cooper-knock@sheffield.ac.uk))

Professor Pamela J Shaw and Dr Johnathan Cooper-Knock,  
Sheffield Institute for Translational Neuroscience.  
385 Glossop Road,  
Sheffield S10 2HQ,  
United Kingdom.  
Tel: +44 114 2222295.

**Abbreviated summary:** Amyotrophic lateral sclerosis (ALS) is determined by a complex gene-environment interaction but environmental risk factors are poorly understood. Mendelian randomization (MR) is a useful tool for identification of environmental risk factors. We review the MR literature in ALS offering methodological and biological insights.

## ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a relatively common and rapidly progressive neurodegenerative disease which, in the majority of cases, is thought to be determined by a complex gene-environment interaction. Exponential growth in the number of performed genome-wide association studies (GWAS), combined with the advent of Mendelian randomization (MR) is opening significant new opportunities to identify environmental exposures which increase or decrease the risk of ALS. Each of these discoveries has the potential to shape new therapeutic interventions. However, to do so rigorous methodological standards must be applied in the performance of MR.

We have performed a review of MR studies performed in ALS to date. We identified 20 MR studies, including evaluation of physical exercise, adiposity, cognitive performance, immune function, blood lipids, sleep behaviours, educational attainment, alcohol consumption, smoking and type 2 diabetes mellitus. We have evaluated each study using gold standard methodology supported by the MR literature [and the STROBE-MR checklist](#). Where discrepancies exist between MR studies, we suggest the underlying reasons. [A number of studies conclude that there is a causal link between blood lipids and risk of ALS; replication across different datasets and even different populations adds confidence. For other putative risk factors, such as smoking and immune function, MR studies have provided cause for doubt.](#) We highlight the use of positive control analyses in choosing exposure SNPs to make up the MR instrument, use of SNP clumping to avoid false positive results due to SNPs in linkage, and the importance of multiple testing correction. We discuss the implications of survival bias for study of late age of onset diseases such as ALS, and make recommendations to mitigate this potentially important confounder.

[For MR to be useful to the ALS field, high methodological standards must be applied to ensure reproducibility. MR is already an impactful tool but poor quality studies will lead to incorrect interpretations by a field which includes non-statisticians, wasted resources and missed opportunities.](#)

## Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and universally fatal late age of onset neurodegenerative disease. ALS is relatively common with a lifetime risk of  $\sim 1/350$ <sup>1</sup>. ALS is also an archetypal complex disease in which 10% of patients suffer monogenic disease but the majority of disease, known as sporadic, is determined by an interaction of multiple environmental and genetic risk factors<sup>2</sup>. Recent years have brought significant progress in delineating the genetic architecture of ALS<sup>3</sup>. However, efforts to understand environmental risk factors and the gene-environment interaction in particular, are in their infancy. To date the only established risk factors for the development of ALS are male sex and increasing age<sup>4</sup>. Our ability to profile and classify specific combinations of genetic and environmental risk factors will determine the future of personalized medicine interventions for the majority of ALS patients.

Mendelian randomization (MR) is a method whereby genetic variation within a population serves as a natural experimental instrument to study the effect of environmental modifiers on an outcome such as risk of a specific disease<sup>5,6</sup>. At meiosis, alleles are randomly assorted according to Mendel's second law. Importantly, this process is independent of disease risk and is not vulnerable to ascertainment bias. In MR, this is exploited to determine whether the risk of developing a specific disease is contingent on genetic liability to be exposed to a risk factor of interest. The randomized nature of allele assortment makes this comparison analogous to a randomized controlled trial (**Figure 1**). *It should be noted that the outcome does not have to be a disease state; for example positioning a disease as an exposure and measurable clinical variables as outcomes has been used effectively for biomarker discovery*<sup>7</sup>.

MR has opened a gateway for analysis of causal relationships in ALS because it avoids many of the challenges that have plagued observational research in this field, in particular ascertainment bias and problems with small sample sizes. Sample size is not a problem because the exposure is typically measured in an independent cohort to the outcome (ALS), providing the cohorts have comparable genetic structure (usually population matched). As a result, there is no requirement to obtain two separate data points (exposure and outcome) in every patient, which is often a critical

bottleneck for observational studies. The use of two independent sample sets is known as two-sample MR.

The most common means of performing MR is to use summary statistics from genome-wide association studies (GWAS). These have become exponentially more accessible for multiple and diverse traits with the advent of large biobanks which incorporate publicly available data, such as UK Biobank, FINNGEN and BioBank Japan. Single nucleotide polymorphisms (SNPs) associated with a proposed risk factor are identified in an exposure GWAS. These SNPs are used as instruments for measuring genetic liability to the exposure of interest. Instrumental SNPs are then measured in a separate outcome GWAS to determine the relationship between the exposure and disease risk. A causal relationship is inferred if there is a linear relationship between the exposure, as measured by the instrument, and the risk of the disease outcome (**Figure 1**).

A valid MR analysis rests upon three key assumptions (**Figure 2**): (1) The instrumental SNPs must be robustly associated with the exposure. (2) The SNPs must be associated with the disease risk outcome only through the exposure of interest, rather than affecting the outcome via multiple pathways or exposures (known as horizontal pleiotropy). (3) The SNPs must not be associated with confounders. The strength of association between instrumental SNPs and the exposure (assumption 1) is usually quantified as a F-statistic which is a measure of bias i.e. the difference between the SNP-derived estimate of the exposure and the actual exposure value. A F-statistic > 10 corresponds to a SNP-derived estimate for which the bias is < 10% of the intragroup variability in the measured exposure<sup>8</sup>. Evidence of violation of the assumptions related to alternative SNP associations (assumptions 2 and 3) is gained through examination of heterogeneity between SNPs, in which the effect on the exposure is not proportional to the effect on the outcome; this forms the basis of a number of sensitivity measures including the MR-PRESSO and Cochran's Q test<sup>9</sup>. An informative paper from Burgess *et al.* summarises gold standard methodology to test these assumptions<sup>10</sup>.

