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Title page

Potential Mechanisms of Mitochondrial DNA mediated Acquired Mitochondrial Disease

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1. Abstract

Mitochondria are cellular organelles which contain mitochondrial DNA (MtDNA) in the form of an extranuclear genome. MtDNA can be present in 100s to thousands to copies per cell in the body depending on the bioenergetic requirements of the host cell. MtDNA encodes subunits of the electron transport chain and therefore is required to produce cellular energy in the form of ATP. However MtDNA can also act as an inflammatory molecule since it resembles bacterial DNA, resulting in activation of pathways leading to enhanced cytokine production and chronic inflammation. In the current chapter, we suggest that MtDNA mediated mechanisms that cause systemic mitochondrial dysfunction are involved in many common diseases not traditionally recognised as mitochondrial disease, and we suggest that such disorders could be considered as “acquired mitochondrial diseases”, distinct from primary and secondary mitochondrial disease. Acquired mitochondrial diseases include cardiovascular and neurodegenerative disease as well as diabetic complications, and are associated with oxidative stress and sterile/chronic inflammation in their pathophysiology. We propose a mechanism of how systemic damage to MtDNA, mediated through oxidative stress, can cause inflammation and bioenergetic deficit. Some recent evidence of the proposed mechanism is provided for diabetic nephropathy, a complication of diabetes, which has not traditionally been regarded as a disease of mitochondrial dysfunction. According to the hypothesis proposed, it may be possible to use MtDNA levels in body fluids or cells to predict risk of acquired diseases of mitochondrial dysfunction, and to design novel therapies targetting the specific MtDNA mediated pathways.

2. Introduction

Mitochondria are double-membrane bound organelles found in all human cells, with the exception of erythrocytes. Mitochondria are often described as the 'powerhouses of the cell' since they produce the majority of cellular energy via oxidative phosphorylation (OXPHOS), a process involving the transfer of electrons along a series of protein complexes in the inner mitochondrial membrane. The process of electron transport results in the production of some reactive oxygen species (ROS) (Boveris *et al.*, 1972), which play an important role in normal intracellular signalling pathways (D'Autreaux and Toledano, 2007). In addition to cellular energy production, mitochondria play many other roles in the cell including homeostasis of cellular calcium, formation of iron-sulphur clusters and regulation of the intrinsic apoptosis pathway. Given their importance in maintaining normal cellular function, it is perhaps no surprise that mitochondria have become a focus of increased attention in terms of their potential impact on the pathogenic mechanisms of human disease.

It is believed that mitochondria originate from the integration of a eubacterium into a host cell, resulting in what we now call eukaryotic cells. Through reductive evolution, the majority of genes were transferred from the eubacterial genome to the nucleus of the host, however a small DNA genome remained within the mitochondrial organelle. The mitochondrial genome (mtDNA) differs from the nuclear genome in terms of size, structure and mode of inheritance. With only 13 proteins encoded by mtDNA, the mitochondrion is under dual control relying on many other proteins encoded by the nuclear genome to function normally. Mutations in these genes can lead to mitochondrial dysfunction, manifesting at a cellular level in various ways, including OXPHOS deficiency, reduced mtDNA copy number or disruption of the mitochondrial network. The clinical symptoms associated with mitochondrial dysfunction fall under the umbrella term of 'mitochondrial disease', which encompasses a clinically and genetically diverse group of conditions with systemic effects on the body's cells and organs (**Figure 1**). These are broadly categorised as either primary or secondary mitochondrial disease, however there is some overlap regarding the definition of these two main groups. In addition, mitochondrial dysfunction has been postulated to play a key role in many other common diseases, which were not traditionally

thought to be mitochondrial disease (Wallace, 1999). To distinguish these from primary or secondary mitochondrial disease, here we will refer to them as “acquired mitochondrial disease” and they are the main topic of the current review (**Table 1**).

