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What Have Advances in Transcriptomic Technologies Taught us About Human White Matter Pathologies?

What do single cell technologies teach us about human white matter pathologies?

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S.J. and A.W. wrote and edited the manuscript. S.J. also designed and prepared the figures.

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Abstract

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For a long time, post-mortem analysis of human brain pathologies has been purely descriptive, limiting insight into the pathological mechanisms. However, starting in the early 2000's, next generation sequencing and the routine application of bulk RNA-sequencing and microarray technologies has revolutionized the usefulness of post-mortem human brain tissue. This has allowed many studies to provide novel mechanistic insights into certain brain pathologies, albeit at a still unsatisfying resolution, with masking of lowly expressed genes and of regulatory elements in different cell types. The recent rapid evolution of single cell technologies has now allowed researchers to shed light on human pathologies at a previously unreached resolution revealing further insights into pathological mechanisms that will open the way for development of new strategies for therapies.
In this review, we will give an overview of the incremental information that single cell technologies have given us for human white matter pathologies, summarize which single cell technologies are available and speculate where these novel approaches may lead us for pathological assessment in the future.

Contribution to the field

This review article gives an overview of the use of modern "omics" approaches in the context of human white matter brain pathologies and how these have contributed to our understanding of disease, with a special focus on novel single cell and single nuclei technologies. As the use of these technologies in human brain tissue has only started to emerge recently and are developing at a rapid speed, this article summarizes very recent work including many papers still in preprint at the forefront of this science. We give an overview of different single cell/single nuclei technologies and possible validation techniques and discuss the advantages and limitations from an experimenter point of view. Hence, the article explains single cell technologies for non-experts to help them better understand how they can implement these in their own research and what to consider when planning an experiment. We also outline how these technologies are changing and how this change will influence human brain pathology in the future.

Funding statement

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2 **human white matter pathologies?**

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1 Abstract

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22 available and speculate where these novel approaches may lead us for pathological assessment in the
23 future.

2 Introduction

25 *Classical approaches to study human white matter pathology*

26 Modern neuropathology has its origins in the late 19th and early 20th century, when famous
27 neurologists or psychologists such as Santiago Ramon y Cajal, Jean-Martin Charcot and Alois
28 Alzheimer started to describe and illustrate the central nervous system (CNS) and its pathological
29 changes. These early, but still accurate and detailed illustrations of the brain and individual cells, were
30 all based on histological stains observed through a simple light microscope. It took many years before
31 pathology could reach another level of detail with the common use of antibodies to develop marker-
32 specific immunological stains that are still a state-of the art in modern research laboratories. Due to the
33 combination with fluorescent labels and the development of better microscopes, this method has
34 become a standard technique to study human pathology and it has helped us to gain a deep
35 understanding of cellular and sub-cellular structures of the brain in health and disease.

36 Multiple Sclerosis (MS), a chronic inflammatory and demyelinating neurodegenerative disease of the
37 central nervous system, is a good example of how this descriptive pathology is still used, but it equally

38 applies to other pathologies. The characteristic lesions in white matter (WM) tracts can be classified
39 into active, chronic active, chronic inactive and remyelinated lesions – so called shadow plaques
40 (Lassmann et al., 1998). This still highly used classification system is based on presence of
41 demyelination and the distribution of infiltrating immune cells in and around the lesions and is carried
42 out with simple histological staining on post-mortem human tissue. So far, there are only limited ways
43 of detecting the different lesion stages during the lifetime of a patient using non-invasive imaging
44 techniques (Bruck et al., 1997;Hemond and Bakshi, 2018). Specific magnetic resonance imaging
45 sequences enables detection of chronic active lesions where acute inflammation is happening at the
46 lesion rim (Absinta et al., 2018) and these are associated with disability and ongoing tissue damage
47 (Absinta et al., 2019) aiding prognosis. These new imaging paradigms are exciting but we are still far
48 from a full picture of MS lesions either by pathology or live imaging. Moreover, we still have limited
49 knowledge about molecular or mechanistic changes in MS lesions which are key to understanding the
50 disease.

51 With the development of new technologies in the 2000s, many labs started to use bulk RNA-sequencing
52 (RNA-seq) or DNA-based microarrays to describe cellular and molecular changes in disease at the
53 transcript level, to gain a deeper insight into functional pathological changes. This was the beginning
54 of a revolution in pathology, helping define molecular markers of disease and raising new hypotheses
55 for disease pathogenesis, to be tested experimentally. This revolution continues with new methods to
56 identify transcripts from single cells or nuclei, and to identify these transcripts spatially on tissue. Here,
57 we describe this revolution, and how this is evolving and will impact our understanding of human WM
58 pathology. Although these techniques are applicable to a wide range of human WM CNS pathologies,
59 this review will mostly use MS as an exemplar and only touch on other diseases where relevant.

3   Modern approaches to study WM pathologies

3.1   What have we learnt from whole transcriptomic approaches?

62 Bulk RNA-seq is a method to detect the entirety of the transcriptome within a sample of interest, which
63 can either be a whole piece of tissue or sorted cells from a tissue. This can be done in an unbiased way
64 where RNA is isolated, fragments transcribed into cDNA, which are further linked with specific
65 adapters making them compatible with next generation sequencing (NGS), which is then
66 bioinformatically analyzed (Fig. 1A). Alternatively, in a more biased way, isolated RNA is loaded onto
67 specific microarray chips containing probes for only predefined gene transcripts. Commercial
68 microarrays contain a large numbers of probes for the most important gene transcripts spread over the
69 whole genome so that it can still be relatively unbiased. However, early experiments also included
70 home-made arrays with lower numbers of genes.

71 The hallmark of WM MS pathology are the clearly distinguishable focal demyelinated lesions where
72 myelin is lost. Therefore, due to its ease of detection and separation from the surrounding normal
73 appearing white matter (NAWM), many groups have performed bulk transcriptome studies on MS
74 tissue comparing these. In addition, probably in part due to its easier accessibility at least in life, many
75 have been performed on body fluids such as blood or cerebrospinal fluid (CSF) but some also used
76 whole brain or spinal cord tissue samples. Several review articles have already summarized these
77 comparisons and discussed the technical challenges (Comabella and Martin, 2007;Kinter et al.,
78 2008;Dutta and Trapp, 2012). In summary (Fig. 4), the major findings are that all analyzed tissue
79 sources express high numbers of inflammatory gene transcripts, although the inflammatory pathways
80 differ. In addition, it became clear that NAWM is not equal to control WM, suggesting that MS is a
81 more global disease than previously thought. Many other findings of transcript differences across these