In this review we have identified 20 MR studies relating to ALS. As such studies become common practice, it becomes necessary for methodological standards to be identified and upheld by the field to facilitate interpretation of results. Currently there is significant variability in methodology utilized by authors, which has led to certain

conflicting results. The STROBE-MR checklist has been developed with a view to improving the quality of reporting of MR studies<sup>11</sup>, but at present is not widely used. In this review, we aim to provide interpretation of findings to allow readers to evaluate past and future MR studies of ALS.

## **Included studies**

This review was prospectively registered to PROSPERO. The registration number for this review is CRD42020221080. A systematic search was performed on 17.11.2020. The search was performed using the PubMed, Scopus and Web of Science electronic databases. For the search, two medical subject heading (MeSH) terms were used. Term A was “motor neuron disease” OR “motor neurone disease” OR “MND” OR “ALS” OR “amyotrophic lateral sclerosis”. Term B was “Mendelian randomisation” OR “Mendelian randomization”. The search terms applied to the title, abstract and keyword fields. No further filters were applied in our search. In order to capture further studies excluded from the aforementioned electronic databases, we searched the references of included studies for further relevant articles and remained vigilant to publications beyond our search date. Articles eligible to be included in this review were required to meet the following criteria: (1) Mendelian randomization study design; and (2) use of outcome SNP data associated with ALS. Articles were excluded for the following reasons: non-original articles, non-human studies, duplicate studies, study protocols, letters, conference proceedings. Rayyan software was utilized to assist with the inclusion/exclusion process<sup>12</sup>.

Our literature search identified a total of 287 studies. During abstract screening, articles were excluded for the following reasons: non-ALS study population (n=158); non-original study, letter or conference proceeding (n=40); does not detail causal inferences using MR (n=17); duplicated study (n=51). 21 studies were therefore reviewed as full-text articles. During full-paper screening, articles were excluded for the following reasons: non-original study (n=1); does not detail causal inferences using MR (n=3); non-ALS study population (n=1). Four further studies meeting the inclusion criteria were identified after the initial search was conducted. Twenty studies were therefore included in our final review. Headline results and a methodology checklist are provided for every exposure measured across all of the twenty studies in **Supplementary Table 1**.

The discussion in our manuscript is centred on areas of consensus and controversy between MR studies focused on risk of ALS. Our aim is to explain disagreements in terms of the underlying methodology and draw attention to recurring issues. Moreover, we aim to draw out biological insights that have been gained from using MR to study ALS. Our approach and findings are summarised in **Figure 3**.

## **What do we know? Findings with some consensus.**

In some instances there is agreement where multiple MR studies have reached the same conclusions regarding an association between ALS risk and a specific environmental exposure. Perhaps the strongest consensus exists regarding a positive relationship between blood lipid levels (LDL cholesterol and total cholesterol) and ALS risk. In this section we also consider related traits linked to blood lipids, such as type 2 diabetes, adiposity, physical exercise and smoking, so that we can discuss evidence for a link between ALS and the ‘metabolic syndrome’<sup>13</sup>. The ‘metabolic syndrome’ is defined as interconnected physiological, biochemical, clinical, and metabolic factors that directly increase the risk of cardiovascular disease, type 2 diabetes, and all cause mortality. There is a long literature linking metabolic syndrome to neurodegenerative disease, particularly Alzheimer’s disease<sup>14</sup>. ALS has been linked to both hyperlipidaemia and insulin resistance<sup>15</sup>. However, ALS has also been linked to physical exercise<sup>16</sup> which is thought to be protective against metabolic syndrome<sup>13</sup>. Epidemiological studies of physical exercise in ALS have largely relied on questionnaire-based quantification of exercise where selection and recall bias have led to inconclusive results. MR is ideally placed to address these limitations.

### *Blood lipids*

Bandres-Ciga *et al.* performed an unbiased MR screen of environmental modifiers of ALS risk using 345 published and 290 unpublished GWAS<sup>17</sup>. Only three exposures were causally linked to ALS risk: LDL cholesterol level (IVW OR 1.12, 95% CI=1.03–1.20, p=0.003); coronary heart disease (IVW OR 1.06, 95% CI=1.0–1.13, p=0.02); and self-reported high cholesterol (IVW OR 2.39, 95% CI=1.48–3.84, p=0.0003). The

authors then used multivariable MR (MVMR) analysis to show that the associations with coronary heart disease and self-reported high cholesterol are underpinned by SNPs linked to LDL cholesterol. MVMR is a means of conditioning between alternative exposures in a MR study to determine which exposure is more closely linked to the disease outcome. Where two or more exposures are correlated this can be a powerful technique to elucidate underlying biological mechanisms.

MR analyses typically report the p-value from the inverse-variance weighted (IVW) measure as the headline result because this has the most statistical power<sup>18</sup>. The IVW is effectively a weighted mean estimate derived from the total set of instrumental SNPs. However, like any mean value, the IVW is vulnerable to outliers, and it is also disproportionately dependent on the assumption that each of the SNPs offers an independent estimate (see discussion of clumping below). Consequently, it is good practice to supplement the IVW estimate for a particular association, with several robust measures which are relatively underpowered but are less vulnerable to outliers. Robust measures include MR robust adjusted profile score (RAPS)<sup>19</sup>, weighted median<sup>20</sup>, weighted mode<sup>21</sup>, and the MR-Egger<sup>22</sup>. Furthermore a leave-one-out (LOO) analysis can be applied to ensure that no single SNP is disproportionately responsible for the headline result. In the study of Bandres-Ciga *et al.*, the results remained significant with deployment of robust measures and were not altered by removing individual SNPs (LOO analysis). The weakness of this study is that there is no correction applied for the multiple testing problem posed by the large unbiased screen that has been employed.

