1 **The mitochondrial epigenome: a role in Alzheimer's disease?**

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4

8 **SUMMARY**

9 **Considerable evidence suggests that mitochondrial dysfunction occurs early in Alzheimer's**

10 **disease, both in affected brain regions and in leukocytes, potentially precipitating**

11 **neurodegeneration through increased oxidative stress. Epigenetic processes are emerging**

12 **as a dynamic mechanism through which environmental signals may contribute to cellular**

13 **changes, leading to neuropathology and disease. Until recently little attention was given**

14 **to the mitochondrial epigenome itself, as preliminary studies indicated an absence of DNA**

15 **modifications. However, recent research has demonstrated that epigenetic changes to the**

16 **mitochondrial genome do occur, potentially playing an important role in several disorders**

17 **characterized by mitochondrial dysfunction. This review explores the potential role of**

18 **mitochondrial epigenetic dysfunction in Alzheimer's disease etiology and discusses some**

19 **technical issues pertinent to the study of these processes.**

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24 Alzheimer's disease (AD) is a chronic, currently incurable, neurodegenerative disorder,

25 accounting for more than 60% of dementia cases, with current estimates predicting more

26 than 135 million dementia cases worldwide by 2050 [1]. The classic neuropathological

27 hallmarks associated with AD include the formation of amyloid beta (Aβ) plaques and

28 neurofibrillary tangles. These are suggested to play a role in the further development of

29 other characteristics of the disease, such as disruption of calcium homeostasis, loss of

30 connectivity, the generation of reactive oxidative species (ROS) and altered plasticity,

31 ultimately leading to neurodegeneration [2-6]. Mitochondrial dysfunction is a consistent

32 feature of AD pathology in both the brain and white blood cells [7-10] although the

33 molecular mechanism(s) mediating this phenomena are yet to be fully elucidated.

34

35 **Mitochondrial dysfunction: a prominent feature of AD**

36 Being the site of ATP generation, mitochondria provide the cell with the energy required to

37 properly function; as such they are often described as 'the powerhouse of the cell'.

38 Mitochondria are cylindrical organelles containing ~16.6kb of DNA (mtDNA) [11], which is

39 separate to the nuclear genome and inherited in a maternal, non-Mendelian fashion. The

40 mitochondrial genome consists of 37 genes, 13 of which encode for polypeptides required

41 for the electron transport chain (ETC) (Figure 1), in addition to two ribosomal RNAs and 22

42 transfer RNAs. The mitochondria play a vital role in a variety of key biological functions,

43 including apoptosis via caspase dependent and independent mechanisms [12], the

44 regulation of calcium homeostasis [13, 14] and the production of ROS [15]. For these

45 reasons, mitochondrial dysfunction has been implicated in the pathogenesis associated with

46 AD [16, 17] and forms the basis of the mitochondrial cascade hypothesis [18]. Proposed by

47 Swerdlow et al, this hypothesis states that an individual's genetic code will determine their

48 basal mitochondrial function and that, throughout ageing, this function will decline due to a

49 combination of genetic and environmental factors, determining an individual's time of

50 disease onset [18].

51

52 Mitochondrial-encoded ETC gene expression has been shown to be altered in both early and

53 late stages of AD, with decreased expression of complex I and increased expression of

54 complexes III and IV [7]. Increased expression of mitochondrial-encoded ETC complex genes

55 has also been associated with aging, with increased expression of complexes I, III, IV and V

56 in 12- and 18-month wild-type mice compared to 2-month mice, which was accompanied

57 by increased oxidative damage [19]. However decreased expression of these genes was

58 seen in older, 24-month old mice. Further evidence for a role of mitochondria in AD

59 pathogenesis comes from a study demonstrating increased levels of mitochondrial gene

60 expression and oxidative damage in a transgenic Amyloid Precursor Protein (*APP*) mutant

61 mouse model of AD [20]. In addition, various components of the mitochondrial permeability

62 transition pore (mPTP), which acts as a voltage-dependent channel regulating mitochondrial

63 membrane permeability, have been shown to interact with Aβ in various murine models of