82   <mark>studies were unique to a specific gene transcript in one study rather than having common ground</mark>
83   <mark>between studies, providing interesting candidates to be investigated.</mark> Most surprisingly and against the
84   common concept in MS that oligodendrocytes are the primary target of attack, it has been suggested
85   that surviving oligodendrocytes around demyelinating lesions in the NAWM are induced by hypoxia
86   to be neuroprotective and anti-inflammatory and are thus more actively involved in disease and perhaps
87   limiting it (Graumann et al., 2003;Zeis et al., 2008). <mark>Very recently, heparan sulphate production by</mark>
88   <mark>mature oligodendrocytes around demyelinating lesions has been shown to be one of the mechanisms</mark>
89   <mark>in limiting demyelinating lesions (Macchi et al., 2020).</mark> Lindberg et al. (2004) came to similar
90   conclusions regarding the NAWM and additionally pointed out that the immune response activation is
91   different in the different compartments with a more cellular response in NAWM and a humoral
92   response in lesions. Looking a bit closer into differences between demyelinated lesions, Tajouri et al.
93   (2003) found that although both acute and chronic lesions share the majority of markers that are
94   changed in MS in comparison to control, the fold change of those gene transcripts is however quite
95   different. More recent publications have used bulk RNA-seq on MS tissue in a more complex way to
96   explore either transcriptomic changes of microglia in the initial phase of MS (van der Poel et al., 2019)
97   or transcriptomic changes in an hormonal context of the hypothalamus-pituitary-adrenaline (HPA)-
98   axis (Melief et al., 2019). The first study reported an increase in transcripts related to lipid metabolism
99   in microglia sorted from NAWM that is similar to those found in active demyelinated lesions, however,
100 whilst maintaining their homeostatic functions. The latter study found that gene expression networks
101 in MS tissue correlate with the activity of the HPA axis and/or disease severity, showing that gene
102 expression in a pathological context is not only regulated by the pathology itself, but also depends on
103 other environmental factors. Thus, careful consideration of the experimental design and the case
104 selection must be part of planning such an experiment.

105 Bulk transcriptomic approaches have brought several advantages to the field, but as ever with evolving
106 technology, also some challenges. In contrast to immunohistochemistry (IHC) or quantitative PCR
107 (qPCR) studies of candidate genes, it is unbiased, or relatively unbiased (with microarrays) allowing
108 detection of new mechanisms rather than only digging deeper into already known ones. It is also not
109 dependent on good primers/antibodies or experimenter choice. Long interfering non-coding RNAs are
110 a good example of this, as most of their roles are relatively understudied and one specific RNA was
111 found to play an important region-specific role in a study on Multiple System Atrophy (MSA), another
112 human WM neuropathology, suggesting regional differences of this RNA to control brain function
113 (Mills et al., 2015). Another advantage, at least in theory, is that studies that are performed by different
114 groups in different tissues should be easy to compare, as all capture RNA in an unbiased way. However,
115 disadvantages are plentiful, limiting comparisons as early studies (at least) used low numbers of
116 individuals as input and findings might thus not be representative for a larger MS cohort. Comparisons
117 chosen have varied and have included: (1) Lesions versus NAWM (Whitney et al., 1999;Whitney et
118 al., 2001;Tajouri et al., 2003), (2) Lesions/NAWM versus Controls (Graumann et al., 2003;Lindberg
119 et al., 2004;Zeis et al., 2008;Zeis et al., 2018), and (3) different lesions and/or different regions of
120 lesions (Lock et al., 2002;Mycko et al., 2003;Hendrickx et al., 2017). Furthermore, MS lesions can
121 occur in all WM regions and transcriptional profiling may be different when the lesions from the
122 different studies come from different regions, for example from cerebellar WM and frontal subcortical
123 WM. Many of these studies used non-standardized RNA isolation methods, different types of
124 microarrays (commercial and homemade) with different probe sets (quantity and type) and also
125 different sensitivities for lowly abundant genes, which may explain why different studies found so
126 many different results. Highly abundant genes may mask more subtle effects, and in MS, this often
127 leads to the discovery that MS lesions are associated with demyelination and inflammation (Kinter et
128 al., 2008) – not quite a surprise for an inflammatory demyelinating disease.

129 A further disadvantage is that bulk transcriptomic studies detect gene expression irrespective of their
130 cellular source within the tissue, so that a signal may be lost if one gene transcript is significantly
131 upregulated in one cell type, but downregulated in another. ==This becomes especially important when==
132 ==studying a tissue with little cellular heterogeneity, as bulk approaches are generally able to detect a==
133 ==shift in cell type proportions (e.g. more inflammatory cells in MS lesions), but are less good in detecting==
134 ==changes within similar cells sharing the majority of transcripts.==

3.2   How can complementary bulk approaches help address WM pathologies?

136 Other technologies, such as proteomics and metabolomics may also illuminate human pathologies (Fig.
137 1B, C). They are either suitable to help validate hypotheses generated by transcriptomics or to generate
138 new hypotheses themselves. Proteomic approaches using different methods of mass spectrometry have
139 been widely used (as summarized in (Farias et al., 2014;Farias and Santos, 2015)). Numerous studies
140 have been performed on human blood and CSF samples, which are easier to obtain, and may allow the
141 development of new disease biomarkers in living patients (Del Boccio et al., 2016) as well as
142 elucidating potential mechanisms of pathogenesis. The scarcity of reliable blood or CSF biomarkers
143 for MS has been quite sobering to date, which might also be due to the technical challenges of highly
144 abundant proteins (e.g. albumin) in the samples that mask smaller changes. Few proteomic studies so
145 far have been performed on human brain tissue itself, (Han et al., 2008;Broadwater et al., 2011;Ly et
146 al., 2011) perhaps as a full proteomic overview of isolated brain tissue is technically challenging. This
147 is due to the high abundance of proteins that cannot be captured by current technologies, mainly
148 because of their dynamic range and the complexity, a reason why further subsampling of the tissue of
149 interest might be helpful (Werner and Jahn, 2010). One ==study== focussed on mitochondria in grey matter
150 (GM), which suggested a dysfunction in the mitochondrial respiratory chain in MS (Broadwater et al.,
151 2011). In line with the findings of bulk transcriptomics data, proteins involved in inflammation and
152 demyelination were upregulated in MS, but in order to find new disease mechanisms that can be
153 targeted for therapies, more specific and sensitive techniques are needed. However, proteomics have
154 been elaborately applied in mouse models of MS and the results from these studies may be worth trying
155 to validate in humans.
156
157 Metabolomics is a relatively newly termed "omics" approach to systemically study metabolites in a
158 sample, and the first metabolomics studies in MS were performed in the 1990s (Lynch et al., 1993).
159 This is useful as metabolites are usually the end product of a biological process allowing us to draw
160 conclusions about function. Despite the novelty of this approach, it has already found a wide usage in
161 MS and in its animal models, to try to identify biomarkers in body fluids like CSF, blood and urine.
162 With this, it might also be possible to observe metabolites in different patients and respond to their
163 individual needs by different drugs, which would be a first step to personalised precision medicine
164 (Bhargava and Calabresi, 2016;Del Boccio et al., 2016).
165
166 Techniques to study brain WM in bulk have greatly shaped understanding of WM diseases, but we
167 now have the technology to examine pathological changes at a single cell level, gaining even deeper
168 and new insights into these diseases.