The role of LDL cholesterol and total cholesterol were also assessed by Chen *et al.*<sup>23</sup>, who combined SNPs associated with either exposure to generate a single measure of genetic liability to cholesterol. Of note this study used different exposure and outcome GWAS to Bandres-Ciga *et al.* Chen and colleagues discovered a statistically significant relationship between increasing levels of LDL cholesterol / total cholesterol and ALS (IVW OR=1.2,  $p \leq 0.001$ ). Notably no robust measures were reported. Zeng and Zhou performed a comprehensive MR study evaluating the roles of HDL cholesterol, triglycerides, LDL cholesterol and total cholesterol in ALS<sup>24</sup>. The major strength of this study is that the results were validated in both European and East Asian populations. In the European population, when the authors applied a stringent Bonferroni correction for multiple testing, only LDL cholesterol was causally linked to



ALS risk (IVW OR=1.14, 95% CI=1.05–1.24,  $p=1.38E-03$ ). LDL cholesterol was also significantly related to ALS risk in the East Asian population (IVW OR=1.06, 95% CI=1.00–1.12,  $p=0.04$ ), including in robust measures: MR Egger ( $p=2.41E-04$ ) and weighted median ( $p=6.29E-04$ ). Neither triglycerides or HDL cholesterol showed a significant relationship with ALS. Finally, van Rheenen *et al.*<sup>25</sup>, using a new larger GWAS (29,612 ALS patients and 122,656 controls), concluded that total cholesterol was positively associated with ALS risk (IVW  $p=0.02$ ), including significance in several robust measures, namely the weighted mode, weighted median and MR Egger. However, only the weighted median remained positive following a stringent Bonferroni multiple testing correction. The authors considered years of schooling a potential confounder, and controlled for it using MVMR, but the result remained significant. In this study LDL cholesterol was also significantly related to ALS risk (IVW  $p=0.03$ ), but this result did not remain significant in robust measures. Again, no causal relationship was demonstrated for triglycerides and HDL cholesterol.

Overall, there is compelling MR evidence for a relationship between both increased LDL cholesterol and total cholesterol and genetic liability to ALS; but not for triglycerides or HDL cholesterol. We can make this conclusion despite lack of significance in some robust measures because by definition robust measures are relatively unpowered and should be interpreted with caution. The convergence between different studies, correction for potential confounders (such as years of education) and outlier SNPs, and significance in a proportion of the robust measures, is good evidence for a causal relationship. Exploration of the link between lipid metabolism and ALS is a promising area for future research and potentially represents a factor amenable to therapeutic intervention.

### *Type 2 diabetes mellitus*

Observational studies have suggested a protective effect of type 2 diabetes mellitus on ALS risk<sup>26</sup>. Zeng *et al.* investigated this relationship using MR in both European and East Asian populations<sup>27</sup> and concluded that type 2 diabetes mellitus was protective against ALS in Europeans (IVW OR=0.93, 95% CI=0.88–0.99,  $p=0.02$ ). However, none of the robust measures showed significance. Moreover, the authors used MR-PRESSO to test for SNP heterogeneity and identified a single outlier SNP; exclusion of this SNP produced a non-significant relationship in all measures including

the IVW ( $p=0.06$ ). Finally, the LOO analysis demonstrated that removal of several individual SNPs invalidated the result. Overall, we conclude that there is no evidence from MR to support type 2 diabetes mellitus as protective against ALS.

### *Adiposity*

Body mass index (BMI) and adiposity have long been proposed to modify ALS risk. It has been observed that ALS patients typically present with normal-low BMI premorbidly, and a raised BMI has been suggested to be protective<sup>28</sup>. The direction of causation is a key question. For example, ALS is associated with hypermetabolic phenotype<sup>29</sup> which could reduce BMI as a byproduct of the disease process. MR is well placed to address this question because of the ability to differentiate upstream assignment at conception from downstream consequences of disease.

Zhang *et al.* used several measures of adiposity in their MR study<sup>30</sup> including body fat percentage, childhood BMI, BMI, birth weight and waist-hip ratio. Therefore, the authors applied a Bonferroni multiple testing correction. There was evidence that genetically predicted increase in body fat percentage is associated with a reduced risk of ALS (IVW OR=0.67, 95% CI=0.54–0.83,  $p=3.25E-04$ ). Importantly, a leave-one-out (LOO) analysis indicated that the relationship between body fat percentage and ALS was driven by two SNPs in particular, which suggests that the IVW estimate may have been biased by SNP outliers. BMI, waist-hip ratio and birthweight were not significantly linked to ALS. Higher childhood BMI was significantly linked to ALS in both the IVW ( $p=0.03$ ) and MR-Egger ( $p=0.04$ ) estimates but did not pass the multiple testing correction.

In a different study, Zeng *et al.* also explored the relationship between ALS and BMI. Using a number of robust MR measures they concluded that increasing BMI is not significantly protective for ALS in cohorts from Europe (IVW  $p=0.28$ ) or East Asia (IVW  $p=0.65$ ). Similarly, van Rheenen *et al.* did not identify a significant relationship between ALS and BMI<sup>25</sup>; and in a letter to the editor, Aziz did not find a significant relationship between body fat percentage and ALS risk, though these findings cannot be appraised extensively due to limited description of employed methodology<sup>31</sup>.

At this point we draw attention to another important methodological consideration in MR analysis known as ‘clumping’. Common SNPs, such as those employed in GWAS

studies and used to create MR instruments, are not independent, but are co-inherited in a process known as linkage disequilibrium (LD). The coinherence of any two SNPs in a population can be measured and used to estimate the probability that the presence of both SNPs in an individual genome represents one or multiple inheritance events. The process of clumping refers to the grouping of significantly correlated SNPs into one independent signal to reduce the risk of measuring the same signal multiple times. Although there is no mandated method of clumping in MR, it is worth noting that relative to other studies in the ALS literature, Zhang *et al.* implement a relatively liberal clumping procedure ( $R^2 < 0.05$ ), which allows for inclusion of SNPs in higher LD with each other compared to other studies and therefore a higher probability of type 1 error (**Supplementary Table 1**). On this basis, and for the other reasons outlined above, we believe that the positive relationship between body fat percentage and ALS identified by Zhang *et al.* should be interpreted with caution. In our own study, we concluded that there is no significant relationship between body fat percentage and ALS risk<sup>32</sup>. We utilized a more stringent clumping criterion ( $R^2 < 0.001$ ) than Zhang *et al.* to remove SNPs in significant LD.

