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

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SUMMARY

Considerable evidence suggests that mitochondrial dysfunction occurs early in Alzheimer's disease, both in affected brain regions and in leukocytes, potentially precipitating neurodegeneration through increased oxidative stress. Epigenetic processes are emerging as a dynamic mechanism through which environmental signals may contribute to cellular changes, leading to neuropathology and disease. Until recently little attention was given to the mitochondrial epigenome itself, as preliminary studies indicated an absence of DNA modifications. However, recent research has demonstrated that epigenetic changes to the mitochondrial genome do occur, potentially playing an important role in several disorders characterized by mitochondrial dysfunction. This review explores the potential role of mitochondrial epigenetic dysfunction in Alzheimer's disease etiology and discusses some technical issues pertinent to the study of these processes.

Alzheimer's disease (AD) is a chronic, currently incurable, neurodegenerative disorder, accounting for more than 60% of dementia cases, with current estimates predicting more than 135 million dementia cases worldwide by 2050 [1]. The classic neuropathological hallmarks associated with AD include the formation of amyloid beta (Aβ) plaques and neurofibrillary tangles. These are suggested to play a role in the further development of other characteristics of the disease, such as disruption of calcium homeostasis, loss of connectivity, the generation of reactive oxidative species (ROS) and altered plasticity, ultimately leading to neurodegeneration [2-6]. Mitochondrial dysfunction is a consistent feature of AD pathology in both the brain and white blood cells [7-10] although the molecular mechanism(s) mediating this phenomena are yet to be fully elucidated.

Mitochondrial dysfunction: a prominent feature of AD

Being the site of ATP generation, mitochondria provide the cell with the energy required to properly function; as such they are often described as 'the powerhouse of the cell'. Mitochondria are cylindrical organelles containing ~16.6kb of DNA (mtDNA) [11], which is separate to the nuclear genome and inherited in a maternal, non-Mendelian fashion. The mitochondrial genome consists of 37 genes, 13 of which encode for polypeptides required for the electron transport chain (ETC) (Figure 1), in addition to two ribosomal RNAs and 22 transfer RNAs. The mitochondria play a vital role in a variety of key biological functions, including apoptosis via caspase dependent and independent mechanisms [12], the

regulation of calcium homeostasis [13, 14] and the production of ROS [15]. For these reasons, mitochondrial dysfunction has been implicated in the pathogenesis associated with AD [16, 17] and forms the basis of the mitochondrial cascade hypothesis [18]. Proposed by Swerdlow et al, this hypothesis states that an individual's genetic code will determine their basal mitochondrial function and that, throughout ageing, this function will decline due to a combination of genetic and environmental factors, determining an individual's time of disease onset [18].

Mitochondrial-encoded ETC gene expression has been shown to be altered in both early and late stages of AD, with decreased expression of complex I and increased expression of complexes III and IV [7]. Increased expression of mitochondrial-encoded ETC complex genes has also been associated with aging, with increased expression of complexes I, III, IV and V in 12- and 18-month wild-type mice compared to 2-month mice, which was accompanied by increased oxidative damage [19]. However decreased expression of these genes was seen in older, 24-month old mice. Further evidence for a role of mitochondria in AD pathogenesis comes from a study demonstrating increased levels of mitochondrial gene expression and oxidative damage in a transgenic Amyloid Precursor Protein (*APP*) mutant mouse model of AD [20]. In addition, various components of the mitochondrial permeability transition pore (mPTP), which acts as a voltage-dependent channel regulating mitochondrial membrane permeability, have been shown to interact with Aβ in various murine models of AD. For example, one recent study found that, in *APP* transgenic mice, Aβ acts to upregulate VDAC1, a component of the mPTP, leading to mPTP blockade. [21]. Interestingly, this study also reports that VDAC1 may interact with hyperphosphorylated tau, suggesting another

mechanism of mitochondrial dysfunction. An earlier study found that Aβ present in mitochondria interacts with CypD, another component of the mPTP, in cortical samples from post-mortem AD patients and *mAPP* transgenic mice [22]. In the mouse model, this was shown to lead to increased ROS production and neuronal cell death. Taken together, this illustrates how mitochondrial-encoded gene expression is altered in AD, a variety of mechanisms by which Aβ interacts with mitochondria in AD, and how mitochondrial dysfunction can lead to changes associated with AD, thus highlighting the need for continued research into the field.