3.3   How do single cell transcriptomic techniques work?

170 Single cell RNA sequencing (scRNA-seq) is a novel technology using the same principle of capturing
171 and sequencing mRNAs in bulk approaches within a tissue, however with the improvement that
172 individual mRNAs can be associated with each cell of origin. This is particularly important for brain
173 pathologies, where not all cell types are equally affected, for example in MS, where oligodendroglia

4

174 are primarily lost. Although scRNA-seq is a relatively young technology – the first paper was published
175 in 2009 (Tang et al., 2009) - many different commercial techniques are already on the market and new
176 ones are emerging at a rapid speed (Svensson et al., 2018;Chen et al., 2019), possibly faster than the
177 publication of this review. Whilst all of them aim to give a snapshot of the transcriptome of individual
178 cells, they are quite different in the technology achieving this. All of them have their advantages and
179 disadvantages depending on the specific scientific question to be answered, so choosing the right
180 technique is an important step in the experimental design. For this, we should consider the number of
181 cells available, the capture efficiency, transcriptome coverage and cost per cell. Several reviews have
182 summarized and compared single cell RNA-seq technologies (Haque et al., 2017;Picelli,
183 2017;Svensson et al., 2018;Chen et al., 2019). Despite the high number of technologies, from an
184 experimental point of view, there are two approaches: studying a high number of cells at a lower
185 resolution (up to tens of thousands of cells) or studying a low number of cells (generally <1000 cells)
186 at a higher resolution.
187 The first approach generally uses droplet-based technologies (Macosko et al., 2015;Zheng et al., 2017),
188 whilst the second approach mainly uses well- or device-based technologies (Fig. 2A,B) to capture
189 single cells (Picelli et al., 2014;Hagemann-Jensen et al., 2020). Droplet-based methods use unique
190 molecular identifiers (UMIs) and/or barcodes to label individual cells and mRNAs during the initial
191 steps, so that the library preparation can be performed in bulk, rather than creating individual libraries
192 in wells. As the droplet-based methods are aimed for high throughput, the costs per cell are much
193 cheaper in comparison to well-based technologies. Also with this barcoding approach, copy numbers
194 of mRNAs within a cell can at least in theory directly be measured, without the need of using additional
195 standards such as External RNA Control Consortium (ERCC) spike-ins (Baker et al., 2005). In order
196 to keep the sequencing costs at a realistic level, usually only the 3' or the 5' ends of the mRNA are
197 amplified and sequenced, which only allows information of whether a gene is expressed or not, with a
198 limited ability to examine splicing variants or SNPs. Conversely, with well- or device-based
199 approaches, it allows study of splice variants of genes, but the cost per cell is higher.
200 Sometimes, a combination of both techniques might be useful. Using unbiased droplet-based
201 techniques to look at the entirety of cells in a tissue of interest helps to get an overview of all cells,
202 including rare cell populations, and to find appropriate markers for these. This is especially useful in
203 understudied tissues such as the human brain, as established markers for rodent cells are not always
204 appropriate in humans. Cell populations of further interest, including rare populations or
205 subpopulations can then be studied at a deeper resolution using a full-length sequencing approach, after
206 isolation or enrichment using the previously identified markers.
207 Currently, scRNA-seq experiments are cutting-edge and popular, generating much data and new
208 hypotheses about the heterogeneity of cell function in all tissues with high impact publications.
209 However, it is essential to validate these data and to keep the research question in mind, as sometimes
210 a more classical approach will lead to an answer quicker, in an easier and cheaper way. Although bulk
211 approaches seem to be outdated at the moment, these technologies have also improved and are still
212 important tools in studying human pathologies.

213 **3.4   What are the challenges and drawbacks of performing human single cell RNA-seq?**

214 All of these single cell technologies were originally developed for cultured cells or rodent tissue, whilst
215 their application to human tissue has only started to boom in recent years. Besides ethical constraints
216 and the limited availability of human tissue (control equally as pathological tissue), there are more
217 technical challenges that delayed the revolution in this field. Single cell technologies were developed
218 for viable single cell suspensions, which is often not possible in WM pathologies, where most tissue is
219 obtained post-mortem and not during biopsies. Hence, cell viability and tissue quality is often already
220 below the accepted threshold to perform the experiments once the tissue arrives in the hands of the

221 researcher. By this time, surviving cells may be highly selected and their transcriptome likely to have
222 changed dramatically, with degraded RNA resulting in bad data quality and lower biological meaning.
223 Moreover, in order to reduce confounding factors, all samples should be run ideally together or at least
224 in as small a number of batches as possible (Hicks et al., 2017), which is impossible when using fresh
225 biopsy or autopsy material, as individual tissue samples are sometimes only available months apart.
226 For these reasons, most research so far in human brain have used single nuclei (sn) RNAseq using the
227 same technologies from archived and frozen tissue samples instead of fresh viable cells. On comparing
228 cells versus nuclei, there is now the general consensus that although nuclei generally yield a lower
229 number of reads, they can add useful information on the biology. This tissue source may even protect
230 from immediate changes in the transcriptome resulting from cell stress during cell isolation and the
231 proportional representation of the *in-vivo* situation may be more preserved, as during a live isolation,
232 vulnerable cells are more likely to die resulting in their underrepresentation (Bakken et al., 2018;Wu
233 et al., 2019). However, a new study suggested that using nuclei is not always a good alternative, as
234 only highly abundant transcripts are detected and the more subtle changes related to the activation state
235 of human microglia could not be distinguished (Thrupp et al., 2020). To get a clear picture of the
236 advantages and disadvantages of using nuclei or cells, more comparative work using the same tissues,
237 experimental setups and sequencing depth will be helpful. However, given the high number of
238 publications, it is already clear that snRNA-seq is an important tool to resolve biological questions,
239 especially for human pathologies when no other tissue source is available.
240 More generally, once the decision has been made to use cells or nuclei, other challenges arise. Although
241 at first sight, plentiful rich data is a clear advantage, it can also lead to data overload, that nobody
242 knows what to do with. Especially with droplet-based technologies, individual sc/snRNA-seq
243 experiments generate so many data points that research groups may only process a small part of it: for
244 example, the experimental design might include all unselected nuclei, however, only a certain cell-type
245 may be analysed. Here is where open access sharing of these data is essential, allowing other groups
246 to use these data to address their own research questions and save a lot of time. This also allows for
247 some mitigation of expense, as the rapid development of these technologies comes at high cost.
248 Although these techniques are becoming cheaper, the costs of commercial kits are still high and the
249 cost per cell must be considered in the experimental plan. Homemade technologies are inevitably
250 cheaper, but require knowledge and time to set up and may not be as robust and comparable between
251 research labs as commercial ones. The costs for the sequencing should not be forgotten, which often
252 equal the cost of the cell capture and cDNA library preparation. The depth of the experimental analysis
253 obtained depends on the depth of sequencing.
254 A major limitation of sc/snRNA-seq is the the number of transcripts that can be detected within a cell.
255 Although the captured transcripts are often treated as representing the entirety of the transcriptome, in
256 reality only about 5-20% of the transcripts are captured depending on the method, leaving about 80%
257 of the biology undiscovered (Islam et al., 2014;Ding et al., 2020). These missing transcripts are usually
258 ones with a low abundance that may represent more subtle changes between cell states. A recent
259 advance is the development of a full-length sequencing method that reaches a significantly deeper
260 transcriptome coverage per cell and thus results in a clearer separation of clusters (Hagemann-Jensen
261 et al., 2020), but unfortunately, this method is not yet suitable for high throughput. Another limitation
262 of these technologies comes from the way in which RNA is captured. Most methods use oligos to
263 capture polyadenylated mRNA only and especially droplet-based methods, additionally only use 3'
264 amplification. Few technologies have been developed to amplify the 5' end of the RNA, however the
265 libraries are also prepared with the polyA-tail, still only accounting for the same type of RNA
266 (Svensson et al., 2018;Chen et al., 2019). Other forms of biologically interesting RNAs, such as many
267 microRNAs are not identifiable using this capture method and detection of splicing variants of genes
268 is more limited (unless using full-length sequencing).