64 AD. For example, one recent study found that, in *APP* transgenic mice, Aβ acts to upregulate

65 VDAC1, a component of the mPTP, leading to mPTP blockade. [21]. Interestingly, this study

66 also reports that VDAC1 may interact with hyperphosphorylated tau, suggesting another

67 mechanism of mitochondrial dysfunction. An earlier study found that Aβ present in

68 mitochondria interacts with CypD, another component of the mPTP, in cortical samples

69 from post-mortem AD patients and *mAPP* transgenic mice [22]. In the mouse model, this

70 was shown to lead to increased ROS production and neuronal cell death. Taken together,

71 this illustrates how mitochondrial-encoded gene expression is altered in AD, a variety of

72 mechanisms by which Aβ interacts with mitochondria in AD, and how mitochondrial

73 dysfunction can lead to changes associated with AD, thus highlighting the need for

74 continued research into the field.

75

76 **Epigenetics and AD**

77 Given the high heritability estimates for AD [23], considerable effort has focussed on

78 understanding the role of genetic variation in disease etiology, although more recently it has

79 been hypothesized that epigenetic dysfunction may also be important [24]. A number of

80 studies have shown reduced global levels of the DNA modifications 5-methylcytosine (5-mC)

81 and 5-hydroxymethylcytosine (5-hmC) in AD brain [25-28] with only a handful of studies

82 have looked at changes occurring at specific loci (reviewed in [24]). Recent methodological

83 advances in microarray and genomic sequencing technologies have enabled researchers to

84 undertake epigenome-wide association studies (EWAS) in AD brain, identifying several

85 consistent differentially methylated regions (DMRs) associated with disease [29-31]. Many

86 of these DMRs are tissue-specific, restricted to regions of the brain associated with AD

87 pathology, and correlate strongly with quantitative measures of neuropathology. As such, a

88 strong case is being built for a role of epigenetics in the etiology of AD.

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Epigenetic regulation of the mitochondrial genome

Although hypotheses about the importance of mtDNA modifications are by no means recent, research in this area has been marred by contradictory results since the 1970s [32-35]. The confirmation in 2011 of both 5-mC and 5-hmC occurring in mtDNA prompted a resurgence of interest in mitochondrial epigenomics [36]. The mitochondrial epigenome has some notable differences compared to the nuclear epigenome, and an overview of the mitochondrial genome, including its CpG sites, can be seen in Figure 1. Unlike the nuclear genome, the mitochondrial genome does not contain classical CpG islands [36], and is not associated with chromatin; instead it is structurally organised by nucleoids [37, 38]. As a result, mtDNA is not associated with histone proteins and relies on transcription factors such as mitochondrial transcription factor A (TFAM) to mediate compaction [39]. Histone modifications do not therefore play a direct role in regulating mitochondrial gene expression, highlighting the potential importance of DNA modifications in the regulation of mitochondrial function [40]. Evidence suggests that mtDNA methylation largely influences mtDNA structure and replication and is affected by factors which influence nucleoid compaction and DNA methyltransferase (DNMT) binding [41]. It has been shown that different areas of mtDNA are packaged differently and that a depletion of the nucleoid protein ATAD3 can reduce mtDNA methylation, resulting in an open circular state mitochondrial genome, although evidence for an effect of TFAM on mtDNA methylation was inconclusive [41].

DNMTs are a family of enzymes that catalyse the removal of a methyl group from methyl donors such as S-adenosylmethionine (SAM) for addition to the 5-position of cytosine.

113   Recently, a DNMT isoform, mitochondrial DNMT1 (mtDNMT1), has been found to contain a

114   mitochondrial targeting sequence allowing it to bind to the D-loop of the mitochondrial

115   genome, which contains the promoter sites for both the light and heavy strand of mtDNA

116   and can therefore influence mitochondrial gene expression by altering transcriptional

117   activity [36]. Furthermore, it has been suggested that the presence of these

118   methyltransferases in mitochondria may be tissue-specific. Although Shock *et al*, did not

119   observe mitochondrial localization of DNMT3a in the two cell lines they investigated, a later

120   paper has found that DNMT3a is present, and in higher levels than mtDNMT1, in the

121   mitochondria of motor neurons [42]. This study also demonstrated significantly higher

122   global levels of both mitochondrial DNMT3a and 5-mC in Amyotrophic Lateral Sclerosis (ALS)

123   motor neurons *in vivo*, suggesting a potential role for mtDNA methylation in motor neurons.

