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Review

Blood mitochondrial DNA copy number: What are we counting?

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ABSTRACT

There is growing scientific interest to develop scalable biological measures that capture mitochondrial (dys) function. Mitochondria have their own genome, the mitochondrial DNA (mtDNA). It has been proposed that the number of mtDNA copies per cell (mtDNA copy number; mtDNAcn) reflects mitochondrial health. The common availability of stored DNA material or existing DNA sequencing data, especially from blood and other easy-to-collect samples, has made its quantification a popular approach in clinical and epidemiological studies. However, the interpretation of mtDNAcn is not univocal, and either a reduction or elevation in mtDNAcn can indicate dysfunction. The major determinants of blood-derived mtDNAcn are the heterogeneous cell type composition of leukocytes and platelet abundance, which can change with time of day, aging, and with disease. Hematopoiesis is a likely driver of blood mtDNAcn. Here we discuss the rationale and available methods to quantify mtDNAcn, the influence of blood cell type variations, and consider important gaps in knowledge that need to be resolved to maximize the scientific value around the investigation of blood mtDNAcn.

1. Introduction

There has been a growing interest for mitochondria among biomedical disciplines including gerontology, epidemiology, environmental health sciences, biological psychiatry, cardiology, oncology, neurology and others. Owing to their documented involvement in multiple health disorders and in relation to aging (Berry and Kaeberlein, 2021; Ferrucci and Zampino, 2020), as well as their central role in biochemical pathways that determine cell life and death, mitochondria have become the most studied organelle across the biomedical sciences (Picard et al., 2016). Because mitochondria are living organelles, like other living creatures, measuring their function generally requires freshly collected cells and tissues. In contrast, DNA is an inert, stable molecule quantified in an often more accessible way, with excellent scalability on stored DNA, in banked samples, or in existing sequencing datasets. One DNA-based measure of mitochondrial biology is mitochondrial DNA copy number (mtDNAcn): the number of copies of the mitochondrial genome per nucleated cell (Filograna et al., 2020) (Fig. 1**A-B**).

The origins of mtDNAcn as a hallmark of dysfunction can be traced to the 1990′s (Moraes et al., 1991), where rare genetic disorders were linked to drastic reductions in mtDNAcn, termed mtDNA depletion syndrome (Moraes, 2001). These observations suggested that low mtDNAcn could be a cause of human disease. However, it was also noted in several genetic mitochondrial disorders that mtDNAcn was not reduced, but elevated (Bai and Wong, 2005; Wei et al., 2001). This likely reflects the upregulation of mtDNAcn to compensate for poor mitochondrial energetics, including in blood cells (Giordano et al., 2014) – a phenomenon termed *compensatory upregulation of mtDNAcn*. As discussed below, mtDNAcn can also change independent of mitochondrial energy production capacity, and it is now well-recognized that additional factors such as cell types and changes in energy requirements (whether cells are more or less metabolically active) can also influence mtDNAcn independent of health or disease states, further complicating the interpretation of mtDNAcn in patient populations.

Since the early notion that altered mtDNAcn could represent an indicator of aberrant mitochondrial health, mtDNAcn studies have expanded outside the primary mitochondrial literature (reviewed in (Malik and Czajka, 2013)). Studies leveraging large population-based datasets have since linked whole blood mtDNAcn to various phenotypes. Low blood mtDNAcn has been associated with neurodegenerative disease (Yang et al., 2021), cardiovascular disease (Ashar et al., 2017), and both cognitive and physical performance in aging (Mengel-From et al., 2014); whereas other disease conditions such as diabetes, major depression, some cancers, and mitochondrial disorders are associated with elevated mtDNAcn (Cai et al., 2015; Hagg et al., 2020; Kim et al.,

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2015). mtDNAcn has also been proposed to exhibit an inverted U-shaped relationship with the dose of exposure to environmental toxicants (Meyer et al., 2018), which have also been associated with either higher or lower mtDNAcn (Roubicek and Souza-Pinto, 2017). These intriguing, statistically robust associations have the potential to inform our models of human health and disease trajectories across the lifespan. Therefore, there is a pressing need to understand the biological significance of changes in blood mtDNAcn and the mechanisms linking those changes to health outcomes.