Primary mitochondrial disease is usually associated with inherited mutations in mtDNA or nuclear DNA which result in impaired OXPHOS (Niyazov *et al.*, 2016). Pathogenic mutations can occur in genes encoding structural subunits of the OXPHOS system, such as the complex I subunits *MT-ND1* in the mtDNA or *NDUFS1* in the nuclear DNA. In addition, there are approximately 1500 nuclear genes encoding proteins indirectly affecting OXPHOS via processes like mtDNA replication, which have been implicated in a number of clinical syndromes. The prevalence of primary adult mitochondrial disease due to nuclear and mitochondrial DNA mutations is estimated at 1 in 4300 (Gorman *et al.*, 2015), although accurately estimating this is challenging due to the genetic and clinical heterogeneity of this group of diseases. Clinically, these conditions can affect any tissue with onset at any age, making diagnosis and management extremely challenging. Furthermore, there is little consistency between the clinical phenotype and the underlying mutation. For example, the m.3243A>G mutation is associated with a severe neurological condition termed MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes), but can also manifest as a much milder CPEO (chronic external ophthalmoplegia) phenotype. Secondary mitochondrial disease arises due to mutations in “genes encoding neither function nor production of the OXPHOS proteins” (Niyazov *et al.*, 2016). These mutations can be inherited, as in the case of Friedreich’s ataxia where a mutation in the frataxin gene (FXN) leads to dysregulation of iron homeostasis (González-Cabo and Palau, 2013). A detailed description of primary and secondary mitochondrial disease is beyond the scope of this chapter and the reader is referred to an excellent recent review by Gorman *et al* (2016). In addition to primary and secondary mitochondrial disease, it has been postulated that mitochondrial dysfunction plays a significant part in the pathogenesis of many common disorders including common metabolic disorders such as diabetes, cardiovascular disease, neurodegenerative diseases and cancers (Schon *et al.*, 2012; Picard *et al.*, 2016). A growing body of evidence implicates changes in MtDNA in cells organs and body fluids in such diseases of acquired mitochondrial dysfunction, including prevalent and chronic health conditions including neurodegenerative disease, obesity, cardiovascular disease, diabetes and its

complications, fatty liver disease, and also include complications arising from drug toxicity (reviewed by Malik & Czajka, 2013, Malik, 2017). Furthermore, emerging evidence indicates that the development of mitochondrial dysfunction can also contribute to chronic inflammation and therefore may also be involved in a number of inflammatory pathways (Boyapati *et al.*, 2017).

In the current chapter, we consider the role of MtDNA in the mechanisms of pathogenesis in acquired mitochondrial disease. Accordingly, we suggest that mitochondrial dysfunction plays a central role in many more diseases than traditionally recognised as mitochondrial disease. We propose that such disorders could be described as putative “acquired mitochondrial diseases” (**Table 1**) and we propose a mechanism of how systemic damage to MtDNA, mediated through processes such as oxidative stress, and contributing to chronic inflammation and bioenergetic deficit, may be involved in the processes leading to acquired mitochondrial disease. To illustrate how the proposed mechanisms may contribute to common diseases, we review some recent evidence using as an example one of the diseases shown in **Table 1**, namely diabetic nephropathy. Diabetic nephropathy has not traditionally been regarded as a disease of mitochondrial dysfunction.

3. The Mitochondrial Genome

In the eukaryotic cell, mitochondria are the only organelle, apart from the nucleus, to harbour a DNA genome. The nuclear genome comprises of linear DS DNA molecules, complexed with histone proteins in the form of chromosomes located within the nucleus, whereas the mitochondrial genome is a circular 16,569bp, double stranded molecule (Anderson *et al.*, 1981), present within the mitochondrial matrix as a protein-DNA complex of approximately 100nm length and known as a nucleoid (**Table 2**). Nucleoids, located at the inner mitochondrial membrane, are estimated to contain between 1.4 to 3 copies of mtDNA (Kukat *et al.*, 2011; Brown *et al.*, 2011) which is complexed with several abundant proteins. The proteins within nucleoids are involved in the mitochondrial replication and transcription machinery, for example the mitochondrial transcription factor A (TFAM), mitochondrial DNA helicase (TWINKLE), single-stranded DNA binding protein (mtSSB) and mitochondrial DNA polymerase (Poly) (Gilkerson *et al.*, 2013).