124   DNMT1 and DNMT3b have also been observed in the mitochondria, with their inactivation

125   reducing methylation at CpG sites [43].

126

127   Recently, it has been debated whether 5-hmC is just an intermediary product of the

128   demethylation process of 5-mC to cytosine or could represent an independent epigenetic

129   mark [44]. Growing evidence now suggests that 5-hmC could be a mark in its own right,

130   produced from the conversion of 5-mC by TET1, TET2 and TET3 [45], with both TET1 and

131   TET2 being present in the mitochondria [43]. Taken together with the presence of 5-hmC in

132   the mitochondrial D-Loop [36], this strengthens the evidence suggesting that demethylation

133   pathways are not only important in nuclear epigenetics, but may also play a role in the

134   mitochondria. Furthermore, recent evidence suggests that 5-mC and 5-hmC exist stably

135   within mtDNA at cytosines not preceding a guanine base, suggesting a role for non-CpG

136   methylation in mtDNA [46]. Further, CpG and non-CpG methylation has been observed in

137   the mitochondrial D-loop at conserved regions associated with DNA-RNA hybrid formation

138   during transcription, suggesting that DNA methylation in mitochondria shares similarities

139   with plants and fungi and that this methylation may play a role in regulating mtDNA

140   transcription and replication in a cell type-specific fashion [43].

141

142   **MtDNA modifications in disease**

143   Despite little being known about the physiological impact of variation in mtDNA

144   methylation, some recent studies have shown that it may be associated with a variety of

145   diseases. The majority of studies have focussed on diseases where mitochondrial

146   dysfunction is known to be prevalent, for example in cancer, which has been previously

147   linked with mitochondrial dysfunction [47] and more recently in Down's Syndrome, where

148   mitochondrial abnormalities have also been reported [48]. Particularly, for the purpose of

149   this review, mitochondrial dysfunction and mtDNA methylation aberrations in Down's

150   syndrome cells (see Table 1) are interesting given that these patients have an increased

151   likelihood of presenting with AD-like phenotypes throughout aging [49, 50] due to

152   possessing an extra copy of *APP*. An overview of studies of mtDNA epigenetics in disease is

153   given in Table 1.

154

155   **MtDNA modifications: evidence for a role in AD and aging**

156   Until recently the role of mtDNA modifications in AD has been largely ignored, despite the

157   evidence that mitochondrial dysfunction is involved in AD [18] and that ncDNA methylation

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158 differences are associated with the disease [29-31]. At a global level, an initial dot blot study

159 showed some evidence for increased mitochondrial 5-hmC in AD superior temporal gyrus

160 tissue, although definitive conclusions could not be drawn given the small number of

161 samples used [51]. Mitochondrial DNA modifications in the brain have been shown to be

162 associated with aging, with global mtDNA 5-hmC levels reduced in the frontal cortex of aged

163 mice and specifically decreased 5-hmC levels being found in the regulatory D-Loop, as well

164 as in two genes encoding ETC complex I polypeptides (*MT-ND2* and *MT-ND5*) [52]. Aging

165 was not only found to be associated with overall decreased mtDNA 5-hmc levels but also

166 with increased cortical expression of the mitochondrial ETC genes *MT-ND2, MT-ND4, MT-*

167 *ND4L*, *MT-ND5*, and *MT-ND6* [52]. A post-mortem study of frontal cortex described

168 differential mtDNA gene expression of these genes, and other mitochondrial-encoded

169 genes, in both early and late-stage AD. [7] Taken together, these findings illustrate that

170 alterations in mitochondrial-encoded genes do occur with aging and in age-related diseases,

171 yet without further studies, the exact role of mtDNA methylation on mitochondrial gene

172 expression in these instances remains uncertain.