Epigenetics and AD

Given the high heritability estimates for AD [23], considerable effort has focussed on understanding the role of genetic variation in disease etiology, although more recently it has been hypothesized that epigenetic dysfunction may also be important [24]. A number of studies have shown reduced global levels of the DNA modifications 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in AD brain [25-28] with only a handful of studies have looked at changes occurring at specific loci (reviewed in [24]). Recent methodological advances in microarray and genomic sequencing technologies have enabled researchers to undertake epigenome-wide association studies (EWAS) in AD brain, identifying several consistent differentially methylated regions (DMRs) associated with disease [29-31]. Many of these DMRs are tissue-specific, restricted to regions of the brain associated with AD pathology, and correlate strongly with quantitative measures of neuropathology. As such, a strong case is being built for a role of epigenetics in the etiology of AD.

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.

Recently, a DNMT isoform, mitochondrial DNMT1 (mtDNMT1), has been found to contain a mitochondrial targeting sequence allowing it to bind to the D-loop of the mitochondrial genome, which contains the promoter sites for both the light and heavy strand of mtDNA and can therefore influence mitochondrial gene expression by altering transcriptional activity [36]. Furthermore, it has been suggested that the presence of these methyltransferases in mitochondria may be tissue-specific. Although Shock *et al*, did not observe mitochondrial localization of DNMT3a in the two cell lines they investigated, a later paper has found that DNMT3a is present, and in higher levels than mtDNMT1, in the mitochondria of motor neurons [42]. This study also demonstrated significantly higher global levels of both mitochondrial DNMT3a and 5-mC in Amyotrophic Lateral Sclerosis (ALS) motor neurons *in vivo*, suggesting a potential role for mtDNA methylation in motor neurons. DNMT1 and DNMT3b have also been observed in the mitochondria, with their inactivation reducing methylation at CpG sites [43].

Recently, it has been debated whether 5-hmC is just an intermediary product of the demethylation process of 5-mC to cytosine or could represent an independent epigenetic mark [44]. Growing evidence now suggests that 5-hmC could be a mark in its own right, produced from the conversion of 5-mC by TET1, TET2 and TET3 [45], with both TET1 and TET2 being present in the mitochondria [43]. Taken together with the presence of 5-hmC in the mitochondrial D-Loop [36], this strengthens the evidence suggesting that demethylation pathways are not only important in nuclear epigenetics, but may also play a role in the mitochondria. Furthermore, recent evidence suggests that 5-mC and 5-hmC exist stably within mtDNA at cytosines not preceding a guanine base, suggesting a role for non-CpG

methylation in mtDNA [46]. Further, CpG and non-CpG methylation has been observed in the mitochondrial D-loop at conserved regions associated with DNA-RNA hybrid formation during transcription, suggesting that DNA methylation in mitochondria shares similarities with plants and fungi and that this methylation may play a role in regulating mtDNA transcription and replication in a cell type-specific fashion [43].

MtDNA modifications in disease

Despite little being known about the physiological impact of variation in mtDNA methylation, some recent studies have shown that it may be associated with a variety of diseases. The majority of studies have focussed on diseases where mitochondrial dysfunction is known to be prevalent, for example in cancer, which has been previously linked with mitochondrial dysfunction [47] and more recently in Down's Syndrome, where mitochondrial abnormalities have also been reported [48]. Particularly, for the purpose of this review, mitochondrial dysfunction and mtDNA methylation aberrations in Down's syndrome cells (see Table 1) are interesting given that these patients have an increased likelihood of presenting with AD-like phenotypes throughout aging [49, 50] due to possessing an extra copy of *APP*. An overview of studies of mtDNA epigenetics in disease is given in Table 1.