A major driver of the popularity of mtDNAcn as a potential marker of mitochondrial health lies in its ease of measure from stored DNA, or in novel methods developed to derive mtDNAcn from genotyping/sequencing data. Compared to direct assays of mitochondrial functions, the scalability of mtDNAcn assessments is appealing for biomarker studies.

2. A typical encounter between interdisciplinary and mitochondrial scientists

In the hallway between two departments or at a coffee break between conference sessions, discussions such as this one frequently occur:

Interdisciplinary scientist (IS): Recently there have been a lot of very interesting papers linking mitochondria to health outcomes and aging. How can we measure mitochondria in our latest epidemiological or clinical study?

Mitochondrial scientist (MS): Do you mean a specific mitochondrial function? There are very good assays of respirometry, which is a measure of oxygen consumption and energy production capacity. Or we could look at specific enzymatic activities of the respiratory chain, which would also get at energy production capacity. Or there are assays for mitochondrial reactive oxygen species production, and other forms of mitochondrial signaling. What is your target sample size?

IS: 1,000 people, and another 1,000 samples for the 2-year follow up.

MS: Ha! The throughput of those live assays is quite low and it's hard to run over 100 samples, so those will not be possible. What kind of samples will you be able to collect?

IS: The samples are already collected and have been in the freezer for 3 years.

MS: OK, so we probably cannot directly measure mitochondrial function.

IS: But we have frozen blood, and leftover extracted DNA used for other assays including telomere length and DNA methylation. In another study, we have also generated whole genome sequencing data.

MS: If you have stored DNA or sequencing data, we could measure mtDNA copy number. That is the number of copies of mtDNA per cell.

IS: That sounds interesting! What will that tell us?

MS: It's difficult to interpret on its own. But if you have DNA available, we can give it a try…

While illustrative, this theoretical conversation highlights the different research perspectives and constraints that may lead to focus investigations of mitochondrial health towards mtDNAcn from whole blood. While seeking maximal scientific value out of existing samples is laudable, the biological or mechanistic interpretation of mtDNAcn may be important to consider. Below we discuss some biological and