173

174 **Two genomes are better than one: interactions between the nuclear and mitochondrial**

175 **genomes**

176 As research into the field of mitochondrial epigenetics gains momentum, studies have

177 focused on a potential *trans*-acting role of mtDNA in the epigenetic regulation of ncDNA,

178 whereby covalent modifications across the mtDNA genome may affect not only the

179 expression of a gene in *cis*, but also have *trans*-acting effects on the transcription of genes in

180 the nuclear genome. Evidence for this is provided by cybrid models, which combine the

181   nuclear genome of one source with the mitochondrial genome of another in an attempt to

182   determine the functional role of the mtDNA. Using Restriction Landmark Genomic Scanning

183   (RLGS) and Rho^{0} cells, a form of cybrid cell line designed for investigating mtDNA depletion,

184   one study found that mtDNA depletion significantly altered DNA methylation at CpG islands

185   in nuclear encoded genes [53], indicating that there are functional interactions between the

186   two genomes. Re-introduction of wild-type mtDNA restored DNA methylation levels, at

187   some RLGS spots, suggesting that, at least for some genes, mitochondria may play a role in

188   nuclear DNA methylation. This is corroborated by a recent study demonstrating that

189   mitochondrial haplotype variation can affect ncDNA methylation, with mtDNA haplotype J

190   exhibiting higher global DNA methylation levels, reduced ATP, and overexpression of the

191   nuclear gene methionine adenosyltransferase I, alpha (*MAT1A*), which is required for SAM

192   production thus regulating methylation patterns in the nuclear genome [54]. Therefore

193   genetic variations in mtDNA are capable of influencing epigenetic modifications in both the

194   mitochondrial and nuclear genomes. As such, it is possible that mitochondrial dysfunction in

195   AD could lead to alterations in mtDNA methylation, affecting nuclear gene expression.

196

197   The mitochondria comprises approximately 1500 proteins, however of these, only 13 are

198   encoded by the mitochondrial genome; the remainder are encoded by the nuclear genome

199   and imported into the mitochondria. A recent study found that >600 of these genes have

200   tissue-specific differentially methylated regions, ultimately leading to changes in

201   mitochondrial function dependent upon tissue type [55]. This suggests that there is an

202   additional level of complexity to consider in the study of mitochondrial epigenetics,

203 whereby epigenetic changes in one genome may affect transcriptional control in another in

204 a tissue-specific manner.

205

206 **Interrogating the mitochondrial epigenome: technical caveats**

207 Despite the potential importance of mitochondrial DNA modifications in AD, there are a

208 number of technical challenges specific to interrogating the mitochondrial epigenome that

209 have hampered widespread studies to date. These issues can be broadly summarized as

210 encompassing genetic issues and specificity issues, which are outlined briefly with potential

211 solutions in Table 2.

212   1. Genetic issues

213   1.1.   Nuclear pseudogenes

214 By far the greatest concern when analyzing mtDNA methylation arises from regions of

215 homology between the mitochondrial genome and nuclear mitochondrial pseudogenes

216 (NUMTs). These genes are nuclear paralogs of mtDNA which have been translocated and

217 inserted into the nuclear genome during evolution of both genomes [56]. This phenomena

218 has been shown to be evolutionarily conserved across many species including cats [57],

219 mice, chimpanzees, rhesus macaques [58] and hominins [59]. These insertions were

220 thought to typically occur in non-coding regions; however, more evolutionary recent

221 translocations have actually been shown to prefer integration into coding regions, thus

222 leading to potential alterations in gene function with implications for disease [60]. NUMTs

223 are generally small and typically comprise ~0.1% of the nuclear genome [61]. However in

224 humans, it has been shown that some NUMTs can be as large as 14.7kb, representing a

225 significant portion of the ~16.6kb human mitochondrial genome [62]. As such, the presence

226 of NUMTs can cause major issues in genomic analyses using pre-sequencing enrichment

227 methods such as custom capture or long-range PCR as the likelihood of NUMT co-

228 amplification, or even preferential amplification, increases due to the strong sequence

