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A perspective on causality assessment in epigenetic research on neurodegenerative disorders

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Epigenetics refers to heritable and reversible processes regulating gene expression that do not involve a change to the DNA sequence. Epigenetic modifications include DNA modifications (e.g. DNA methylation and hydroxymethylation), histone modifications, and non-coding RNAs such as micro RNAs and long-coding RNAs (Holtzman and Gersbach, 2018). Amongst others, epigenetic mechanisms play a vital role in cell proliferation and development, to ensure the correct genes are being expressed in a differentiating cell type. However, epigenetic mechanisms are also influenced by environmental cues, where they are subject to change during life, and may even mediate transgenerational inheritance (John and Rougeulle, 2018). In the last decades, research on epigenetics has expanded to study the role of these mechanisms in a plethora of diseases, such as neurodegenerative disorders (Lardenoije et al., 2018).

The most studied epigenetic modifications are DNA modifications, in particular DNA methylation. DNA methylation refers to the process of adding methyl groups to DNA molecules, in particular at the level of CpG dinucleotides, i.e. where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases. Recent technological advances have led to epigenome-wide association studies (EWAS), such as methylome-wide association studies, allowing for an in-depth analysis of epigenetic changes associated with the disease. While EWAS/methylome-wide association studies represent an important tool to establish a candidate list of genetic loci associated with a specific disorder, they remain purely correlational. Even with robust replicated findings highlighting the same differentially methylated loci and/or regions and showing functional correlations with gene expression, it remains difficult to infer a cause-effect relationship. This notion is especially problematic when studying disorders that are poorly understood. In fact, any epigenetic difference between diseased and healthy subjects could represent a cause or consequence of risk factors, the disease itself, its treatment, or an epiphenomenon, or a combination of one or more of these features. While this limitation is often acknowledged in research across the field, it is rarely addressed properly.

In the last couple of years, epigenetic editing, i.e. altering the epigenome by reversing or restoring e.g. DNA methylation at a specific site, has grown as a powerful tool to further study the involvement of epigenetics in various diseases, especially in view of addressing causality (Xu and Heller, 2019).

This perspective proposes a guideline on how to thoroughly investigate potential cause-and-effect relationships for epigenetic alterations in neurodegenerative diseases taking Alzheimer's disease (AD) and multiple sclerosis (MS) as examples.

Major concerns in inferring cause-and-effect relationships in neurodegenerative diseases: Cause-and-effect relationships between observed biological changes and disease-associated phenotypic variation are challenging to infer. Neurodegenerative diseases are particularly suffering from this limitation for a number of reasons. Firstly, these diseases are progressive in nature, posing an enormous challenge to assess the exact disease state. This notion limits the signal-to-noise ratio in EWAS when comparing neurodegenerative patients to healthy controls, while it also makes it difficult to identify those epigenetic changes involved in the early stages of the disease, which often emerge years if not decades before the presentation of its symptoms. Age as such may also interfere in this respect, exerting its own epigenetic imprint (Benayoun et al., 2015). Secondly, these diseases are often multi-factorial, with a complex etiology, concomitant with secondary psychological and behavioral changes, or comorbidity, all of which in turn can affect the epigenome. Thirdly, treatment (e.g. pharmacological) interventions can have an impact upon epigenetic changes. Finally, it is of crucial importance to consider the cellular heterogeneity of bulk tissue, on which most of the EWAS studies are being conducted. Such sample heterogeneity does not only limit the reproducibility of the observed data, but can also lead to biased conclusions. Novel techniques, such as single-cell sequencing, could be an ideal strategy to cope with this issue, yet unfortunately such an approach is not yet standardized for DNA methylation sequencing.

In this perspective, we focus on two neurodegenerative disorders that are both poorly understood, devastating, yet fundamentally different in terms of their etiology, are AD and MS. While the former is characterized by toxic protein aggregates leading to neuronal degeneration and loss, the latter leads to neuronal loss due to the demyelination of axons (Dal Bianco et al., 2008). We focus on these disorders to showcase how flexible and versatile this approach to investigate causality is.

AD: AD is a fatal progressive neurodegenerative disease and the most common form of dementia. It is characterized by intracellular neurofibrillary tangles and extracellular amyloid depositions, leading to memory loss, often accompanied by changes in affective behavior and, eventually, death. AD has devastating implications for patients and care-takers due to rapid cognitive decline. To date, advances in the field have not led to new treatment methods, as the etiology of AD is multi-factorial and remains poorly understood. Approximately 10% of AD cases are considered familial, whereas over 90% are considered sporadic. Sporadic AD cases are most likely caused by a combination of different genetic, environmental and epigenetic factors, such as DNA hypermethylation, deacetylation of histones, and repressed chromatin states (Lardenoije et al., 2018). While a recent meta-analysis has highlighted numerous genome-wide significant neuropathology-associated DNA methylation differences in AD, annotated to 121 genes, a causality of those genes has not yet been assessed (Smith et al., 2021).