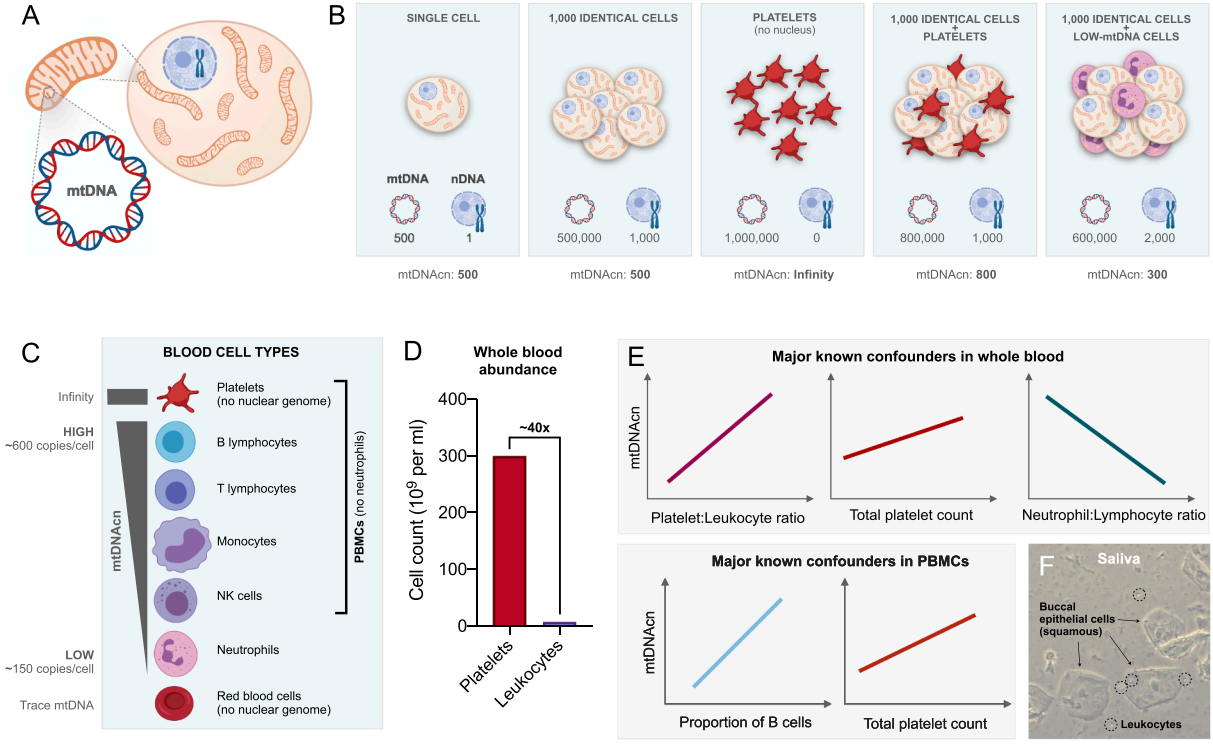


Fig. 1. Mitochondrial DNA copy number (mtDNAcn) and relevant cellular contributors in human blood. (**A**) Cell schematic with a nucleus (blue) and cytoplasmic mitochondria of various sizes and shapes. Most mitochondria contain one or more copies of mtDNA, all maternally inherited; each nucleus contains one pair of the nuclear genome, two copies or alleles, one from each parent. (**B**) The concept of mtDNA copy number (mtDNAcn), represented for different scenarios. Note that if all cells are identical, increasing the number of cells does not change the mtDNAcn. Platelets have mitochondria and mtDNA but do not have a nucleus, making it impossible to determine mtDNAcn in pure platelets (mtDNA copies divided by 0 = infinity). Contamination of white blood cells with platelets or cells with low mtDNAcn (e.g., granulocytes) can alter mtDNAcn. (**C**) Summary of mtDNAcn in different blood-derived cells. (**D**) Platelet count relative to all leukocytes in human blood (data from Rausser et al. 2021). (**E**) Summary of the direction and effect size of known influence of cell type composition for mtDNAcn in human blood, see text for details. (**F**) Micrograph of human saliva showing a similarly heterogenous cellular composition with several leukocytes (circled) and squamous buccal epithelial cells (arrows) that are shed from the inner lining of the cheeks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

technical considerations related to mtDNAcn measurements in human blood.

3. Why study mitochondria?

Dozens of recent clinical, epidemiological, cross-sectional and longitudinal studies have quantified mtDNAcn from existing genomic material (i.e., extracted DNA) originally collected for other purposes. In general, these secondary analyses aspire to quantify the association between *mitochondrial health (energy production capacity, dynamics, signaling, oxidative stress, and others aspects of mitochondrial behavior)* and one of the following categories of variables:

i) *Exposures*: to test the hypothesis that mitochondria are a target of psychosocial or stress exposures, behavioral interventions, environmental or drug exposures (e.g., (Cote et al., 2002; Tyrka et al., 2016)).
ii) *Downstream health outcomes*: to test the potential role of mitochondria as a predictor of future health outcomes, such as future incident cardiovascular disease or age-related decline (e.g., (Ashar et al., 2017; Mengel-From et al., 2014)).
iii) Other *biological or psychological measures*: to examine potential mechanistic pathways positioning mitochondrial dysregulation as a mediator or moderator for medical, psychiatric, or psychological state or traits (e.g., (Picard et al., 2018; Saenen et al., 2019)).

In most cases, addressing such hypotheses requires a non-invasive, valid measure of mitochondrial health that can be obtained from reasonably large cohorts of participants. Unfortunately, no measure is currently available that globally assesses *mitochondrial health*, defined as the ability of mitochondria to perform a variety of normal biological functions. Mitochondrial functions include but are not limited to oxidative phosphorylation and energy production in the form of ATP, reactive oxygen species (ROS) production, cell death signaling, as well as steroid hormone synthesis (Selvaraj et al., 2018), and systemic signaling (Forsstrom et al., 2019; Lehtonen et al., 2016).