MtDNA modifications: evidence for a role in AD and aging

Until recently the role of mtDNA modifications in AD has been largely ignored, despite the evidence that mitochondrial dysfunction is involved in AD [18] and that ncDNA methylation

differences are associated with the disease [29-31]. At a global level, an initial dot blot study showed some evidence for increased mitochondrial 5-hmC in AD superior temporal gyrus tissue, although definitive conclusions could not be drawn given the small number of samples used [51]. Mitochondrial DNA modifications in the brain have been shown to be associated with aging, with global mtDNA 5-hmC levels reduced in the frontal cortex of aged mice and specifically decreased 5-hmC levels being found in the regulatory D-Loop, as well as in two genes encoding ETC complex I polypeptides (*MT-ND2* and *MT-ND5*) [52]. Aging was not only found to be associated with overall decreased mtDNA 5-hmc levels but also with increased cortical expression of the mitochondrial ETC genes *MT-ND2, MT-ND4, MT-ND4L*, *MT-ND5*, and *MT-ND6* [52]. A post-mortem study of frontal cortex described differential mtDNA gene expression of these genes, and other mitochondrial-encoded genes, in both early and late-stage AD. [7] Taken together, these findings illustrate that alterations in mitochondrial-encoded genes do occur with aging and in age-related diseases, yet without further studies, the exact role of mtDNA methylation on mitochondrial gene expression in these instances remains uncertain.

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

As research into the field of mitochondrial epigenetics gains momentum, studies have focused on a potential *trans*-acting role of mtDNA in the epigenetic regulation of ncDNA, whereby covalent modifications across the mtDNA genome may affect not only the expression of a gene in *cis*, but also have *trans*-acting effects on the transcription of genes in the nuclear genome. Evidence for this is provided by cybrid models, which combine the

nuclear genome of one source with the mitochondrial genome of another in an attempt to determine the functional role of the mtDNA. Using Restriction Landmark Genomic Scanning (RLGS) and Rho^0 cells, a form of cybrid cell line designed for investigating mtDNA depletion, one study found that mtDNA depletion significantly altered DNA methylation at CpG islands in nuclear encoded genes [53], indicating that there are functional interactions between the two genomes. Re-introduction of wild-type mtDNA restored DNA methylation levels, at some RLGS spots, suggesting that, at least for some genes, mitochondria may play a role in nuclear DNA methylation. This is corroborated by a recent study demonstrating that mitochondrial haplotype variation can affect ncDNA methylation, with mtDNA haplotype J exhibiting higher global DNA methylation levels, reduced ATP, and overexpression of the nuclear gene methionine adenosyltransferase I, alpha (*MAT1A*), which is required for SAM production thus regulating methylation patterns in the nuclear genome [54]. Therefore genetic variations in mtDNA are capable of influencing epigenetic modifications in both the mitochondrial and nuclear genomes. As such, it is possible that mitochondrial dysfunction in AD could lead to alterations in mtDNA methylation, affecting nuclear gene expression.

The mitochondria comprises approximately 1500 proteins, however of these, only 13 are encoded by the mitochondrial genome; the remainder are encoded by the nuclear genome and imported into the mitochondria. A recent study found that >600 of these genes have tissue-specific differentially methylated regions, ultimately leading to changes in mitochondrial function dependent upon tissue type [55]. This suggests that there is an additional level of complexity to consider in the study of mitochondrial epigenetics,

whereby epigenetic changes in one genome may affect transcriptional control in another in a tissue-specific manner.

Interrogating the mitochondrial epigenome: technical caveats

Despite the potential importance of mitochondrial DNA modifications in AD, there are a number of technical challenges specific to interrogating the mitochondrial epigenome that have hampered widespread studies to date. These issues can be broadly summarized as encompassing genetic issues and specificity issues, which are outlined briefly with potential solutions in Table 2.