269 Once the sequencing data are generated, data storage is another often unconsidered problem. As
270 datasets become bigger, in terms of sample number, cell number and sequencing depth, the output data
271 files become bigger and too large to be stored on a standard computer, instead requiring big data servers
272 or cloud storage, which come at a further cost. Data handling capacity challenges go hand in hand with
273 increasing data size and depth, and analysis of these datasets generally requires a high-end workstation
274 or, better, access to a computational cluster with the respective expertise. Associated with the fast
275 development of scRNA-seq technologies, there is a large expansion in the available tools for data
276 analysis, which are evolving all of the time and are generally open source and therefore free. This can
277 gives a bewildering variety of options of how to analyse the dataset. The challenge here is to find the
278 right tool that is suitable for the data of interest, as not all available tools are. Helpful comparisons have
279 emerged, for example in an overview of 45 current tools to calculate pseudotime trajectories (Saelens
280 et al., 2019), as not all of them are equally suited for every dataset. Another example would be the
281 availability of different clustering methods, with Seurat (Satija et al., 2015), Monocle (Trapnell et al.,
282 2014) and Conos (Barkas et al., 2019) as highly used examples. Each of them uses a different algorithm
283 and clustering approach, as outlined in (Duò et al., 2018) and might thus result in different final clusters
284 of which all may be valid. These are only examples, but every step in the experimental and the analysis
285 part has many options. One study outlined this problem and showed that using only a minimum of
286 options in different steps and combining it in a different way already results in ~3000 different pipelines
287 for analysis (Vieth et al., 2019). This field is as experimental as wet lab work, and it may be useful to
288 use several analysis tools purporting to do the same thing on one dataset, in order to determine how
289 robust the analysis is. However, ultimately, the only way to discover if the analysis is correct is to
290 validate the results using other methods.

3.5 What have we learnt about WM pathologies using single cell/nuclei transcriptomic approaches?

293 Pioneering scRNA-seq analysis in rodent brain tissue clearly showed detection of all brain cells that
294 were distinguishable by specific markers (Zeisel et al., 2015). Of interest to MS, a key study in mouse
295 oligodendrocytes (Marques et al., 2016) first used scRNA-seq to report their heterogeneity suggesting
296 different inherent functions of oligodendrocytes not only between the brain and the spinal cord, but
297 even in the same region of the brain. The first studies using snRNA-seq on normal human brain tissue
298 were proof of principle that this method was suitable in such a tissue and that there is cellular and
299 regional heterogeneity (Habib et al., 2017;Lake et al., 2018) and were the starting point of many
300 following studies. Not surprisingly, it did not take long before this technology was used to study brain
301 pathologies including MS (Fig. 4).

302 With this hitherto unreached resolution, cellular heterogeneity in MS tissue was demonstrated in
303 oligodendrocytes, neurons, microglia and astrocytes (Jakel et al., 2019;Masuda et al., 2019;Schirmer
304 et al., 2019;Wheeler et al., 2020) with disease-specific cell types or different heterogeneous states
305 present in different proportions in MS compared to controls. In their study, Jakel et al. (2019) found
306 heterogeneous oligodendroglial states in non-pathological brain tissue and contrary to the current idea
307 that all oligodendrocytes in MS lesions are equally vulnerable, reported that some of these states were
308 over- and some underrepresented. Although the functional role of this cellular heterogeneity is not yet
309 clear, this skew in the proportions of different oligodendrocyte states seen in MS was present in both
310 NAWM and in MS lesions, again adding to the evidence that NAWM is indeed not normal, as
311 previously shown for microglia (van der Poel et al., 2019). Furthermore, these data were able to identify
312 a small population of previously unknown oligodendroglia with immunological functions (Falcao et
313 al., 2018;Jakel et al., 2019) which may influence disease pathogenesis as it suggests that
314 oligodendrocytes may be an active player in the disease as well as a vulnerable target. This is of

315 importance, as therapeutic approaches simply aiming to increase differentiation of oligodendrocytes to
316 improve remyelination may need to be reconsidered, as replacing the 'correct' type may be preferred.
317 Another study using a similar approach to address the cellular composition of MS lesions found
318 specific signatures for stressed oligodendrocytes, reactive astrocytes and activated microglia,
319 especially at the rim of demyelinated lesions. As this study also included cortical grey matter tissue,
320 the authors reported a selective loss of CUX2-expressing upper layer excitatory projection neurons in
321 the grey matter both in demyelinated and partially remyelinated lesions (Schirmer et al., 2019). In line
322 with the previous study, they also found that some stressed oligodendrocytes seem to be capable of
323 antigen presentation. This again confirms that damage does not affect all cells equally and that there is
324 still a large gap of knowledge about disease mechanisms in MS. These studies are mostly descriptive,
325 but a recent study in zebrafish has demonstrated that two distinct subgroups of oligodendrocyte
326 precursor cells (OPCs) identified by scRNA-seq are confirmed to be functionally distinct with one
327 primarily making networks and the other primarily differentiating into oligodendrocytes to make
328 myelin (Marisca et al., 2020). Although this study was performed on normal zebrafish, this may also
329 be important as if this is similar in humans, it may again force us to rethink our therapeutic
330 remyelination strategies in MS, aiming to stimulate differentiating OPCs selectively. With a focus on
331 microglia, Masuda et al. (2019) described microglial heterogeneity for the first time in non-
332 pathological human brain and additionally found clusters of disease-related microglia in MS patients
333 that were similar to rodent animal models of MS, but with a high inter-personal variability. A very
334 recent study has directed its focus on astrocytes in MS showing that astrocytes in mice and human are
335 also heterogeneous. The authors found a clear MS-associated astrocyte cluster actively promoting CNS
336 inflammation by the regulation of gene expression (Wheeler et al., 2020).

337 These technologies have also reached other human brain pathologies such as Alzheimer's disease (AD)
338 (Grubman et al., 2019;Mathys et al., 2019;Zhou et al., 2020), Huntington's disease (Al-Dalahmah et
339 al., 2020) and other psychiatric disorders (Renthal et al., 2018;Velmeshev et al., 2019;Nagy et al.,
340 2020). Although AD is usually considered a neuronal disease mostly of the GM, it has been surprisingly
341 found that oligodendrocytes in the WM do show a significant transcriptional change in the disease
342 apparently adapting their metabolism to neuronal degeneration (Mathys et al., 2019;Zhou et al., 2020).
343 OPCs also seem involved, as in AD, OPCs repress apolipoprotein E (APOE), which is a genetic risk
344 factor for this disease, strengthening the hypothesis that oligodendroglia actively contribute to
345 pathogenesis (Grubman et al., 2019). This study used the known AD risk genes to study how these
346 contribute to disease in a cell-specific manner, as a relevant strategy to focus the analysis of the wealth
347 of data. Another recent study found that besides neurons, OPCs are majorly disturbed in major
348 depressive disorder and this seemed to be coupled with their interaction with neurons rather than their
349 ability to differentiate and myelinate (Nagy et al., 2020), which demonstrated that using this technology
350 is important to disentangle the functions of subsets of cells. Most importantly, these new studies have
351 started to shed new light on neurodegenerative and psychiatric diseases, moving away from a
352 neurocentric view of these diseases with new recognition of the importance of glial cells in their
353 pathogenesis - a shift in the research landscape.