In total, the 16.5kb mtDNA contains 37 genes, encoding 22 transfer RNAs (mt-tRNAs), 13 hydrophobic proteins involved in the OXPHOS system and two ribosomal RNAs (mt-rRNAs). Most of these genes are located on the outer, guanine-rich heavy strand, whereas the inner,

cysteine-rich light strand holds only 8 tRNA genes and one protein-encoding gene (*MT-ND6*) (Andrews *et al.*, 1999). Unlike nuclear DNA, mtDNA is compact and contains no introns. One or two non-coding base-pairs separate genes, except in the case of *MT-ATP6* and *MT-ATP8*, where there is a region of overlap between these two genes. There is only one non-coding region, known as the D-loop. This region, extending approximately 1kb in human mtDNA, contains the major control elements for mtDNA transcription and is the site of origin for heavy strand replication (Kasamatsu *et al.*, 1971; Shadel and Clayton, 1997).

In terms of genome copy numbers, there is a vast excess of MtDNA relative to nuclear DNA in the human body. With the exception of erythrocytes, human cells contain multiple mitochondria, each carrying mtDNA. The number of mitochondria within a cell, and therefore the number of mtDNA copies, can vary depending upon the cellular energy requirements of the cell (Lightowlers *et al.*, 1997). For example a cardiac muscle cell may have around 7000 copies of mtDNA (Miller *et al.*, 2003), a white blood cell may have less than a hundred (Selak *et al.*, 2011) whereas oocytes may contain several hundred thousand copies (Shoubbridge and Wei 2007). The number of mitochondria can also change in response to the cell's physiological environment (Trinei *et al.*, 2006), consequently, the amount of MtDNA found in different cell types varies depending on the mitochondrial content and bioenergetic requirements of the cell, as well as the physiological status of the cell.

The inheritance of mtDNA follows a non-Mendelian pattern and is instead strictly maternally inherited (Giles *et al.*, 1980), although there is one documented case of paternal inheritance of mtDNA with a deletion in *MT-ND2* (Schwartz and Vissing, 2002), yet this has not been reported by others. Paternal mtDNA is thought to be eliminated by selective proteasomal degradation or autophagy post-fertilization, although degradation prior to fertilisation has also been reported (Luo *et al.*, 2013). Mutations in mtDNA are passed from mother to child, however there is great variability in the heteroplasmy levels and phenotypic expression across generations (Taylor and Turnbull 2005).

4. Mitochondrial DNA Content in different cells and organs

Organs and cells in the body have differing levels of mitochondrial content, reflected by their MtDNA content. Mitochondrial content in different cells and organs varies depending on the bioenergetic requirements of the cells, with organs requiring high amounts of energy usually having a higher mitochondrial mass, therefore it follows that with altered mitochondrial mass there will be altered MtDNA content. Currently there is little consensus in the literature on

MtDNA content is different cells and organs. We have previously shown that in the mouse, the heart contains the highest MtDNA content followed by the kidney (**Figure 2A, Malik *et al.*, 2016**), this observation correlates with earlier proteomic quantification of mitochondrial proteins which found the highest level of mitochondrial cytochrome C in the heart followed by the kidney (**Figure 2B, Pagliarini *et al.*, 2008**). The observation of high mitochondrial content in heart and kidney is further supported by the assessment of metabolic rate of different organs where two reports of very similar levels with heart and kidney showing the highest metabolic rates (**Figure 2C, McClave and Snider 2001; Wang *et al* 2010**).

Oxidative stress and acquired mitochondrial dysfunction

The key role of oxidative stress in the chronic disorders we designate as acquired mitochondrial disease has long been recognised and many therapeutic strategies are focused around the correction of redox impairment and scavenging of ROS (Halliwell and Gutteridge, 2007). The process of energy production via OXPHOS uses oxygen and metabolic substrates to produce energy in the form of ATP in mitochondria, and can directly result in oxidative stress in the cell, a condition in which the redox balance of the cell has shifted towards an oxidative state. It is largely accepted that oxidative stress is a common feature in many diseases including diabetes complications, cardiovascular disease, neurodegenerative disease, cancer, renal disease and others.