Overall, MR studies do not consistently support a causal role for adiposity in ALS, which may suggest that there is a confounder unaccounted for in the observational literature.

### *Physical exercise*

The role of physical exercise as a risk factor for ALS has historically been controversial<sup>16</sup>. With a view to resolving this conflict, we performed a MR study under the hypothesis that intense and frequent exercise increases the risk of ALS<sup>32</sup>. To test this hypothesis, an instrument capturing frequent participation in strenuous sport and other exercise (SSOE<sup>33</sup>, [defined as spending two or more days per week performing SSOE for a duration of 15-30 minutes or greater](#)) was used as the exposure variable. [Of note the control group in the SSOE GWAS was individuals who reported no time spent performing SSOE in the past four weeks.](#)

An important choice in the design of an MR study is the p-value threshold (for the association between genetic variation and the exposure of interest) used to identify instrumental SNPs used to measure the exposure. A stringent p-value can lead to too

few SNPs and an underpowered test. A liberal p-value increases the risk of including invalid SNPs which violate the assumptions underpinning MR. To avoid these pitfalls, we used a positive control (body fat percentage) to show that a conservative measure of SSOE consisting of only genome-wide significant ( $p < 5E-08$ ) SNPs is underpowered to detect a causal effect, but a more liberal threshold ( $p < 5E-06$ ) performs as expected (SSOE is shown to reduce body fat percentage). A liberal threshold was then used to select instrumental SNPs to show that genetic liability to participation in SSOE is associated with increased risk of ALS (IVW  $p = 0.01$ ). This result was consistent in multiple robust measures including MR RAPS, weighted median and weighted mode.

Our findings have been contested in an analysis by Zhang *et al.* who utilised a conservative ( $p < 5E-08$ ) p-value threshold for selection of instrumental SNPs to measure SSOE and a different ALS GWAS<sup>34</sup>. In this study, Zhang *et al.* found no significant relationship between SSOE and ALS (IVW  $p = 0.49$ ). As described above, our positive control analysis of the SSOE exposure suggests that this instrument is underpowered.

A further study evaluated the effect of “days per week moderate activity” and “days per week vigorous activity”<sup>25</sup>. Despite use of both a liberal ( $p < 5E-05$ ) and a conservative ( $p < 5E-08$ ) p-value threshold to select instrumental SNPs, neither measure was significantly linked to ALS risk (IVW  $p = 0.89$  and  $0.19$  respectively). However, neither of these exposures included a consideration of time spent exercising, unlike the SSOE measure. Moreover, it is interesting that SSOE has been shown to be negatively associated with workplace activity, unlike other UK Biobank measures such as “moderate to vigorous physical activity” (MVPA) and “vigorous physical activity” (VPA)<sup>33</sup>. This is an important distinction, given that occupational and leisure time activities are dissimilar in their respiratory and neuromuscular requirements<sup>32</sup>. Zhang *et al.* showed that neither VPA or MVPA are significantly linked to ALS risk (IVW  $p = 0.17$  and  $0.19$  respectively). [Leisure-time activity more often includes the kind of dynamic and anaerobic activity<sup>35</sup> which selectively recruits motor neurons supplying type IIb fast twitch muscle fibres; the same motor neurons which degenerate early in the pathogenesis of ALS<sup>36</sup>.](#)

Our study<sup>32</sup> is in agreement with Zhang *et al.* that overall physical activity (i.e. not quantified as being strenuous or frequent) alone is not a risk factor for ALS.

Specifically, Zhang *et al.* showed a non-significant relationship between overall activity and ALS (IVW  $p=0.45$ ), and we discovered that average accelerations were not associated with ALS risk in either liberal or conservative analysis (IVW  $p=0.31$  and  $0.37$  respectively). Similarly, both studies agree that sedentary behaviour is not protective for ALS (IVW  $p=0.29-0.81$ ).

Overall, the MR evidence is clear that sedentary behaviour is not protective for ALS and that lower intensity or infrequent exercise does not increase risk. There is conflict between studies with respect to the role of activity which is both strenuous and frequent activity in development of ALS. A key point from this discussion is the importance of the precise measure used to quantify the exposure of interest. Subtly different measures, such as workplace versus leisure-time physical activity, can be associated with non-overlapping or even anticorrelated instrumental SNPs<sup>33</sup>. Each measure must be evaluated to determine relative advantages and disadvantages; for example average accelerations measured by accelerometer is likely to be the most objective, but there is no way of knowing if subjects performed their usual behaviour during the short testing period. We would recommend the use of a positive control to optimise the choice of instrumental SNPs as a means of increasing confidence in both negative and positive results<sup>10</sup>.

## *Smoking*

Cigarette smoking is amongst few risk factors confidently depicted as a causative factor in the development of ALS<sup>37</sup>. Despite this assertion, studies continue to produce contradictory findings and it has been suggested by some investigators that the relationship is a result of confounding factors such as military service, education, exercise participation and socio-economic status<sup>38</sup>. Moreover, because smoking is known to have a large effect on life expectancy, there is the potential for a spurious protective effect because smokers may be significantly less likely to reach the age at which disease manifests<sup>39</sup>. This effect, known as survival bias, is a potential problem for performing MR in any disease population with late age of onset such as ALS. Various corrections have been proposed for this issue<sup>40</sup> but all risk a loss of power. An obvious solution is to measure the disease outcome in a birth cohort where survival cannot have impacted selection. The use of unaffected family members of deceased

patients has also been proposed as a method for reducing survival bias in sample recruitment<sup>41</sup>.