MS: MS is an (auto)immune-driven demyelinating and neurodegenerative disorder of the central nervous system (CNS), caused by autoreactive insults to the myelin sheath. MS is characterized by a sustained toxic pro-inflammatory environment within the CNS parenchyma, both due to resident and infiltrated reactive immune cells, as well as oligodendroglial degeneration and demyelination. The loss of the isolating capsule around the axons does not only affect electrical impulse conduction, but the lack of trophic support also leads to axonal damage, ultimately contributing to the progressive and neurodegenerative aspect of the disease (Garg and Smith, 2015). The primary and most studied factor associated with MS pathology is the immune-driven attack in the CNS, accompanied by the breakdown of the myelin sheath. Both innate and adaptive immune cells have been shown to be involved in inflammation observed in MS, yet it remains unclear how these immune cells become autoreactive. The so-called 'outside-in hypothesis' suggests that immune cells acquire a pathogenic phenotype in the periphery, possibly due to environmental and epigenetic factors, causing them to invade the CNS where they attack the oligodendrocytes and myelin sheath. In contrast, however, the 'inside-out hypothesis' states that MS pathology starts with oligodendrocyte dysfunction and cell death, which eventually triggers an autoimmune response (Sen et al., 2020). This discrepancy, together with the heterogeneity of the disease, are complicating factors when defining causality.

Causality assessment of epigenetic signatures – a proposed workflow: While EWAS studies are highly relevant as they provide new insights into the disease and allow researchers to explore new avenues, they do not give an indication about the cause-and-effect relationship of the studied genes. We, therefore, propose a workflow to aid in assessing causality of candidate epigenetic signatures in neurodegenerative diseases, such as AD and MS (**Figure 1**).

A general starting point is an EWAS study on a power-based sample size discovery sample cohort to stratify candidate signatures associated with the phenotype of interest. Such candidate signatures can be further validated using targeted sequencing technologies, like pyrosequencing. To control for bulk tissue bias, it would be ideal to consider cell-specific methylation analysis, which can be achieved by technologies such as fluorescent-activated cell sorting or laser-captured microdissection. Once promising candidate signatures are determined and validated, an epigenetic editing toolbox could be applied. The recent introduction of new epigenetic editing tools, such as the clustered regularly interspaced short palindromic repeats-deactivated CRISPR associated protein 9 (CRISPR-(d) Cas9) based system, has opened a new avenue to investigate the potential causal associations between epigenetic modifications and the pathogenesis of neurodegenerative disorders (Waryah et al., 2018). Based on the nature of the DNA (hydroxy)-methylation signature, one can opt for either a DNMT3a- or TET1-based CRISPR-dCas9 vector and design an optimal sgRNA to the desired genomic region. The epigenetic editing construct can then be transfected into cells of interest, to assess the functional consequence both *in vitro* and *in vivo*. This proposed workflow allows for higher throughput due to a standardized approach, a higher chance to identify biologically relevant targets and, therefore, a higher chance to translate findings to patients.

Specific considerations – AD: One of the many challenges that epigenetic research in AD has to handle is on how to proceed with differentially methylated loci and regions. Firstly, it is unclear if a gene displaying differential methylation when comparing AD and control individuals exerts a causal effect or is differentially methylated as a consequence of its pathophysiology. Secondly, it also has to be assessed if normalizing the degree of methylation of the differentially methylated region has any biological relevance in terms of halting or reversing the disease pathology and functional phenotype. Thirdly, as AD is progressive and involves numerous genes and associated pathways, one has to consider that disease heterogeneity is a highly complicating factor in interpreting the relevance of differential methylation and selecting candidate genes to investigate further.

Currently available epigenetic editing systems offer an ideal toolbox to investigate whether the identified differentially methylated regions represent potential key players in the development and/or progression of AD (**Figure 1**). As a proposed workflow, one could investigate the effects of inducing hypo- and/or hypermethylation in specific target genes *in vitro* and studying the effects on different parameters, such as cell viability, neuronal growth, plasticity, and metabolic activity in neuronal cells. Here, it is important to consider the potential role of glial cells as effects can be cell type-specific. Focusing on a single cell type increases the signal-to-noise ratio, concomitant with an increase in power, and allows assessing causality in a more reliable manner. A subsequent step could be to culture cells in the presence of amyloid-beta (Aβ) oligomers to investigate if the altered methylation of the candidate genes would exacerbate the toxic effects of Aβ. This approach is not limited to Aβ exposure, but can be extended to pretty much any relevant neuropathological (tau, cytokines, etc.) or environmental (e.g. stress) factor that is relevant to the disease. Furthermore, chemical long-term potentiation in *ex vivo* brain slices could be applied as a functional validation as well. However, *in vitro* models suffer from some limitations, such as insufficiently mimicking the neurodegenerative process, which occurs over many years. Alternatively,