1. Genetic issues

1.1. Nuclear pseudogenes

By far the greatest concern when analyzing mtDNA methylation arises from regions of homology between the mitochondrial genome and nuclear mitochondrial pseudogenes (NUMTs). These genes are nuclear paralogs of mtDNA which have been translocated and inserted into the nuclear genome during evolution of both genomes [56]. This phenomena has been shown to be evolutionarily conserved across many species including cats [57], mice, chimpanzees, rhesus macaques [58] and hominins [59]. These insertions were thought to typically occur in non-coding regions; however, more evolutionary recent translocations have actually been shown to prefer integration into coding regions, thus leading to potential alterations in gene function with implications for disease [60]. NUMTs are generally small and typically comprise ~0.1% of the nuclear genome [61]. However in humans, it has been shown that some NUMTs can be as large as 14.7kb, representing a

significant portion of the ~16.6kb human mitochondrial genome [62]. As such, the presence of NUMTs can cause major issues in genomic analyses using pre-sequencing enrichment methods such as custom capture or long-range PCR as the likelihood of NUMT co-amplification, or even preferential amplification, increases due to the strong sequence similarity between the two segments of genome [63]. As such, this sequence similarity can lead to the misclassification of NUMTs as mtDNA during analysis, and has led to a number of publications wrongly describing NUMTS as mtDNA [64, 65]. NUMT misclassification has also been observed in AD genetic studies whereby amplification of the NUMT sequence has led to false heteroplasmies (see below) being reported [66, 67]. One potential solution is to separate mitochondria prior to DNA extraction in an attempt to reduce the risk of contaminating the mitochondrial and nuclear genomes. However, despite extensive research being dedicated to mtDNA analysis, existing methods for mitochondrial isolation and mtDNA extraction via the use of fractional precipitation or gradient ultracentrifugation remain time consuming and labour intensive [68] and often leave residual nuclear DNA contamination following mitochondrial isolation [69].

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

Each mitochondrion contains between 2-10 copies of mtDNA. However, not all mtDNA in each mitochondrion share the same DNA sequence. Indeed, mutations in some copies of mtDNA mean that the cell itself may be made up of a mixture of different sequences. This phenomenon is known as mitochondrial heteroplasmy and has been linked to various mitochondrial diseases, [70]. It is a potential confounder in studies of mitochondrial 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

clear tissue-specific epigenetic differences across brain regions [74, 75]. There is an added level of complexity with respect to the mitochondrial epigenome because each mitochondrion contain between 2-10 copies of mtDNA and each cell contains varying levels of mitochondria; therefore the amount of mtDNA copies in each cell can vary between 100-10,000 dependent upon cell type. In neurodegenerative diseases such as AD, the issue becomes more complicated in that the disease itself is characterized by the loss of neuronal cells and the activation of glia, a process that has been associated with changes in mitochondrial morphology and fission [76, 77]. A recent study using laser capture microdissection demonstrated that alterations in mitochondrial 5-hmC are seen with age in dissected mouse cerebellar purkinje cells, which was not evident in whole cerebellar tissue [52], demonstrating the importance of cell-specific analyses in heterogeneous tissue, particularly when investigating functional impact.

Currently, the most common method of measuring DNA methylation is via the conversion of DNA with sodium bisulfite followed by subsequent sequence analysis. However, these approaches are unable to distinguish between 5-mC and 5-hmC [78], an important limitation given recent studies confirmed the presence of 5-hmC in mitochondria in brain tissue [52]. Studies have found that although both DNA modifications are present in the mitochondria, they occur at much lower levels compared to in ncDNA [36, 51], and thus methods used for quantification may need to be more sensitive. Furthermore, variation in mitochondrial copy number may lead to the dilution of signals and reduce detection if the tissue is largely heterogeneous. Importantly, the mitochondrial genome is not interrogated using tools such as the Illumina Infinium 450K methylation array, the current gold standard

for methylomic analyses in large numbers of samples; thus methods for detecting mtDNA modifications across the entire mitochondrial genome are largely restricted to antibody-based enrichment, such as MeDIP-Seq, which may be less sensitive for detecting low levels of modified cytosine and does not interrogate methylation levels at single base resolution [79].