229 similarity between the two segments of genome [63]. As such, this sequence similarity can

230 lead to the misclassification of NUMTs as mtDNA during analysis, and has led to a number of

231 publications wrongly describing NUMTS as mtDNA [64, 65]. NUMT misclassification has also

232 been observed in AD genetic studies whereby amplification of the NUMT sequence has led

233 to false heteroplasmies (see below) being reported [66, 67]. One potential solution is to

234 separate mitochondria prior to DNA extraction in an attempt to reduce the risk of

235 contaminating the mitochondrial and nuclear genomes. However, despite extensive

236 research being dedicated to mtDNA analysis, existing methods for mitochondrial isolation

237 and mtDNA extraction via the use of fractional precipitation or gradient ultracentrifugation

238 remain time consuming and labour intensive [68] and often leave residual nuclear DNA

239 contamination following mitochondrial isolation [69].

240 1.2. Variation in mtDNA: haplogroups and genetic and epigenetic

241 heteroplasmy

242 Each mitochondrion contains between 2-10 copies of mtDNA. However, not all mtDNA in

243 each mitochondrion share the same DNA sequence. Indeed, mutations in some copies of

244 mtDNA mean that the cell itself may be made up of a mixture of different sequences. This

245 phenomenon is known as mitochondrial heteroplasmy and has been linked to various

246 mitochondrial diseases, [70]. It is a potential confounder in studies of mitochondrial

247 diseases, because inter- and intra-individual heteroplasmic variation can confuse the

248 association between a haplogroup with its corresponding phenotype. The importance of

249 this issue, in the context of this review, is highlighted by a recent study demonstrating that

250 mitochondrial heteroplasmy alters DNA methylation across the nuclear-encoded

251 mitochondrial genes TFAM and POLMRT [71]. Finally, if mtDNA methylation is altered across

252 different mtDNA in the same mitochondrion, it could create an epigenetic mosaic within the

253 mitochondrion, the cell and across the tissue, whereby each copy of mtDNA may possess its

254 own methylation profile. If this 'methylomic heteroplasmy' were to occur it could be very

255 difficult to tease apart the effects of such a mosaic in functional studies.

256

257 On a larger scale, mutations in mtDNA can be used to help group cohorts or "haplogroups".

258 Throughout evolution, mutations in mtDNA may be conserved and passed on through

259 maternal inheritance, thus allowing for the tracing of common ancestral lineage by

260 comparing haplogroups. Numerous studies have identified both contributory and protective

261 effects of different haplogroups in AD. For example, haplogroup K reduces the risk of

262 developing sporadic AD in Apolipoprotein ε4 (*APOε4*) carriers in an Italian population [72]

263 but not in the Polish population [73]. This presents an additional potential caveat in

264 mitochondrial epigenetics, as mitochondrial haplogroups have been found to affect global

265 levels of DNA methylation [54]. As such, extra care should be taken to account for

266 haplogroup variability in AD mitochondrial epigenetic studies.

267   2. Specificity and technical issues

268 The brain is a complex, heterogeneous organ with numerous functionally-distinct sub-

269 regions, each with their own different composition of cell types. Unsurprisingly, there are

270 clear tissue-specific epigenetic differences across brain regions [74, 75]. There is an added

271 level of complexity with respect to the mitochondrial epigenome because each

272 mitochondrion contain between 2-10 copies of mtDNA and each cell contains varying levels

273 of mitochondria; therefore the amount of mtDNA copies in each cell can vary between 100-

274 10,000 dependent upon cell type. In neurodegenerative diseases such as AD, the issue

275 becomes more complicated in that the disease itself is characterized by the loss of neuronal

276 cells and the activation of glia, a process that has been associated with changes in

277 mitochondrial morphology and fission [76, 77]. A recent study using laser capture

278 microdissection demonstrated that alterations in mitochondrial 5-hmC are seen with age in

279 dissected mouse cerebellar purkinje cells, which was not evident in whole cerebellar tissue

280 [52], demonstrating the importance of cell-specific analyses in heterogeneous tissue,

281 particularly when investigating functional impact.