Using MR, Zhan and Fang presented weak evidence that “ever smokers” have a higher risk of genetic liability to ALS than non-smokers (IVW OR=1.25; 95% CI=1.01-1.55; p=0.04)<sup>42</sup>. The relationship between smoking and ALS did not persist in robust measures. Similarly, Opie-Martin *et al.* concluded that “ever smoker” was weakly associated with ALS (IVW OR=1.10, 95% CI=1.00-1.23, p-value=0.05) but this result was also non-significant in all robust analyses<sup>43</sup>. Van Rheenen *et al.* tested for an effect of both “age of smoking initiation” and “cigarettes per day” but concluded that neither were significantly linked to ALS using a conservative or liberal p-value threshold to choose instrumental SNPs (IVW p-value=0.78 and 0.33 respectively)<sup>25</sup>. Overall, the current MR literature does not support a causal role for smoking in the development of ALS.

Although there is consensus that LDL cholesterol and total cholesterol are positively linked to risk of ALS, there is more controversy or even negative findings for the related traits of type 2 diabetes, adiposity, physical exercise and smoking. The evidence does not support a simple correlation between ALS and a metabolic syndrome, for example strenuous and frequent physical exercise should reduce risk of the metabolic syndrome but appears to increase risk of ALS. [It seems likely that multiple pathogenic pathways exist leading to neurotoxicity and perhaps different pathways are relevant for different sub-groups of patients<sup>44</sup>. Currently it is not clear whether patients vulnerable to high cholesterol are the same patients who are vulnerable to strenuous physical exercise; it could be that these groups are distinct, perhaps determined by genetic background. Lifestyle recommendations to reduce ALS risk may need to be individually tailored, rather than uniform.](#)

### **What don't we know? Negative studies and lack of power**

A number of reported MR studies did *not* identify a relationship between an exposure of interest and ALS risk and set this forth as evidence regarding the underlying biology. The problem with this assertion is that it is difficult to distinguish between lack of power and a true negative result. This is another example where a positive control can be helpful, for example genetic instruments for smoking earn confidence

because they have been used to show a significant effect on other diseases such as coronary artery disease<sup>45</sup>. The power problem is illustrated by discussion of a series of MR studies considering a link between various immune exposures and ALS including blood leukocyte count and blood levels of soluble receptors for various interleukins: IL-1, IL-2 and IL-6. For total leukocyte count a smaller study reports a positive association and a larger study reports a negative finding; we consider whether the larger study should always take precedence because of theoretical superior power.

### *Leukocyte count*

Dysregulated immunity is considered to be a common feature of neurodegenerative diseases<sup>46</sup>. Li *et al.* explored the relationship between peripheral leukocytes and ALS using MR<sup>47</sup>. Exposures tested were monocyte count, neutrophil count, eosinophil count, basophil count, lymphocyte count and total leukocyte count. The authors used a conservative p-value threshold to choose instrumental SNPs ( $p < 5E-08$ ), removed potentially pleiotropic SNPs which were associated with BMI or smoking, and used stringent clumping criteria ( $R^2 < 0.001$ ). It should be noted that no information is provided regarding the use of proxy SNPs, and this is a potential weakness in the methodology of this study. Proxy SNPs are utilized when matching between exposure and disease outcome. GWAS leads to loss of SNPs simply because of differences in the experimental design *i.e.* different GWAS experiments measure different SNPs. This can lead to loss of important information, but can be partially overcome by use of a “proxy”, defined as a SNP present in both datasets which is in linkage disequilibrium (LD) with the SNP which cannot be matched.

Li *et al.* suggested that there is a causal relationship between genetic liability to increased total leukocyte count and reduced risk of ALS (IVW OR: 0.9, 95% CI: 0.84–0.97,  $p=0.007$ ). Of note, this result would remain significant after a Bonferroni multiple testing correction and was supported by the MR-Egger analysis, whereas the weighted median showed the same direction of effect with a borderline non-significant p-value ( $p=0.06$ ). Increasing neutrophil count was also shown to decrease ALS risk using the IVW (OR: 0.93, 95% CI: 0.86–1.00,  $p=0.049$ ) but not with any robust measures.

These findings were not replicated by van Rheenen *et al.*, who use a new and larger GWAS for both ALS and blood cell traits<sup>25,48</sup>. In this analysis, no significant relationship was identified between ALS and eosinophil, lymphocyte, monocyte, neutrophil or total leukocyte counts. Although total leukocyte count was near-significant in IVW analysis ( $p=0.06$ ), this did not persist in robust measures (weighted mode  $p=0.76$ , weighted median  $p=0.23$  and simple mode  $p=0.76$ ) with the exception of the MR-Egger ( $p=0.04$ ), and the relationship remained non-significant when using a more liberal  $p$ -value threshold to choose instrumental SNPs ( $p<1E-05$ ).

It is tempting to suppose that the negative result reported by van Rheenen *et al.* represents the true underlying biology because of the larger sample size in both exposure and outcome GWAS. A larger exposure GWAS should deliver more powerful instrumental SNPs which can account for a greater proportion of variability in the exposure of interest. Underpowered instruments have led to false positive conclusions in the past. For example, small-scale MR studies using limited instrumental SNPs confined to a single locus suggested that plasma urate was protective against Parkinson's disease (PD), but this was not reproducible. A larger MR study, using instrumental SNPs across multiple loci with more collective power to measure variability in urate, did not show a protective effect and moreover, a clinical trial of intervention to elevate urate in PD patients was terminated due to lack of efficacy<sup>49</sup>. Similarly, a larger outcome GWAS should provide a more precise estimate of SNP-effect size on disease risk. However, we argue that the result reported by Li *et al.* meets gold standard methodological criteria including robust tests and sensitivity analyses and should not be dismissed entirely, particularly when the total leukocyte measure was actually close to significance in both studies. Complexity can result when a larger GWAS is derived from a more heterogeneous population structure. Measurement of genetic liability rests upon portability of genetic instruments between exposure and outcome GWAS. Instrumental SNPs and even LD structure vary significantly across population groups<sup>50</sup>.