Unlike inert biomarkers like proteins (e.g., interleukins, c-reactive protein) or metabolites (cortisol, lactate) that have one of two states (high or low), mitochondria are complex living organelles that have dozens to hundreds of states. Asking about "cellular function" or "human function" would lack specificity, because most cells and the human organism perform multiple functions, and these functions are quantified with very different tests and measurements. For the same reason, because mitochondria have multiple relatively independent functions, different tests are required to tap into different aspects of mitochondrial health. The available toolkit of measurements for mitochondrial health in human tissues includes over a dozen measurements that range in biological specificity and in the complexity of the mitochondrial behavior or function assessed (discussed below in *Section 12*).

4. Nature and function of the mtDNA

The mitochondrial genome is a circular DNA molecule, consistent with its bacterial ancestry (Sagan, 1967) – all bacteria have circular genomes. The human mtDNA is present in multiple copies per cell and is maternally-inherited (Giles et al., 1980). The mtDNA is 16,569 base pairs long in humans and encodes information to produce protein subunits of the respiratory chain (RC). Thousands of RC units, also known as the electron transport chains, are embedded inside each mitochondrial inner membrane (Nicholls and Fergusson, 2013). In the RC, the electrons derived from food activate proton pumping across the inner mitochondrial membrane, and are ultimately combined with the oxygen we breath to generate the membrane potential – the life-giving charge that allows mitochondria to generate ATP and to perform other functions. Therefore, when inside the mitochondrial matrix, the mtDNA play a crucial role as a *template* for RC synthesis.

But the fact that the mtDNA is the template for proteins that participate in energy production does not mean that mtDNAcn directly reflects RC function or energy production capacity. There are at least three intermediate biological steps between the mtDNA and energy production capacity (Fig. 2). *First*, the mtDNA must be transcribed into the intermediate RNAs (i.e., transcription). *Second*, the messenger RNAs must be translated into 13 functional subunit proteins of the RC. And *third*, mtDNA-encoded proteins must assemble, along with proteins encoded in the nucleus, into functional RC complexes (complexes I, III, IV, V): large multi-protein complexes that transfer electrons and pump protons. In total, the mitochondrion is made of $>$ 1,300 proteins (Rath et al., 2021), only 13 of which are encoded in the mtDNA. Membrane integrity and availability of vitamins and co-factors are also necessary to animate the assembled RC. Thus, rapid changes in either transcription, translation, RC assembly or other factors may uncouple mtDNAcn from actual downstream energy production capacity.

Importantly, the number of mtDNA copies is redundant and does not scale linearly with RC capacity. Cells can lose a significant proportion of functional mtDNA yet still produce sufficient mRNA and proteins to sustain normal energy production capacity (e.g., (Picard et al., 2014)). According to the theory of "biochemical threshold", only when mtDNAcn decreases by 60 to 80% of normal levels does RC function and energy production capacity decrease (Boulet et al., 1992; Rossignol et al., 2003). This means that in many cases 20–40% of the baseline mtDNAcn is sufficient to produce the 13 proteins necessary to sustain normal respiratory capacity. But this level of mtDNA depletion seems to occur only in rare mitochondrial diseases (Basel, 2020) or in isolated single cells in diseased organs (Grunewald et al., 2016). The uncoupling of mtDNAcn and respiratory capacity may be accounted for by the fact that upregulation of transcription and translation from pre-existing mtDNA copies can increase the levels of mRNA, protein subunits, RC function, and energy production capacity, *without* a change in mtDNAcn. This notably occurs in response to exercise, where mitochondrial content and RC activity in human muscle increases within days to weeks without a change in mtDNAcn (Egan et al., 2013; Puente-Maestu et al., 2011). Thus, in human tissues, mtDNAcn is not directly coupled to, and does not directly reflect mitochondrial bioenergetics.

5. How is mtDNAcn related to plasma cell-free mtDNA (cf-mtDNA)

Beyond its role as a template for the respiratory chain, mitochondria also actively engage in signaling (Wu et al., 2021). The mtDNA can be released outside the mitochondrion, into the cytoplasm or in the bloodstream. When released in the cytoplasm, the mtDNA engages DNA sensing receptors that trigger innate immune signaling (Nakahira et al., 2011) (for excellent reviews, see (Riley and Tait, 2020; West and Shadel, 2017)). When released in the bloodstream as cell-free mtDNA (cf-mtDNA), or possibly as whole mitochondria (Al Amir Dache et al., 2020; Stephens et al., 2020), it predicts mortality in critically ill individuals (Nakahira et al., 2013; Scozzi et al., 2021), reflecting its potential physiological significance. Even in the absence of injury, psychological stress can induce cf-mtDNA in plasma and serum (Hummel et al., 2018; Trumpff et al., 2019). But the physiological significance and function of cf-mtDNA in humans largely remains to be defined (Miliotis et al., 2019; Trumpff et al., 2021).