282

283 Currently, the most common method of measuring DNA methylation is via the conversion of

284 DNA with sodium bisulfite followed by subsequent sequence analysis. However, these

285 approaches are unable to distinguish between 5-mC and 5-hmC [78], an important

286 limitation given recent studies confirmed the presence of 5-hmC in mitochondria in brain

287 tissue [52]. Studies have found that although both DNA modifications are present in the

288 mitochondria, they occur at much lower levels compared to in ncDNA [36, 51], and thus

289 methods used for quantification may need to be more sensitive. Furthermore, variation in

290 mitochondrial copy number may lead to the dilution of signals and reduce detection if the

291 tissue is largely heterogeneous. Importantly, the mitochondrial genome is not interrogated

292 using tools such as the Illumina Infinium 450K methylation array, the current gold standard

293   for methylomic analyses in large numbers of samples; thus methods for detecting mtDNA

294   modifications across the entire mitochondrial genome are largely restricted to antibody-

295   based enrichment, such as MeDIP-Seq, which may be less sensitive for detecting low levels

296   of modified cytosine and does not interrogate methylation levels at single base resolution

297   [79].

298

299   **Future Perspective: the potential for biomarkers in AD**

300   Two important goals of research into the etiology of AD are a) a fast, non-invasive,

301   inexpensive and reliable biomarker and b) an effective treatment that targets the underlying

302   neuropathology. A potential utility for DNA methylation biomarkers has been proposed for

303   diseases in which traditional biomarkers are either too expensive, invasive, unspecific or

304   insensitive for clinical purposes [80]. Epigenetic modifications have been widely studied in a

305   variety of different cancers and other conditions such as preeclampsia to check for their

306   suitability as prognostic and/or diagnostic biomarkers [81-83]. Differential methylation of

307   mtDNA has yet to be examined with respect to its potential utility as an AD biomarker, but

308   certainly warrants further investigation.

309

310   **Conclusion**

311   With mitochondrial epigenetics only recently emerging as a focus for biomedical research,

312   the role of the mitochondrial epigenome in AD has yet to receive much attention. However,

313   it is possible that deregulation of the mitochondrial methylome may lead to aberrant

314   changes in many of the intricately controlled processes that it helps to govern, such as

apoptosis, which may play a key role in pathogenesis. Furthermore, as mitochondrial dysfunction occurs early in AD pathogenesis, it is plausible that alterations in the mitochondrial methylome may play a major role in the onset and development of the disease. Despite the field presenting numerous challenges the links between mitochondrial epigenetics and AD provide good bounds for future research directions.

EXECUTIVE SUMMARY

Mitochondrial dysfunction: a prominent feature of AD

- The mitochondrial genome plays a vital role in a variety of key biological functions, including apoptosis via caspase dependant and independent mechanisms, regulating calcium homeostasis and production of ROS.
- Mitochondrial dysfunction is reported to occur in both the brain and blood of AD patients.

EWAS and AD

- Studies focussing on global levels of 5-mC and 5-hmC have found a reduction in levels of both marks in AD brain.
- Three recent EWAS studies have found differential methylation at specific loci in AD brain.

Epigenetic regulation of the mitochondria genome

- Despite early controversial results, both 5-mC and 5-hmC have been recently reported in mitochondria.

336   • MtDNA is not tightly wrapped by histones and is instead condensed by nucleoids,

337   suggesting methylation could play an important role in gene regulation.

338   • DNMT1 can bind to the D-Loop of the mitochondrial genome and can influence gene

339   expression.

340   • MtDNA methylation occurs at both CpG sites and non-CpG site in the mitochondrial

341   genome.

MtDNA methylation: a key player in AD?

343   • Very few empirical studies have examined the role of mtDNA methylation in brain.

344   • Decreased mtDNA 5-hmC levels and increased expression of some mitochondrial-

345   encoded genes has been seen in the pre-frontal cortex of aged mice.

Technical caveats

347   • NUMT misclassification has been observed in AD genetic studies whereby

348   amplification of the NUMT sequence has led to false heteroplasmies being reported.

349   • MtDNA methylation could be altered in different mitochondria, creating a

350   methylomic heteroplasmy.