354 **3.6   What other techniques can we use to complement the sc/snRNAseq approach?**

355 RNA-seq at a single cell/nuclei level is only the start, with the fast development of other single cell
356 resolution technologies, including epigenetic methods. Assay for Transposase-Accessible Chromatin
357 using sequencing (ATAC-seq) has already long been used to assess the bulk chromatin accessibility
358 and the chromatin signature of cellular DNA (Buenrostro et al., 2013) including the human brain in
359 health and disease (Corces et al., 2017;Bryois et al., 2018;Fullard et al., 2018), but can now also be
360 done at the single cell/nuclear level (Fig. 2C). This adds information about transcriptional regulation

361 of different cell types and has already widely been used on rodent (Preissl et al., 2018;Sinnamon et al.,
362 2019), but also on human brain tissue (Zhong et al., 2020). A very recent study used this method to
363 identify new single nucleotide polymorphisms (SNPs) with a more functional annotation than
364 classically found with GWAS and found new risk-factors for Parkinson's and Alzheimer's disease
365 (Corces et al., 2020). Bioinformatics tools are emerging to integrate scRNA-seq with scATAC-seq data
366 in order to get a deeper understanding of the transcriptional and genomic landscape within one
367 individual cell. Further explorations of the epigenomic landscape at a single cell level include DNA
368 methylation profiling to detect methylation marks identifying regulatory programmes in different cell
369 populations (summarized in (Fiers et al., 2018)). It is already possible to detect epigenetic marks and
370 gene expression in the same cell, not only bioinformatically but also experimentally, as shown with the
371 sc-GEM (single cell analysis of genotype, expression and methylation) assay on cultured human
372 fibroblasts (Cheow et al., 2016) and its use on human tissue would be another highly valuable method
373 to understand its pathology.

374 Clearly, single cell DNA/RNA changes can imply function but protein detection adds a further level
375 of information to determine a cell's behaviour. Single cell technologies have also entered the protein
376 field with Cytometry by time of flight (CyTOF), although this is not yet unbiased but requires a
377 selection of markers of interest. This method uses metal-labelled antibodies to detect cellular antigens
378 that are then analysed by mass cytometry. This approach is similar to classical Fluorescence-activated
379 cell sorting (FACS), but with a broader separation of metals which overcomes the limit of the overlap
380 of fluorophores and allows the use of around 40 antibodies together (up to 100 when considering
381 isotopes as well). With a bioinformatics analysis approach, high numbers of single cells can be
382 thoroughly profiled. This approach has been used to characterize a change in the populations of
383 peripheral immune cells of MS patients (Bottcher et al., 2019a) as well as to characterize multiple
384 different region-dependent populations of microglia in human brain that are clearly distinguishable
385 from peripheral cells (Bottcher et al., 2019b). CyTOF can also directly be applied to histological tissue
386 sections – called imaging mass cytometry – and can be used to profile individual cells whilst
387 maintaining the spatial information. Although still a fledgling technique at the spatial level, this has
388 already successfully been applied in MS brain tissue to characterize astrocytes and peripheral cells in
389 MS lesions (Park et al., 2019) and to characterize the immune cell landscape within different lesions
390 from an individual MS patient (Ramaglia et al., 2019).

391 For a disease such as MS, spatial information is clearly very important, due to the focal nature of
392 demyelinated lesions, but there may also be pathological changes in more restricted areas in other
393 neurodegenerative pathologies as well, e.g. in AD. Although not yet at a single cell resolution, spatial
394 transcriptomics technologies aim to capture the whole transcriptome of each of very small areas of a
395 tissue section in an unbiased way in combination with histological analysis (Stahl et al., 2016). Using
396 the same principle of capturing and barcoding mRNA as droplet-based methods, this technology allows
397 location of the origin of an individual mRNA to a defined spot on a predefined grid on which the tissue
398 has been placed, thus maintaining the spatial information. This technology is already being used on
399 human tissue (Maynard et al., 2020), and with an earlier version in ALS (Gregory et al., 2020). With
400 the clear advantage of capturing the transcriptome at a high resolution whilst maintaining spatial
401 information, spatial transcriptomics technologies are clearly at the forefront of development and may
402 in the future be more widely used than current sc/snRNA-seq technologies. They are either based on
403 sequencing the transcripts *in situ* after having been barcoded (Ke et al., 2013;Lee et al., 2014;Wang et
404 al., 2018;Gregory et al., 2020;Lundin et al., 2020;Maynard et al., 2020), or use highly multiplexed
405 single molecular fluorescent *in situ* hybridisation probes detectable using confocal microscopy (Lubeck
406 et al., 2014;Shah et al., 2016). The current limitation of sequencing-based methods is the low detection
407 of transcripts. Multiplexed *in situ* methods on the other hand are restricted by the number of probes
408 (hundreds to thousands) due to the limited availability of fluorophores and optical resolution of
409 individual molecules, making them less suitable for an unbiased discovery-driven research approach.

410 However, recent developments have combined the methods, using sequential hybridisation with *in situ*
411 sequencing to theoretically cover the whole transcriptome with only few fluorophores (Shah et al.,
412 2016;Eng et al., 2019). As a result, a higher number of transcripts per cell can be detected. Although
413 these methods have not yet been implemented on human brain tissue, which will be challenging due to
414 its high autofluorescence, this high resolution of individual mRNAs will not only allow the localization
415 of cells within a tissue, but also will allow study of the subcellular localization of mRNAs, clear
416 advantages in comparison to scRNA-seq methods. Unfortunately, these methods do only work well on
417 thin tissue sections, limiting the information we gain from a three-dimensional point of view.
418 Sequencing-driven spatial methods in particular are still expensive and are thus tend to be performed
419 on small tissue pieces with few sections from an individual, which may introduce some bias to the
420 biological findings.
421 Validation of results, preferably on a separate cohort of tissue, is essential by classical
422 immunohistochemistry/fluorescence and in situ hybridisation, and/or these burgeoning multiplexing
423 technologies, mentioned above. These have allowed spatially detection of 100 different transcripts by
424 in situ sequencing (Lundin et al., 2020) or around 100 proteins and 1000 genes using oligonucleotide
425 labelling in a tissue section (Geiss et al., 2008;Kulkarni, 2011). Although imaging mass cytometry is
426 usually used to characterize novel cell populations, it can clearly serve as a validation method for
427 transcriptomic data as well. These require analysis tools to distinguish signals in different cells, but
428 appear very useful and are likely to become standard to address human pathologies in the future.