OXPHOS involves the oxidation of nutrients by oxygen, mediated via the transfer of electrons through protein complexes known as the electron transport chain in the inner mitochondrial membrane. However, not all of the oxygen being used during OXPHOS is utilised to produce ATP, since ~1-3% is converted into free radicals, known as reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007). ROS are highly reactive molecules with unpaired electrons and can oxidise and impair the function of cellular DNA, proteins and lipids. In the case of DNA, ROS damage can cause mutations, deletions and oxidation. Since MtDNA is found located close to the electron transport chain, it is more likely to incur ROS-induced damage than nuclear DNA.

Under normal physiological conditions, most cells use cellular proteins such as glutathione peroxidase, catalase, and superoxide dismutase (Nordberg and Arnér, 2001) as antioxidants to sequester ROS by accepting electrons and becoming oxidised. These endogenous antioxidant proteins are highly abundant in cells and are recycled by donating their electrons to chains of acceptors such as reduced nicotinamide adenine dinucleotide phosphate

(NADPH) (Rydström, 2006). However, in conditions of chronic ROS production, the cell's ROS levels exceed their detoxification and cause a shift in the redox balance resulting in oxidative stress. Free radicals which escape the cells' antioxidant response are highly reactive molecules which oxidise proteins, lipids and DNA molecules within the cell, leading to altered properties and cellular damage. Many common drugs cause mitochondrial oxidative stress (reviewed by Mehta et al., 2008), and many common diseases such as diabetes and its complications, cancer, neurodegenerative disorders as well as ageing have been shown to have redox impairment (Halliwell and Gutteridge, 2007; Wallace, 1999; Ying, 2008).

Mitochondria are directly affected by oxidative stress, which can impair their functioning at different levels, both by direct damage to MtDNA, mitochondrial lipids and proteins, and also indirectly by altering cellular signalling. Oxidative stress induced MtDNA damage is a central concept in the "free radical theory of ageing", which proposes that oxidative stress and ROS directly lead to damage to MtDNA in the form of single mutations or deletions.

Mutations and damage to MtDNA are clearly involved in ageing and several acquired diseases, although damage to MtDNA repair and replication errors are also likely to be involved in MtDNA damage rather than direct ROS induced damage.

Abnormal signalling has been shown to result in an adaptive response through enhanced production of mitochondria (Michel et al., 2012). We have shown that oxidative stress induced by hyperglycemia can cause an increase initial increase in MtDNA, however in cells with oxidative stress the increased MtDNA is not functional as such cells show decreased viability, decreased cellular respiration, and decreased transcription of MtDNA (Czajka et al, 2015). The effect of the shifted redox balance can also impact on mitophagy, the degradation and removal of damaged mitochondria. Reduced removal results in the accumulation of damaged mitochondria (Kim et al., 2007) as is the case for diabetes where blockage of the electron transport chain at complex III results in accumulation of excess ROS (Giacco and Brownlee, 2010; Newsholme et al., 2007). As MtDNA is located close to the source of ROS production, the DNA itself can become damaged resulting in accumulation of deletions and mutations (Croteau and Bohr, 1997).

Theoretically, the maternally inherited mitochondrial genome in an individual should be the same in all cell types unless it has been damaged via mutations or deletions either before or after conception, in which case cells may exhibit heteroplasmy in their mitochondria (Taylor & Turnbull 2005). Since most cells contain many copies of MtDNA, ranging from 100s to 1000s

depending on their bioenergetic requirements, a small percentage of damaged MtDNA may not have much functional impact, and further MtDNA repair mechanisms and mitophagy may suffice to contain any impact of such damage. However, once a functional threshold is crossed, damage to MtDNA could result in metabolic perturbation in the tissue. Cells need sufficient energy supplies for cellular processes and a fully functional ETC is crucial especially in tissues with high energy requirements such as the heart, kidney and the brain. Damage to MtDNA, which encodes 37 genes, all crucial for mitochondrial function, in the form of acquired mutations, could lead to energy deficit by affecting the function of the ETC (Wallace 1999). The inability to produce sufficient energy to carry out cellular processes is likely to have significant effects on cells, impairing cellular repair and growth and could lead to cell loss.