### *Interleukins*

Inflammatory responses mediated by interleukin-1 and interleukin-2 (IL-1 and IL-2) have been suggested to have a role in the development of ALS<sup>51</sup>. Consequently, Yuan *et al.* used MR to test for a causal relationship between circulating levels of IL-1



receptor antagonist (IL-1Ra) and IL-2 receptor antagonist (IL-2Ra) and ALS risk<sup>52</sup>. The authors chose to use a conservative p-value threshold ( $p < 5 \times 10^{-8}$ ) which resulted in a very small number of instrumental SNPs: two SNPs for IL-1Ra and one SNP for IL-2Ra. In this study, it was shown that for a 1-SD increase in circulating IL-1Ra levels, the OR of ALS was 0.64 (95% CI, 0.46–0.88; IVW  $p = 0.005$ ). A borderline significant effect was found for an inverse relationship between circulating IL-2Ra levels and ALS (Wald ratio, OR, 0.91; 95% CI 0.83–1.00;  $p = 0.06$ ). As so few genome-wide significant SNPs are available in this analysis, robust measures do not serve a role and therefore only the IVW and Wald ratio are presented. Further, the two genome-wide significant SNPs for IL-1Ra were in LD ( $R^2 = 0.11$ ), and had only the most significant of these two SNPs been analysed then no significant relationship would have been identified (Wald ratio,  $p = 0.08$ ).

Similarly, Zhang *et al.* investigated the relationship between soluble IL-6 receptor (s-IL-6R) level and ALS using a single genome-wide significant SNP derived from a small GWAS of 1650 individuals<sup>53</sup>. This study found that there was no significant relationship between s-IL-6R levels and ALS (IVW OR = 1.00; 95% CI 0.92–1.10;  $p = 0.94$ ). However, the small number of SNPs suggests that this study, like that of Yuan *et al.*, may be underpowered.

In conclusion, there is not currently any MR evidence to support an association between inflammatory exposures, particularly interleukins, and ALS. Reported positive findings rest upon underpowered instruments and have not been validated in larger, better powered studies. However, we have some concern over population matching in the largest study of total leukocyte count which would benefit from future study. We note that a recent genome-wide study of DNA methylation in ALS discovered a significant association between ALS and white blood cell proportions<sup>54</sup>. Underpowered instruments mean that even negative findings should be interpreted cautiously. A larger concern is that the immune response is a complex derivative of interaction between multiple cells and cytokines; the suggestion is that the simple measures used in the studies described here are not an accurate measure of immune function.

## **Controversies: where MR studies disagree**

We have discussed several MR studies where the headline results disagree and have outlined possible reasons based on the methodology. Alcohol intake and cognitive performance represent two important and modifiable environmental risk factors which have been consistently linked to ALS. For both exposures there is disagreement in the MR literature which remains unresolved and highlights a need for more work.

### *Alcohol consumption*

Studies in the observational literature detailing the relationship between alcohol consumption and ALS risk have produced directly contradictory findings<sup>55</sup>. Yu *et al.* explored the relationship between gram per day intake of alcohol and risk of ALS in an MR study<sup>56</sup>. The authors carefully controlled for a potential confounding effect of smoking using multivariable MR (MVMR)<sup>57</sup> and discovered a significant relationship between alcohol consumption and ALS risk (IVW OR=2.48 for each ~10g/day increase in alcohol consumption, 95% CI 1.38–4.44,  $p=0.002$ ). Robust measures were performed but were not significant. In contrast van Rheenen *et al.* did not discover a relationship between “alcoholic drinks per week” and ALS in their study<sup>25</sup>, in which neither the IVW ( $p=1.00$ ) nor robust measures are significant. Though this finding contradicts the conclusions of Yu *et al.*, it is important to recognise that van Rheenen tested “alcoholic drinks per week” without quantification of units/grams of ethanol, which is a less precise measure of actual ethanol consumption.

### *Cognitive performance*

Zhang *et al.* explored the relationship between ALS and genetic liability to a range of academic and cognitive performance measures including educational attainment (EA), highest mathematics class, self-reported mathematics ability, intelligence and cognitive performance<sup>58</sup>. SNPs were selected from large GWAS at a conservative  $p$ -value threshold of  $p<5E-08$ , but clumped using a relatively liberal threshold of  $R^2<0.1$ . Following a Bonferroni correction for multiple testing, this study concluded that there was a significant protective effect on ALS risk of higher EA (IVW OR=0.79 per year of education completed, 95% CI 0.73–0.86;  $p=8.46E-08$ ) and higher mathematics class (IVW OR=0.81, 95% CI 0.72-0.91;  $p=3.76E-4$ ). Both results were supported by robust tests in the form of MR-Egger, weighted median and simple median for EA, and

weighted median and simple median for highest mathematics class. Mode-based estimation was not performed in this analysis, although this is generally considered a core measure<sup>10</sup>.

The potential weakness of the above analysis is highlighted by our own study<sup>32</sup>, which did not show a significant relationship between EA and ALS ( $p=0.68$ ). In this study, the same EA GWAS<sup>59</sup> was used, but a more stringent clumping criterion of  $R^2 < 0.001$  was employed to ensure independence between instrumental SNPs. Stringent clumping dramatically reduced the number of SNPs included in the exposure instrument (298 versus 1271 SNPs), suggesting that the instrument employed by Zhang *et al.* may have contained non-independent signals.