3.7   What does the future hold?

430 The outputs of all of these technologies applied to human white matter pathologies are still no more
431 than descriptive pathology – although on a much deeper level than was ever possible before and at least
432 implying function. This work however, is just the start of a new era of single cell resolution techniques
433 that will revolutionize human pathology and will most likely become a standard technology for
434 pathological assessment. The richness of these data will allow us to take the next step, which is to
435 address more functional changes to gain a deeper understanding of the diseases. For example, snRNA-
436 seq will allow us to study transcriptomic changes in a high numbers of cells in many different types of
437 MS lesions which then may allow us to reclassify them on a functional level, namely their regenerative
438 potential rather than using the classical degenerative description. Moreover, these data may allow us
439 to determine lesion markers that can be used for PET-imaging and will thus be an invaluable tool for
440 disease diagnosis, prognosis and response to therapies. Furthermore, as most of the data are gained in
441 a similar way and deposited with its metadata on open-source databases, it is then easier to compare
442 many brain regions (such as WM and GM, or brain and spinal cord) or diseases with each other, to
443 gain a much clearer picture of the cellular architecture of our brain. The Human Cell Atlas is a
444 collaborative effort to exactly achieve this aim (https://www.humancellatlas.org/) not only for the
445 brain, but for the entire human body.

446 So far, most of these technologies are used individually by different groups but in the future,
447 complimentary but different technologies will be used in the same experimental setup, and their outputs
448 integrated, as recently shown from the Allen Brain Institute (Bakken et al., 2020). Maybe in ten years
449 from now it will be possible to look at the transcriptome, the epigenome, the proteome and the
450 metabolome on a single cell level from the same tissue source at once, as suggested in Fig. 3. Attempts
451 to achieve this have already been made in recent preprint manuscripts where the authors were able to
452 simultaneously study either proteins and mRNA (Vistain et al., 2020) or chromatin accessibility and
453 gene expression (Ma et al., 2020) in single cells. This will allow us to look at the same data from many
454 different perspectives to gain a deeper understanding of individual cells in health and disease and
455 further explore pathological mechanism. The options here seem endless with money as the only limit!

<mark>**4   Concluding remarks**</mark>

Analysis and understanding of human WM pathologies has come a long way from being purely descriptive histological analysis to gaining cell type-specific information at a single-cell resolution <mark>(Fig. 4).</mark> This development goes hand in hand with the development of novel technologies, although their use in human tissue is inevitably more challenging than in animal models. We can now sequence the RNA of a cell, examine the state of the chromatin and it is likely that current proteomic tools will become more sensitive as well, which will allow us to explore the proteome of individual cells. Although all of these technologies have developed separately, we will need to link them together in order to see the full picture in the future. This will allow us to gain a deeper insight into human pathologies and will become important tools not only for basic science, but will also revolutionize diagnostics and may pave the way for developing new therapies. However, even though these technologies are developing at an exciting and rapid speed, it is important to retain a clear focus on the research question to be answered, to avoid distraction by an ocean of data and high costs, and to ensure that we advance biology.

5   Author Contributions

S.J. and A.W. wrote and edited the manuscript. S.J. also designed and prepared the figures.

6   Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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828

829   **10   Figure legends**

830   **Figure 1**: **Schematic overview of the workflow of common bulk approaches to address human
831   pathology.** (A) Standard workflow of bulk RNA-sequencing: the entirety of mRNA/RNA is isolated
832   from a tissue/cell type of interest and prepared for next generation sequencing, which is followed by
833   bioinformatics data analysis. (B) Standard workflow of bulk proteomics: the entirety of proteins is
834   isolated from a tissue/cell type of interest and prepared for mass spectrometry, which is followed by
835   bioinformatics data analysis. (C) Standard workflow of bulk metabolomics: the entirety of metabolites

836 is isolated from a tissue/cell type of interest and prepared for mass spectrometry, which is followed by
837 bioinformatics data analysis. In all approaches (A-C) the information about the cell of origin and the
838 spatial distribution are lost.

839 **Figure 2: Schematic overview of the workflow of common single cell/nuclei (sc/sn) approaches to**
840 **address human pathology.** (A) Standard workflow of droplet-based sc/sn RNA-sequencing: tissue is
841 dissociated into a single cell/nuclei suspension and the mRNA of each cell is captured and barcoded
842 individually and prepared for next generation sequencing, which is followed by bioinformatics data
843 analysis. This can be done for up to ten thousands of cells. (B) Standard workflow of well-based sc/sn
844 RNA-sequencing: tissue is dissociated into a single cell/nuclei suspension and the cells are captured in
845 individual wells where each cell is prepared for next generation sequencing, which is followed by
846 bioinformatics data analysis. This is usually done for less than 1000 cells. (C) Standard workflow of
847 droplet-based ATAC-seq: tissue is dissociated into a single cell/nuclei suspension and the open
848 chromatin regions of each cell are cut and barcoded individually and prepared for next generation
849 sequencing, which is followed by bioinformatics data analysis. This can be done for up to ten thousands
850 of cells. In all approaches (A-C) the information about the cell of origin is maintained, but the spatial
851 distribution is lost.

852 **Figure 3: Schematic overview of an imaginative holistic workflow of common single cell/nuclei**
853 **(sc/sn) and validation approaches to address human pathology.** Tissue is dissociated into a single
854 cell/nuclei suspension and sc/snRNA-seq, sc/snATAC-seq, scCyTOF, scProteomics and
855 scMetabolimics are performed in parallel on the same tissue source with their respective workflows.
856 After individual and comparative/integrated bioinformatics data analysis, the results are validated,
857 ideally on a different tissue source by standard immunohistochemistry (IHC), in-situ hybridization
858 (ISH) and other high throughput multiplexed techniques (100-1000 genes/proteins of interest) where
859 IHC and ISH can be combined. With such a possible workflow, first the information about the cell of
860 origin is maintained, and with the validation techniques, the spatial resolution can be analyzed as well.
861 This holistic approach would allow to thoroughly exploring human pathology from different angles to
862 gain deeper information.

863 **Figure 4: Summary of the biological findings in Multiple Sclerosis (MS) that were gained through**
864 **advances in technology.** Classical histology has helped to classify MS lesions based on their
865 immunological status. The development of bulk transcriptomic approaches has helped to unravel many
866 transcriptional pathways that are changed in MS, with the most important common theme being
867 inflammation. More recent studies using sc/snRNA-seq has started to unravel cellular heterogeneity
868 and changes in the representation of these cells in disease. Combining different sc/sn approaches and
869 using spatial technologies in the future will help to deepen our understanding of the functionality of
870 the heterogeneous clusters and the underlying pathological mechanisms.