351   • MtDNA methylation patterns could be cell-specific and is an important consideration

352   when investigating heterogeneous tissues such as brain.

357

Figure 1: The structure of the mitochondrial genome showing genes encoded by the mitochondria. 3358 mtDNA genetic variants are shown in red and black lines highlight the predicted CpG sites relative to mutations that define the mitochondrial haplogroup. P_H and P_L represent the heavy and light strand promoter regions and O_H and O_L represent the origins of heavy-strand and light-strand replication respectively. Image taken from [84].
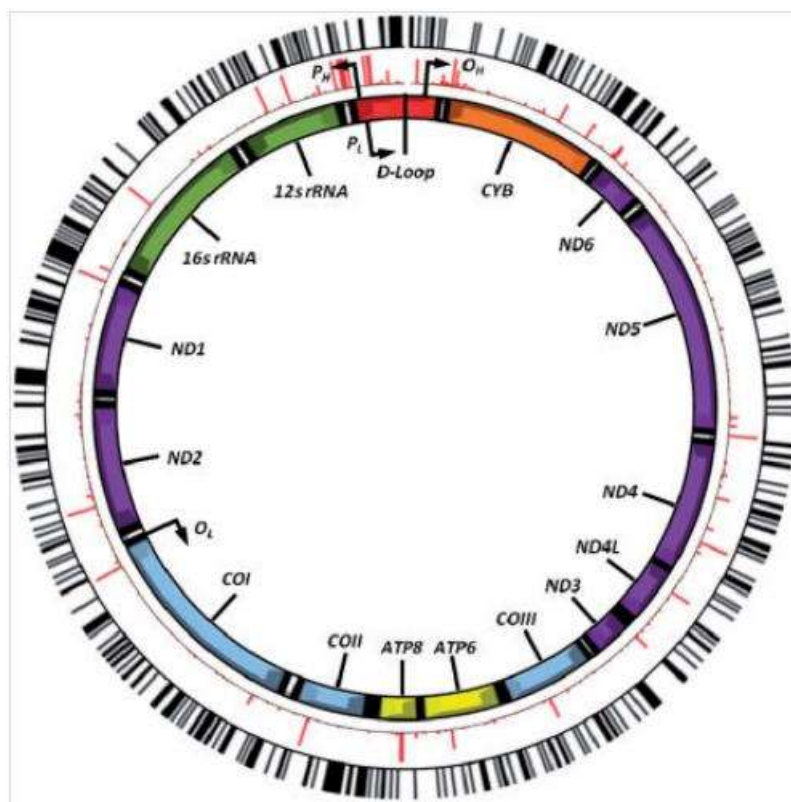
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369 **Table 1: An overview of current studies of mitochondrial epigenetics in disease.**

370 Abbreviations: Quantitative Real-Time PCR (qRT-PCR), mitochondrial DNA (mtDNA), simple

371 steatosis (SS), non-alcoholic steatohepatitis (NASH), S-adenosylmethionine (SAM) , Liquid

372 chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS), Liquid

373 chromatography mass spectrometry (LC-MS), Immunofluorescence (IF), Amyotrophic lateral

374 sclerosis (ALS).