Finally, van Rheenen *et al.* explored the relationship between “years of schooling” and ALS risk<sup>25</sup>. They identified a significant protective relationship by the IVW method ( $p=0.01$ ), but this was not supported by any robust measures. Further, the instrumental SNPs were significantly heterogeneous (Cochran’s  $Q$   $p=3.90E-09$ ), suggesting that the underlying assumptions of MR may have been violated. As discussed above, the IVW measure is particularly vulnerable to instrument heterogeneity and outlier SNPs<sup>20</sup>.

In summary, whilst there is evidence to support a protective role for educational factors in ALS, there are weaknesses and conflicts in the available evidence which urges caution in interpretation.

## **Larger MR studies lead to more multiple testing problems**

The advent of large biobanks releasing huge numbers of GWAS studies relating to diverse phenotypes has brought the opportunity for ever larger MR studies addressing multiple phenotypes simultaneously. This opportunity for unbiased, data-driven work is likely to lead to significant advances, but requires proper multiple testing correction. It has been argued that less stringent requirements for multiple testing should be applied in MR due to the relative lack of power of the tests used<sup>10</sup>. However, we point to the early days of genetic association testing where similar arguments led to irreproducible results and a significant waste of resources<sup>60</sup>. We have already discussed several studies where this has been an issue, notably the work of Bandres-

Ciga *et al.*. Nonetheless, findings with respect to blood lipids have been validated in independent datasets. For other exposures a lack of proper multiple testing correction means that no firm conclusions can currently be drawn. In relation to this we discuss serum metabolites and sleep behaviours.

Metabolites which make up the metabolome are the intermediates and end products of cellular regulatory processes. Changes in the levels of metabolites are influenced by both genetic background and environmental stimuli and therefore an MR study of the metabolome is an effective integrator of multiple exposures. For example, the serum concentration of a given metabolite may be lower because of genetic mutations within enzymes responsible for key steps in metabolite synthesis, or because of a limited supply of substrates from the diet. ALS has been associated with a number of metabolic defects including deficits in production of nicotinamide<sup>61</sup> and inosine<sup>62</sup>. Indeed metabolites such as branched chain amino acids (BCAA)<sup>63,64</sup> and vitamin B12<sup>65</sup> have been administered to ALS patients as part of experimental medicine trials.

ALS has a strong association with sleep disorders, primarily as a result of muscle weakness leading to hypoventilation or other dysfunction which can disturb sleep. However, there is also evidence that ALS is associated with non-motor sleep disorders including insomnia and rapid eye movement (REM) parasomnia<sup>66</sup>. There is some suggestion that such disturbances may be causal, for example sleep-wake disturbances are more common in patients with a known genetic cause of ALS<sup>67</sup>. MR is an ideal tool to disentangle this question and reveal whether or not sleep disturbance contributes to the risk of ALS.

### *Serum metabolites*

Yang *et al.* performed an unbiased screen of the human metabolome using a GWAS of high-throughput metabolic profiling performed by Shin *et al.*<sup>68,69</sup>. The authors use a liberal p-value threshold for choosing instrumental SNPs ( $p < 1E-05$ ) and relatively loose clumping criteria ( $R^2 < 0.1$ ). A total of 18 metabolites were significantly linked to ALS in the IVW analysis including 6 lipids, 4 amino acids, 3 peptides, 2 xenobiotics, an energy metabolite, and 2 unknown metabolites. However, just one metabolite remained significant in robust tests: gamma-glutamylphenylalanine (IVW OR 1.94,

95% CI OR 1.03-3.74,  $p=0.048$ , MR Egger  $p=0.04$ ; Weighted-median  $p=0.01$ , and MR-PRESSO  $p=0.04$ ). Other metabolites which did not withstand all robust test but did show promising results were gamma-glutamylleucine (IVW OR 1.98, 95% CI 1.04-3.75,  $p=0.04$ ), gamma-glutamylthreonine (IVW OR 0.50, 95% CI 0.26-0.99,  $p=0.05$ ), lathosterol (IVW OR = 1.43, 95% CI=1.05–1.93,  $p=0.02$ ) 1-stearoylglycerophosphoethanolamine (IVW OR= 0.54, 95% CI=0.35–0.83,  $p=0.005$ ), 2-methylbutyrylcarnitine (IVW OR=1.96, 95% CI=1.06–3.64,  $p=0.03$ ), 4-acetamidobutanoate (IVW OR = 0.48, 95% CI=0.24–0.94,  $p=0.03$ ), and 2-methoxyacetaminophen sulphate (IVW OR = 0.98, 95% CI=0.96–0.99,  $p=0.0009$ ). The authors comment that their results may indicate that dysfunction of glutathione metabolism plays a role in the development of ALS. Although the results of this paper are interesting, none of the results would have passed a Bonferroni multiple testing correction. In total 486 metabolites were tested which would require a corrected p-value threshold of 0.0001; the smallest p-value reported in the study was 0.0009. Moreover, closely linked metabolites, such as those within the same metabolic pathway, may be tightly correlated, in which case it can be difficult to determine the biological basis of an observed causal inference. In such cases further experimental validation may be necessary, but we note the utility of MVMR for determining dependence between traits and estimating the direct causal effect of each exposure on the outcome of interest<sup>57</sup>. We conclude that further validation is required to interpret the metabolic data reported to date.

### *Sleep behaviours*

Cullell *et al.* performed an MR study investigating the hypothesis that sleep/wake cycles are implicated in neurodegenerative disease<sup>70</sup>. As such, the authors identified instrumental variables for a large number of traits including insomnia, daytime sleepiness, subjective and objective (using accelerometer device measures) chronotype, sleep duration and obstructive sleep apnoea (OSA). It should be noted that the OSA measure is derived from individuals of diverse ancestries which may invalidate the measurement of ALS risk with this instrument. Only daytime sleepiness was significantly linked to risk of ALS (IVW beta=1.17,  $p=0.02$ ). The study demonstrated that there was not significant heterogeneity between SNPs in the instrument, and the relationship remained significant in the weighted median ( $p=0.04$ )

and a LOO analysis. [The result was not significant in MR Egger or mode based estimate.](#) Importantly, the relationship would not have remained significant if any kind of multiple testing correction had been applied (Bonferroni corrected p-value threshold would be 0.008) and should be interpreted with caution.