5. Inflammation and acquired mitochondrial dysfunction

It is increasingly being reported that MtDNA can directly cause inflammation in the body (reviewed by Malik & Czajka, 2013). Unlike eukaryotic nuclear DNA which is methylated, MtDNA resembles bacterial DNA in that it is largely un-methylated. Therefore, when MtDNA is “out of place”, i.e not contained within the mitochondria, for example in damaged cells where MtDNA may leak into the cytosol or into circulation, it can be recognised by the immune system as foreign DNA. Therefore MtDNA can cause immune responses for example via the activation of the intracellular Toll like receptor 9 (TLR) 9 which normally recognises exogenous bacterial DNA released from pathogen. (Zhang et al 2010). Activation of TLR9 by MtDNA can cause the activation of an inflammatory cascade via myeloid differentiation primary response gene 88 (MYD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and results in activation of cytokines such as TNF α leading to an inflammatory response which can persist in the absence of any exogenous infection (McCarthy et al., 2015). Indeed, MtDNA mediated inflammation may be the cause of phenomena described as “sterile inflammation”, where an enhanced inflammatory response occurs in the absence of infection (Chen and Nunez, 2010).

The diseases listed as “acquired mitochondrial diseases” (Table 1) share the feature of chronic or sterile inflammation since an enhanced inflammatory response ensues in the absence of any detectable pathogens or infection. There is overwhelming evidence showing a causal role of immune-inflammatory responses in cardiovascular disease where the presence of low grade inflammation in patients has been postulated to be causative of and to precede adverse

cardiovascular events (Welsh et al., 2017). There are many convincing studies using both animal models and human populations showing that in diabetes inflammation plays a significant role, and increased inflammatory markers are associated with diabetic nephropathy (Donate-Corea et al., 2014) and diabetic retinopathy (Semeraro et al., 2015). complications. Therefore it is tempting to speculate that inflammation present in these diseases may be mediated by MtDNA.

As well as playing a potential role in chronic inflammatory responses, the release of large amounts of MtDNA can result in acute inflammatory responses with serious consequences. For example, blocking of MtDNA degradation in cardiac cells led to heart failure in a mouse model (Oka et al., 2012). Cell free MtDNA levels have been shown to strongly correlate with risk of mortality in intensive care units and in patients with sepsis. (Nakahira et al., 2013; Yamanouchi et al., 2013).

6. Emerging evidence of MtDNA changes in a large number of chronic and other human diseases.

MtDNA lends itself to rapid detection and quantification via methods such as qPCR and digital PCR, making it an attractive high-throughput biomarker. MtDNA levels can be measured in human clinical samples and disease associated changes can be detected in populations. There is widespread interest in using MtDNA as a biomarker in human populations and in the last decade, the evidence for reported alterations in MtDNA in body fluids, cells, or of human subjects in correlation with many diseases has grown rapidly and strongly supports the view that alterations in MtDNA levels are associated with many human diseases. MtDNA levels have been measured in numerous types of human samples such as blood cells (e.g. lymphocytes, PBMCs), body fluids (urine, plasma, serum, saliva, cerebrospinal fluid), , and in tissues or biopsies (normal and diseased e.g. cancer). Alterations in MtDNA levels have been described in numerous different types of cancers, cardiovascular disease, diabetes and its complications and others. A detailed description of this rapidly growing evidence base is beyond the scope of the current chapter (Reviewed by Malik and Czajka, 2013) however it is clear that many human diseases are associated with measureable changes in MtDNA. Furthermore, there is increasing evidence showing a link between patient drug response and circulating MtDNA levels showing that there is strong potential for the future use of MtDNA in the field of personalised medicine (Malik, 2017).

7. Proposed mechanisms of Mitochondrial DNA mediated damage in acquired

mitochondrial disease.

In 2013 we proposed a novel hypothesis stating that acquired damage to MtDNA, distinct from inherited genetic mitochondrial disease, can result in mitochondrial dysfunction in a specific pathway involving an early increase in MtDNA levels (Malik and Czajka 2013; Czajka et al., 2015). According to this hypothesis, changes in MtDNA may impact on cellular health via two distinct but interconnected processes: (a) Bio-energetic deficit in cells caused by mutations in MtDNA leading to a defective electron transport chain resulting in cellular damage, and release of damaged MtDNA into circulation (b) Accumulation of damaged MtDNA in the cytosol (instead of within mitochondria) and of cell free MtDNA in circulation could lead to chronic inflammation. Therefore, this hypothesis predicts an early adaptive increase in MtDNA in response to oxidative stress, and subsequently a decrease in levels of MtDNA as well as damage to MtDNA, followed by increased inflammation and reduced bioenergetic capacity (see figure 3). In order to detect the changes predicted by this hypothesis, we have used experimental model systems and clinical samples for the disease diabetic nephropathy.