Future Perspective: the potential for biomarkers in AD

Two important goals of research into the etiology of AD are a) a fast, non-invasive, inexpensive and reliable biomarker and b) an effective treatment that targets the underlying neuropathology. A potential utility for DNA methylation biomarkers has been proposed for diseases in which traditional biomarkers are either too expensive, invasive, unspecific or insensitive for clinical purposes [80]. Epigenetic modifications have been widely studied in a variety of different cancers and other conditions such as preeclampsia to check for their suitability as prognostic and/or diagnostic biomarkers [81-83]. Differential methylation of mtDNA has yet to be examined with respect to its potential utility as an AD biomarker, but certainly warrants further investigation.

Conclusion

With mitochondrial epigenetics only recently emerging as a focus for biomedical research, the role of the mitochondrial epigenome in AD has yet to receive much attention. However, it is possible that deregulation of the mitochondrial methylome may lead to aberrant 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.

- MtDNA is not tightly wrapped by histones and is instead condensed by nucleoids, suggesting methylation could play an important role in gene regulation.
- DNMT1 can bind to the D-Loop of the mitochondrial genome and can influence gene expression.
- MtDNA methylation occurs at both CpG sites and non-CpG site in the mitochondrial genome.

MtDNA methylation: a key player in AD?

- Very few empirical studies have examined the role of mtDNA methylation in brain.
- Decreased mtDNA 5-hmC levels and increased expression of some mitochondrial-encoded genes has been seen in the pre-frontal cortex of aged mice.

Technical caveats

- NUMT misclassification has been observed in AD genetic studies whereby amplification of the NUMT sequence has led to false heteroplasmies being reported.
- MtDNA methylation could be altered in different mitochondria, creating a methylomic heteroplasmy.
- MtDNA methylation patterns could be cell-specific and is an important consideration when investigating heterogeneous tissues such as brain.

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].

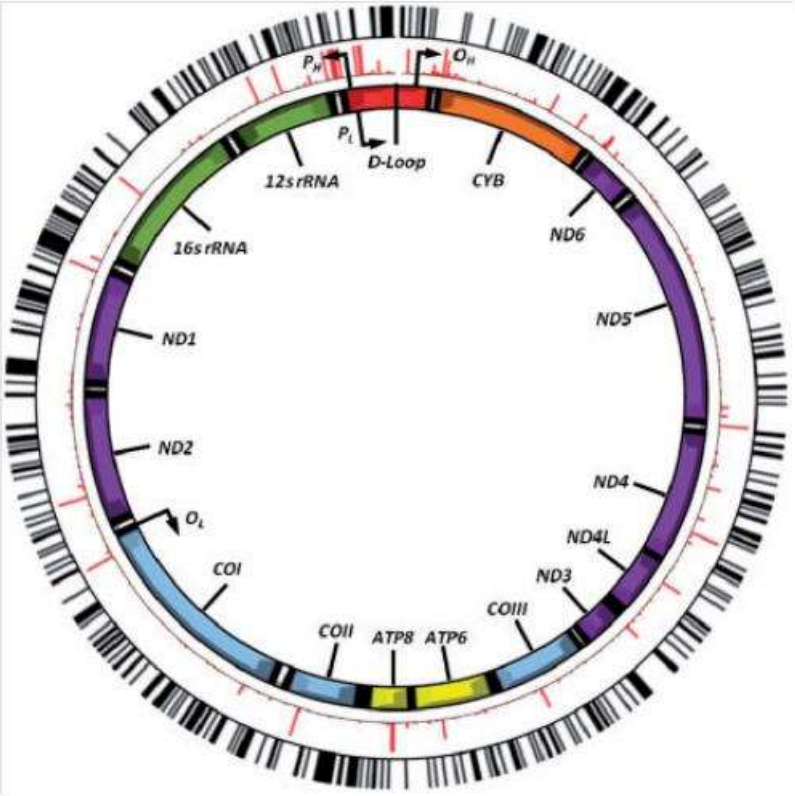

Table 1: An overview of current studies of mitochondrial epigenetics in disease. Abbreviations: Quantitative Real-Time PCR (qRT-PCR), mitochondrial DNA (mtDNA), simple steatosis (SS), non-alcoholic steatohepatitis (NASH), S-adenosylmethionine (SAM) , Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS), Liquid chromatography mass spectrometry (LC-MS), Immunofluorescence (IF), Amyotrophic lateral 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]

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.

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