871

Figure 1.JPEG
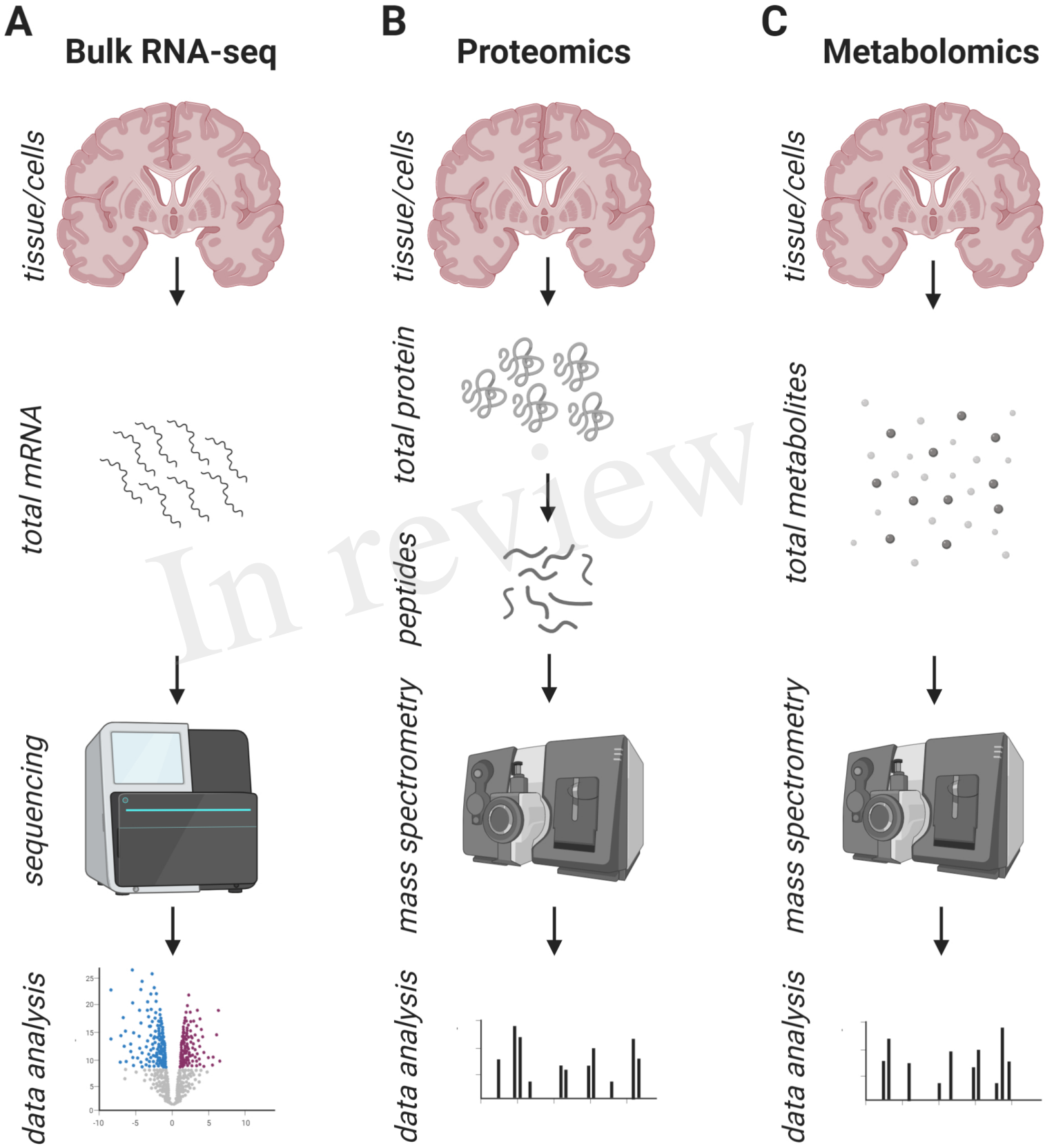
Figure 2.JPEG

Figure 3.JPEG

tissue/cells

cells/ nuclei

sc technologies

scRNA-seq   scATAC-seq   scCyTOF   scProteome   scMetabolome

sequencing   *mass spectrometry*

data analysis

integrated data analysis

validations

immunohistochemistry   *in-situ hybridisation*   *multiplexed high-throughput technologies*
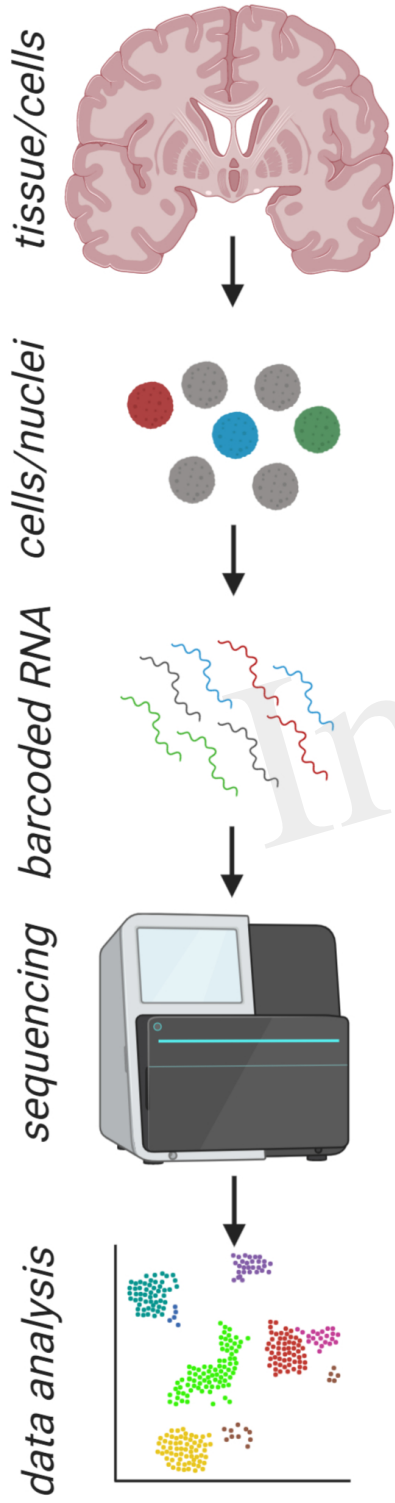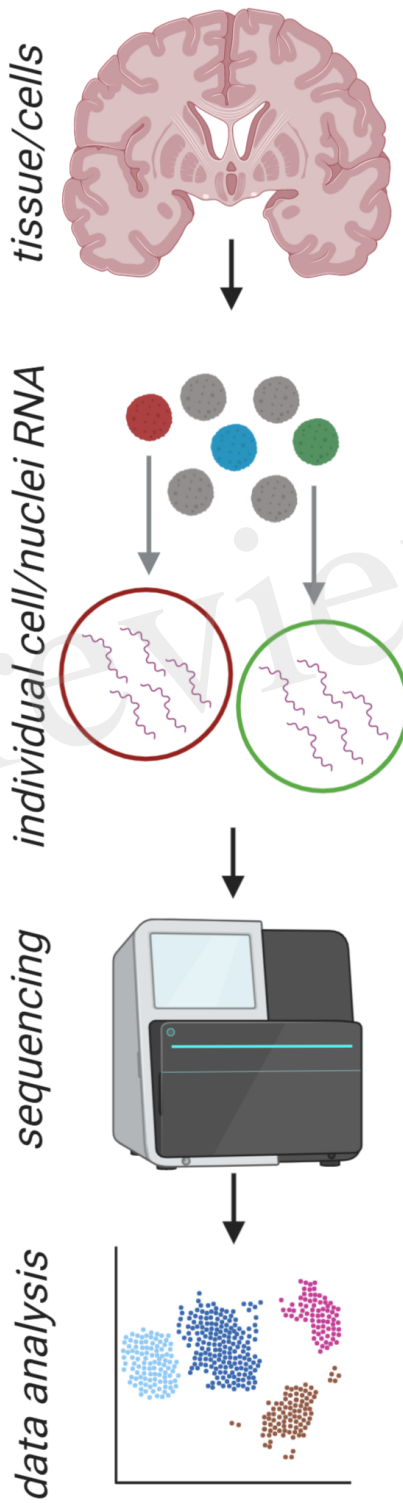
Figure 4.JPEG