Research Question	Techniques	Main Findings	Reference
The effect of different environmental exposures (metal-rich particulate matter, air benzene levels and traffic derived elemental carbon levels) on mitochondria	Pyrosequencing qRT-PCR	Increased exposure to particulate matter increases *MT-RNR1* and *MT-TF* gene methylation Increased *MT-RNR1* methylation is associated with a significant increase in mtDNA copy number.	[85]
The effect of mtDNA methylation in the mitochondrial D-Loop on gene expression in colorectal cancer cells.	Methylation-specific PCR Western blotting	An increased level of demethylated sites in the D-Loop of tumour cells is strongly associated with increased *MT-ND2* expression and mtDNA copy number.	[47]
The effect of methylation in the D-Loop, *MT-ND6* and *MT-CO1* on disease progression in simple steatosis (SS) and non-alcoholic steatohepatitis (NASH)	Methylation-specific PCR qRT-PCR	Increased *MT-ND6* methylation and decreased *MT-ND6* protein levels in NASH compared to SS. Physical activity reduced *MT-ND6* methylation in NASH.	[86]
The effect of decreased S-adenosylmethionine (SAM) on mtDNA methylation in Down's Syndrome lymphoblastoid cells	LC-ESI-MS LC-MS/MS	Decreased SAM availability in Down's syndrome lymphoblastoid cells reduces methyl uptake to mitochondria and leads to mtDNA hypomethylation.	[87]
The tissue specificity of DNMTs and 5-mC in the mitochondria in relation to ALS models.	IF Pyrosequencing	Increased methylation at six cytosine sites in the *16S rRNA* gene in the spinal cord of an ALS mouse cell line. Reduced levels of mtDNMT3a protein in skeletal muscle and spinal cord early in disease.	[88]
The effect of mtDNA methylation on mtDNA copy number in gastric cancer.	qRT-PCR Pyrosequencing	Reduced mtDNA copy number levels in late clinicopathological stages. Demethylation of mtDNA increases mtDNA copy number.	[89]

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Table 2: A summary of the major issues and potential solutions in the field of mitochondrial epigenetics. Abbreviations: nuclear mitochondrial pseudogenes (NUMTs), fluorescence-activated cell sorting (FACS), laser capture microdissection (LCM).

Caveat	Potential Issues	Potential Solutions
Genetic Issues	~~Wrongful~~ Incorrect determination of pseudogenes as mtDNA affects the validity of results.	1. Isolate mitochondria before mtDNA extraction to avoid nuclear contamination 2. Specific primers designed with the consideration of NUMT amplification [90]. 3. BLAST search to identify known NUMTs
	Genetic mutations in mtDNA may have specific associated methylation signatures.	Haplogroup and heteroplasmy studies should consider mtDNA methylation as a potential variable
Cell Specificity and Technical Issues	Different brain regions have differential methylation patterns and different cell population compositions.	1. Larger samples sizes in specific brain subregions will improve statistical significance 2. FACS or LCM to separate cell types such as glia and neurons prior to analysis.
	Reduced methylation levels in mitochondria and variation in mtDNA copy number may increase noise and dilute signals.	Comparative analysis of techniques for their suitability to mitochondrial methylation studies should be considered.
	Bisulfite based methodologies cannot distinguish between 5-mC and 5-hmC.	Using oxidative bisulfite-sequencing allows for the distinction of 5-mC and 5-hmC at single base resolution[91].

REFERENCE ANNOTATIONS

- Bellizzi et al., 2013 –reported both 5-mC and 5-hmC in mtDNA at both CpG and non-CpG sites. The study also found that inactivation of DNMT1, DNMT3a and DNMT3b reduced CpG methylation levels markedly, but failed to impact non-CpG methylation to the same extent. As such, this study poses the question as to whether DNMT activity is important for mitochondrial methylation, or whether other factors may also be important.

- Chestnut et al., 2011 – found that DNMT3a was localized in the mitochondria of motor neurons, potentially indicating tissue-specific localization of this methyltransferase. This study also found 5-mC in mitochondria *in vivo*, suggesting that mitochondrial methylation may play a role in motor neurons.

- Dzitoyeva et al., 2012- found that the global levels of 5-hmC in mtDNA show an age-associated decrease in murine frontal cortex and that this was inversely correlated with the expression of some mitochondrial genes, suggesting a potential role of mtDNA methylation in aging.

- Lunnon et al., 2014 – used Illumina Infinium 450K methylation beadarray to demonstrate DNA methylation changes in AD cortex.

- Manczak et al., 2004 –investigated expression levels of mitochondrial-encoded ETC genes in AD, reporting decreased expression of complex I and increased expression of complex III and IV in early and late-stage disease.

- Shock et al., 2011 – reported 5-mC and 5-hmC in mtDNA, leading to a resurgence of interest in the field of mitochondrial epigenetics. This paper also identified an isoform of DNMT1, mtDNMT1, in the mitochondria.

ACKNOWLEDGMENTS

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