## Conclusion

MR is a powerful method capable of addressing many of the confounding factors which have previously limited study of environmental risk factors for ALS including ascertainment bias and small sample sizes. Our review of the present literature illustrates several examples where MR has delivered clarity including a causal links between blood lipids and ALS. [Replication across multiple studies using different instrumental SNPs and/or different outcome ALS GWAS add confidence.](#) Further, despite some difficulty in interpreting negative studies, MR studies have provided cause for doubt concerning some putative risk factors including smoking. The potential is that MR studies, combined with genetic segregation of ALS patients, could lead to individualized predictions and personalized medicine for the majority of ALS patients.

Where MR studies disagree, we have identified key methodological discrepancies, most often involving the choice of instrumental SNPs with which to measure an exposure. Inadequate clumping of SNPs in particular appears to have led to what we conclude are unreliable results. In future we would recommend that a stringent threshold of  $R^2 < 0.001$  is maintained to ensure that truly independent signals are measured. We have observed that relaxing this threshold is a key determinant of discrepancy between studies. [An alternative is to incorporate a population-specific LD matrix to correct for correlated SNPs but, while such methods have been proposed,<sup>71</sup> they are not widely used currently.](#) To overcome the arbitrary nature of choosing SNPs for an instrument, we advocate for the use of a positive control where one is available, in order to measure instrument power and performance. We<sup>32</sup> and others<sup>72</sup> have successfully used this method to achieve an adequately powered measure of genetic liability.

[In addition to these specific concerns, there are certain general problems which apply to MR results in general. We have already referred to the possibility that MR](#)

measurements may be invalidated by mismatch between exposure and outcome GWAS populations; this will be even more of a concern as future GWAS study cohorts become larger and more heterogeneous. This issue also illustrates a general principle, because MR relies on instrumental SNPs which are themselves a function of population specific LD structure, then instrumental SNPs may not be applicable in multiple populations. It is noteworthy that a focus on Europeans in GWAS studies has limited tools available for causal inference in other population groups and is a potential source of ongoing health inequality. A way to counter this and to simultaneously add confidence to an MR result is to perform a trans-ancestry study whereby the same exposure and outcome are tested in different populations using population-specific GWAS data; examples of this practice are described above<sup>24,27</sup>.

Another general issue is that of time-varying exposures: MR is usually used to infer the effect of lifetime exposure. However, instrumental SNPs are derived from a GWAS performed at a fixed time point in a specific set of individuals. SNPs correlated with an exposure may be different for individuals of different ages<sup>73</sup>. The implication is that instrumental SNPs used to measure a time-varying exposure may not be relevant over the entire lifetime of an individual. Instead the MR measurement will be biased by the age distribution of the cohort used to construct the exposure GWAS. Moreover, behaviours change over time in response to changes in the environment. For example, SNPs which correlate with smoking behaviour may have changed significantly before and after the introduction of widespread indoor smoking bans. This is particularly true when one considers that there is evidence that smoking behaviour interacts intimately with personality<sup>74</sup>.

MR has immense potential for interrogating complex diseases such as ALS. However, for MR to be useful, particularly in a field which includes non-experts in MR, high methodological standards must be applied in peer review to prevent publication of false positive and false negative results. The alternative is irreproducible results, wasted resources and missed opportunities.

## **Acknowledgements and funding**

This work was supported by the National Institutes of Health (CEGS 5P50HG00773504, 1P50HL083800, 1R01HL101388, 1R01-HL122939, S10OD025212, P30DK116074, and UM1HG009442 to M.P.S.), the Wellcome Trust (216596/Z/19/Z to J.C.-K.) and NIHR (NF-SI-0617-10077 to P.J.S.). We also acknowledge support from a Kingsland fellowship (T.M.), and the NIHR Sheffield Biomedical Research Centre for Translational Neuroscience (IS-BRC1215-20017).

### **Competing interests**

M.P.S. is a cofounder of Personalis, Qbio, Sensomics, Filtricine, Mirvie, and January. He is on the scientific advisory of these companies and Genapsys. No other authors have competing interests.



## Figure and Table legends

**Figure 1: Mendelian randomization (MR) is analogous to a randomized control trial.** Individuals with and without genetic liability to exposure to a risk factor (**A**) are compared against each other with respect to disease outcome (**B**). Causation is implied when there is a linear relationship between the effect of a SNP on an exposure and the effect of that SNP on an outcome (**C**), provided that the assumptions detailed in Figure 2 are not violated.

**Figure 2: Conditions for a valid genetic instrument in Mendelian randomization (MR).** In a valid MR analysis, SNPs must be significantly related to the exposure of interest (**A**). However, the SNPs must not be significantly associated with the disease/outcome in question other than through the exposure of interest (**B**). Finally, SNPs should not be associated with potential confounders which are associated with both the exposure and the disease (**C**).

**Figure 3: Summary of Mendelian randomization (MR) studies of ALS including measured exposures.** Important aspects of methodology which have led to consensus or controversy are highlighted. T2DM = Type 2 diabetes mellitus.

**Supplementary Table 1: Headline results and study methodology.** Studies should aim to report all elements addressed in the table, though this will not be possible in all cases (particularly positive controls use and validation across populations, which may be limited by the availability of appropriate GWAS data). “N/A” indicates that an item is either not possible or not necessary within the study in question. The negative results of extensive screens without an underpinning hypothesis for the primary analysis are not detailed in the table. Blue highlighting indicates best practice whereas red highlighting indicates failure to implement best practice or insufficient information provided. When referring to best practice, we have adhered to the guidance provided in the literature including the STROBE MR checklist, and key studies <sup>5,10,11,19,20,22,57,75–</sup>

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