Diabetes results in increased risk of numerous other complications which affect major organs, including eyes (retinopathy), heart (diabetic cardiomyopathy), blood vessel (peripheral vascular disease) and brain (dementia) (Figure 1) and therefore resembles genetic forms of mitochondrial disease. Diabetic nephropathy is a kidney disease which affects approximately 30% of patients with diabetes. The disease, detected using microalbuminuria, the appearance of protein in the urine, develops over a period of clinical silence and can lead to gradual loss of renal function resulting in end stage renal failure. With the epidemic rise in the incidence of diabetes currently affecting more 350 million people worldwide, more than 100 million people are at risk of DN and approximately 30% of these are likely to progress to end stage renal failure despite therapy . Kidneys are organs requiring large amounts of energy in form of ATP due to the re-absorption processes and although their mass account of less than 1% of total body mass, they use almost 10% of the body's oxygen which is utilized in cellular respiration via OXPHOS, and therefore they are rich in mitochondrial content and MtDNA. We used human primary renal glomerular mesangial cells (HMCs) as well as patient samples to detect the changes predicted by the hypothesis described in Figure 3 (Malik and Czajka, 2013; Czajka and Malik, 2016). We showed that when HMCs are grown in diabetic conditions, the high glucose leads to increased ROS and at the same time we observed more than 200%

increase in cellular MtDNA (Figure 4A), however this MtDNA was damaged (Figure 4B) , and moreover continued growth in high glucose led to damaged cellular respiration in parallel with increased ROS (Figure 4E). We also found evidence of similar changes in blood samples taken from diabetes patients with and without diabetic kidney disease (See Czajka et al., 2015) and in patients with diabetic retinopathy, where circulating MtDNA changes correlated with inflammation (Malik et al., 2015). Specific MtDNA mutations can lead to diabetes and kidney disease in patients with mitochondrial genetic disease (Wallace et al 1999; Mazzaccara et al 2012; Seidowsky et al 2013; D'Aco et al 2013). These genetic forms of diabetes and kidney disease support our hypothesis as they show that MtDNA damage can cause these specific pathologies.

8. Concluding remarks

In the current chapter we have described potential mechanisms by which changes in MtDNA levels may mediate mechanisms of disease common diseases which we have termed acquired mitochondrial diseases. The proposed mechanisms centre around two attributes of MtDNA. Firstly, MtDNA encodes subunits needed for a functional electron transport chain in order to make cellular energy via OXPHOS, and secondly MtDNA is a potentially pathogenic molecule as it can elicit inflammatory responses in cells through its resemblance to bacterial or foreign DNA. Therefore, in diseases of oxidative stress, damage to MtDNA, initially causes a maladaptive response in the form of increased cellular MtDNA levels, and subsequently cause a bioenergetic deficit through impaired OXPHOS. Lack of cellular energy in cells results in release of damaged MtDNA which then would cause an inflammatory response. In diseases we describe as acquired mitochondrial diseases, both oxidative stress and inflammation have been strongly implicated. However targeted therapies to correct redox impairment and/or chronic inflammation have had limited clinical success. It is possible that a more targeted approach, which takes into account the potential timeline of events with early oxidative stress related changes and importantly, their impact on MtDNA, as well as later inflammatory event may be required. The goal therefore is to identify methods for early detection of mitochondrial dysfunction to allow intervention and treatment before irreversible damage to mitochondria in different regions of the body can lead to disease (Figure 5). Ideally, a biomarker which can detect mitochondrial dysfunction at an early stage is required. We

suggest the MtDNA has the potential to be such a biomarker.