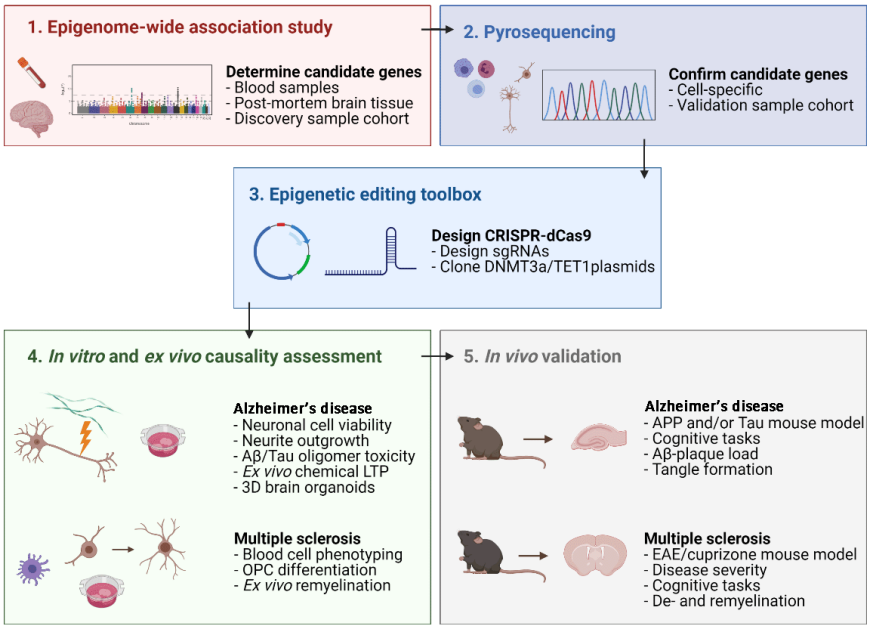

Figure 1 | **Proposed workflow to aid in assessing causality of candidate epigenetic signatures in neurodegenerative diseases using AD and MS as showcases.**
Candidate genes, determined in epigenome-wide association study, can be validated in a cell-specific manner using targeted sequencing techniques, such as pyrosequencing. As a functional validation, the epigenetic editing toolbox can be applied to assess the effect of specific epigenetic modifications of the candidate signatures both *in vitro* and *in vivo*. Aβ: Amyloid-beta; AD: Alzheimer's disease; APP: amyloid-beta precursor protein; CRISPR: clustered regularly interspaced short palindromic repeats; dCas9: deactivated CRISPR associated protein 9; DNMT3A: DNA methyltransferase 3a; EAE: experimental autoimmune encephalitis; LTP: long term potentiation; MS: multiple sclerosis; OPC: oligodendrocyte precursor cell; sgRNAs: single guide RNAs; TET1: Tet methylcytosine dioxygenase. Created with BioRender.com.

3D brain organoids, generated from embryonic stem cells or induced pluripotent stem cells can be generated to study the pathophysiology of AD. Finally, an *in vivo* approach making use of an AD mouse model could be used to explore the effects of (site and locus-specific) hyper- or hypomethylation, in order to identify the potential functional (e.g. cognitive), and hence putatively even therapeutic, consequences of targeting this locus. Spatiotemporal control of epigenetic modulation in causality assessment can be mimicked using stereotactical injection and cell type-specific promoters.

Specific considerations – MS: Investigating disease causality in MS would be ideally performed on samples from patients at symptom onset. Since the prodromal MS phase is gaining attention, it would be of great interest to investigate those epigenetic alterations occurring at such an early phase in order to identify individuals at-risk (Tremlett and Marrie, 2021). Furthermore, longitudinal blood samples, obtained from MS patients over time, could be of great value to investigate epigenetic alterations acquired as the disease progresses. The identified target genes can then be epigenetically edited to investigate disease causality. However, even though the epigenetic signature of different subsets of peripheral blood mononuclear cells of MS patients is already widely investigated by independent research groups, the data does not always reveal reproducible findings (Ewing et al., 2019). This discrepancy could be the result of limited sample size or methods of sample selection, methylation measurements, or data analysis. An overarching meta-analysis of these studies, could potentially correct for methodological dissimilarities and reveal interesting targets that can be further assessed for their potential causative role in MS disease pathology (Smith et al., 2021).

Investigating epigenetic changes in post-mortem brain tissue and taking into account the differences between lesion types could also potentially reveal new markers or targets for remyelination, neuroprotection, and disease progression in MS. In order to study causation of the observed DNA methylation pattern in MS, the previously mentioned epigenetic editing tools such as CRISPR-dCas9 could be utilized. An interesting approach would be to make use of the CRISPR-dCas9-DNMT3a/TET1 tool to induce DNA (de)methylation at specific loci, which have been associated with oligodendrocyte function. Primary *in vitro* oligodendrocyte cultures could then be transfected with the epigenetic editing plasmid to assess the effects on oligodendrocyte survival and differentiation. Furthermore, *in vivo* epigenetic editing of these genes in for instance cuprizone animal models could reveal whether targeted (de)methylation of these genes does influence remyelination capacity.

In conclusion, causality assessment in epigenetic research remains a challenge. This workflow aims to aid researchers on how to assess candidate epigenetic signatures in neurodegenerative diseases, taking AD and MS as an example. While a single gene is unlikely to be the main contributor to these diseases, it allows for a more thorough understanding of the role a single gene can play in neurodegenerative disorders, allowing to identify whether the epigenetic signature is a cause or merely a bystander or consequential imprint of the pathology. This proposed workflow can be applied to other neurodegenerative disorders as well.

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