Technological advances in MS pathology over time

present   *future*

Histology | Bulk transcriptomics | sc/sn transcriptomics | Spatial/combined approaches

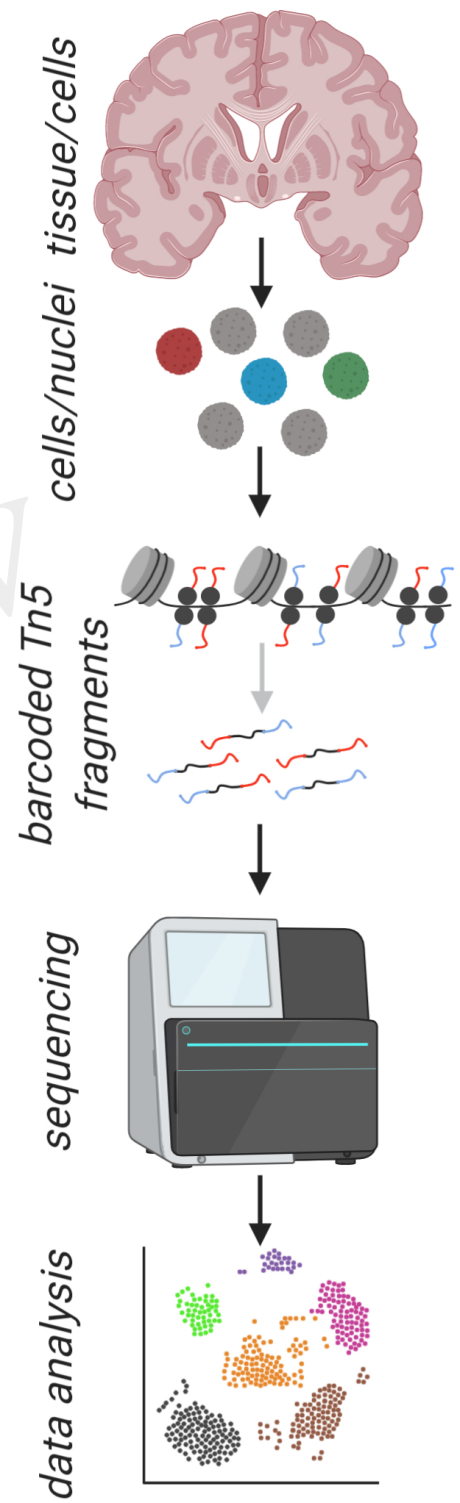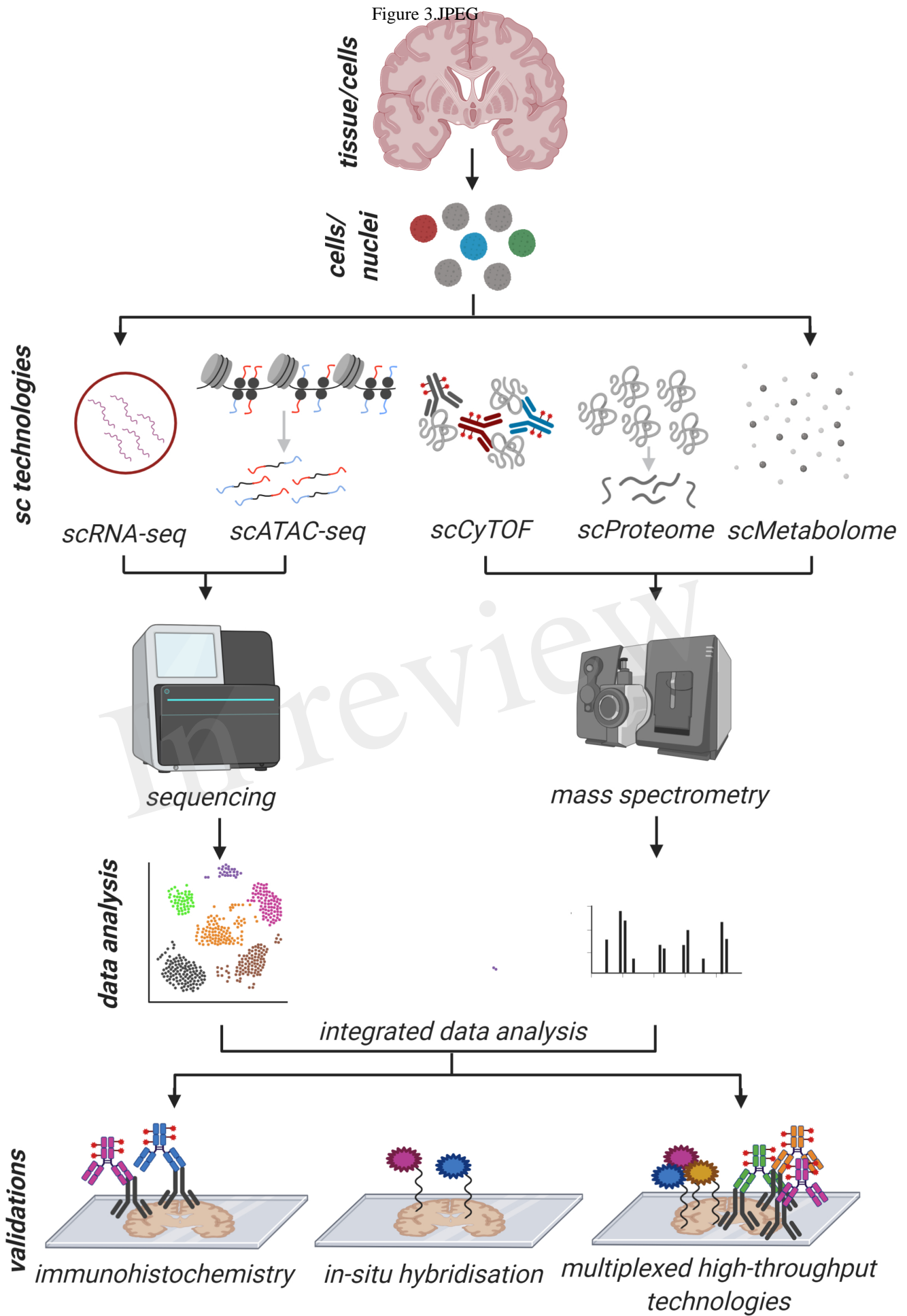|			
MS lesions classified based on immunological status (*Lassmann et al. 1998*)	• NAWM not "normal" (*Common finding*) • MS lesions highly inflammatory (*common finding*) • OL are anti-inflammatory (*Graumann et al., 2003, Zeis et al., 2008*) • Lesion types mostly differ in number of transcripts (*Tajouri et al., 2003*) • Microglia change metabolism in NAWM (*van der Poel et al., 2019*) • Pathological gene regulation influenced by environment (*Melief et al., 2019*)	• OL heterogeneity skewed in MS (*Jakel et al. 2019*) • Disease-specific immune OL (*Falcao et al. 2018, Jakel et al., 2019*) • MS-specific signature of glia and selective neuronal loss (*Schirmer et al., 2019*) • Microglia heterogeneity and MS-associated clusters (*Masuda et al., 2019*) • Astrocyte heterogeneity with MS-associated cells (*Wheeler et al., 2020*)	• Spatial resolution of cellular heterogeneity • Functionality of cell clusters • Understanding of mechanisms