Evidence for dysregulated MtDNA levels in numerous diseases continues to grow at a fast pace. Since the year 2000, almost 800 papers per annum have been published on some aspect of human MtDNA, and a large percentage of these relate to altered MtDNA in chronic disease in human body fluids or cells. Interestingly, our data suggests that early changes in MtDNA, caused by altered redox balance in the cell, may provide a predictive biomarker (Figure 4), since we found changes in MtDNA were detectable early, and preceded bioenergetic deficit.

Therefore one potential way to identify the stage of events is to use non invasive methods to measure MtDNA content in human subjects. However, although the wider availability of qPCR as a methodology has led to a substantial increase in publications reporting changes in MtDNA content in human body fluids and tissues, methodological issues remain. Protocols for accurately quantifying MtDNA in small volumes of body fluids remain variable and affected by problems. Such methodology issues have hindered the successful use of MtDNA as a biomarker and led to conflicting and unreproducible findings in many cases. Of particular note in this regard is the presence of nuclear mitochondrial DNA segments (NUMTs) in the nuclear genome which can skew data by co-amplifying nuclear genes when MtDNA levels are being assessed. In addition, assays currently in use seldom distinguish between cell free and cellular MtDNA, the former is of importance as it may be an indicator of inflammation, and the latter is important as it may be an indicator of bioenergetic deficit in the cell. Therefore there is a growing need to accurately measure MtDNA levels in peripheral blood, compartmentalised as PMBCs for cellular and plasma for cell-free, as well as in urine, compartmentalised as urinary pellet for cellular debris and cell free urinary supernatant, or other body fluids, such as saliva, semen, or cerebrospinal fluid. The field of MtDNA may hold the potential for the elucidation of many more diseases than traditionally thought of as mitochondrial genetic disease.

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Figures/Tables

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Figure 5 - Early detection of mitochondrial dysfunction to prevent chronic disease

Table 1: Primary, secondary and acquired mitochondrial disease

Group and definition	Definition	Example: Genes if known: Effects	References
Primary mitochondrial disease	Mostly inherited mutations which result in impaired OXPHOS	Leigh Syndrome: Progressive neurological disorder with infantile onset > 75 nDNA and mtDNA genes implicated. Affects thalamus, basal ganglia and brain stem.	Gorman <i>et al</i> , 2016
		Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS): mtDNA mutation m.3243A>G (MT-TL1) accounts for 80% cases. Affect many systems including brain, heart, eyes, ears, stature, balance.	
		Leber Hereditary Optic Neuropathy (LHON) – predominantly caused by three mt-DNA mutations in OXPHOS genes. Organ-specific condition associated with vision loss.	
Secondary mitochondrial disease	Mostly inherited mutations which affect mitochondrial function (not in genes encoding OXPHOS)	Friedreich’s Ataxia - Neurodegenerative condition caused by mutation in the Frataxin gene resulting in impaired iron metabolism. Cerebellar ataxia and cardiomyopathy are common symptoms.	González-Cabo & Palau, 2013
		Charcot-Marie-Tooth type 2A – Peripheral neuropathy associated with mutation in MFN2 gene involved in mitochondrial fusion dynamics.	Cartoni & Martinou, 2009
		Wilson’s disease – Mutations in ATP7B cause abnormal copper metabolism, resulting in a multifactorial condition primarily affecting the liver.	Wu <i>et al</i> , 2015
Acquired mitochondrial disease	Somatic mutations/deletions in MtDNA caused by environmental factors (e.g oxidative stress) affecting specific target tissues	Neurodegenerative Disease (e.g. non-familial forms of Alzheimer’s disease ¹ , Parkinson’s disease ² , multiple sclerosis and others)	¹ Wilkins & Swerdlow, 2016; ² Bose & Beal, 2016
		Cancer (e.g. colorectal cancer, breast cancer and others)	Chen <i>et al</i> , 2016
		Cardiovascular disease (e.g. heart failure, ischaemic heart disease)	Murphy <i>et al</i> , 2016
		Diabetes¹ and its complications (Diabetic nephropathy ² , retinopathy, and others)	¹ Morrow <i>et al</i> , 2017; ² Higgins & Coughlan, 2013
		Drug induced toxicity¹ (e.g HIV treatment induced toxicity ²)	¹ Walker, 2008; ² Gardner <i>et al</i> , 2013

Table 2 – Comparison of Human Mitochondrial and Nuclear DNA Genomes

	Mitochondrial DNA	Nuclear DNA
Structure		
Size (nt)	16,569	~30,000,000,000
Major Function	Information storage DNA replication and transcription, signalling	Information storage DNA replication and transcription
Organisation	Double-stranded circular DNA molecule complexed with TFAM	Double-stranded duplex linear DNA molecules (chromosomes) complexed with histones to form chromatin
Genetic code	Different use of start and stop codons	Universal
Replication	Bidirectional from a single origin of replication	Numerous origins of replication
Transcription	Poly-cistronic mRNAs from 2 promoters	Highly regulated and mostly individual mRNA transcription from thousands of individual promoters
Introns/exons	No introns, very few non-coding regions, contiguous and overlapping	Contain introns and large stretches of non-coding regions
Inheritance	Maternal	Bi-parental
Replication	Independent of the cell cycle	Dependent on the cell cycle
Number of copies per cell	10s to many 1000s –variable and can change in response to physiological stimuli	2 (1 in the case of the sex chromosomes)-fixed
Methylation	Resembles bacterial DNA (less methylated)	Methylated
Sequence identity	Contains very few regions which are unique (>90% is duplicated in the nuclear genome)	Contains variable regions known as NUMTs (nuclear mitochondrial DNA segments) which are identical to MtDNA scattered across most chromosomes

Figure 1. Systemic impact of mitochondrial disease. Common symptoms of mitochondrial disease are shown alongside examples of primary¹, secondary² and acquired³ mitochondrial diseases. Abbreviations: MELAS = Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes; CPEO = Chronic Progressive Ophthalmoplegia; LHON = Leber's Hereditary Optic Neuropathy; GI = gastrointestinal.

Figure 2: Mitochondrial content in different tissues. A) Mitochondrial DNA copy number in mouse tissue (Malik et al., 2016). B) Mitochondrial content, represented by cytochrome c levels, in mouse tissues (Pagliarini et al., 2008). C) Metabolic rates of human tissues and organs (McClave and Snider, 2001; Wang et al., 2010).

Figure 3: Proposed mechanism of mitochondrial DNA mediated damage in acquired mitochondrial diseases. A trigger such as hyperglycemia leads to increased intracellular reactive oxygen species (ROS). Under normal conditions, the cell's endogenous antioxidant response recovers the redox balance by scavenging the ROS. However, under conditions of chronic ROS, the cell's antioxidant response is overwhelmed resulting in oxidative stress. Increased intracellular ROS leads to increased mitochondrial biogenesis, an adaptive response, which can be detected by measuring Mt/N. Over time the oxidative stress would cause damage to mitochondrial membranes, proteins and DNA leading to mitochondrial dysfunction. Accumulation of damaged MtDNA would lead to an inflammatory response as MtDNA is un-methylated and resembles bacterial DNA. According to this hypothesis changes in Mt/N would precede mitochondrial dysfunction. Figure adapted from Malik & Czajka 2013.

Figure 4: Changes in cellular MtDNA precede metabolic dysfunction in conditions of oxidative stress. Growth of human mesangial cells (HMCs) in high glucose led to a significant increase in cellular MtDNA within 24 hours, (A) after 4 days ($n=12$, $P<0.001$), however the MtDNA was damaged as illustrated by reduced amplification of an MtDNA 8.6kb fragment (B). Cells showed normal bio-energetic profile at 4 days (C), however after 8 days, maximal respiration and reserve capacity were significantly reduced in hyperglycemia cells but unaffected in normoglycemic cells (D, E). See Czajka et al., 2015 for the full data.

Figure 5: Early detection of mitochondrial dysfunction to prevent chronic disease. Environmental/lifestyles triggers such as high fat and/or glucose or drugs can result in oxidative stress and altered signaling which in turn damages mitochondria in organs (e.g. kidney, heart, liver), cells (blood cells, adipocytes), and blood vessels, the damage may take decades to manifest itself and cause pathology. Identification of biomarkers for the early detection of the metabolic and bioenergetic changes associated with these pathologies could allow intervention and prevention of irreversible bioenergetic dysfunction.