So in contrast to *intra*-mitochondrial copies of mtDNA that contribute to mtDNAcn discussed above, cf-mtDNA residing outside functional mitochondria or in the bloodstream does not serve as a template to contribute to energy production capacity inside the cell. Therefore, cf-mtDNA levels are biologically and quantitatively distinct from tissue mtDNAcn. Accordingly, in human blood there is no consistent correlation between mtDNAcn in blood leukocytes and cf-mtDNA levels in plasma (Lindqvist et al., 2018; Rosa et al., 2020). Below we exclusively focus on mtDNAcn in its normal environment, inside cellular

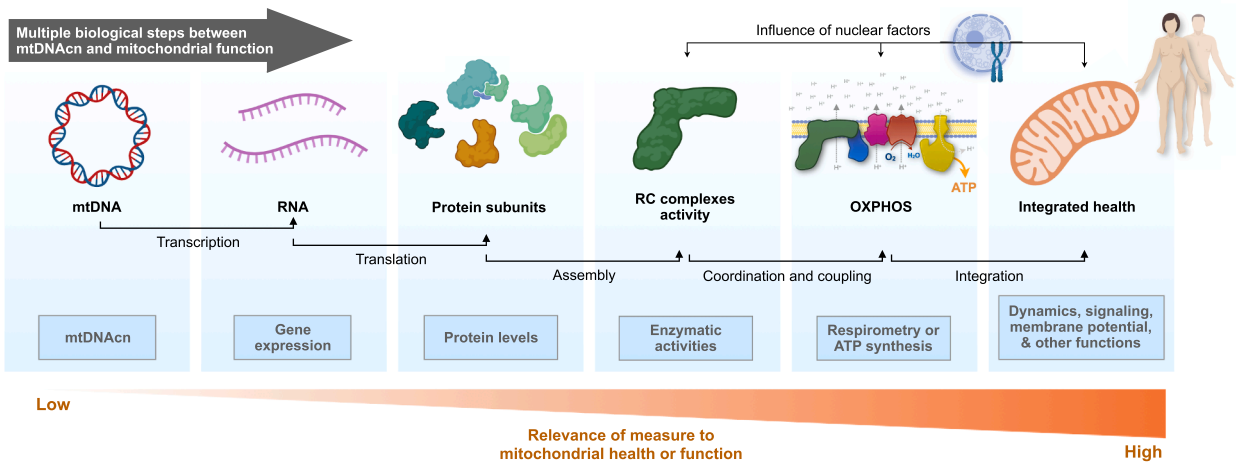


Fig. 2. Hierarchy of biological processes linking mtDNA to mitochondrial health. The mtDNA is the template for 13 mRNAs, which are translated into proteins in the mitochondrial matrix. All mtDNA-encoded proteins are subunits that assemble together with nDNA-encoded subunits into respiratory chain (RC) complexes within the inner mitochondrial membrane. RC complexes are subject to post-translational regulation that influence their ability to pump protons across the IMM and to respire (i.e., consume oxygen by complex IV). This generates the proton motive force that provide the driving force for ATP synthesis and other aspects of mitochondrial behavior. ATP synthesis by the OXPHOS system along with other dynamic processes of mitochondrial fusion/fission, signaling, and biogenesis, determine the integrated state of mitochondrial health that regulate cellular and organismal health. Thus, several independently modifiable biological steps separate the mtDNA template (mtDNAcn) from the final integrated function of mitochondria, explaining why *in vivo* mtDNAcn is not tightly coupled to energy production capacity nor with other physiologically-relevant aspect of mitochondrial health. Abbreviations: *OXPHOS*, oxidative phosphorylation; *RC*, respiratory chain.

mitochondria.

6. How is mtDNAcn measured?

The most common approach to measuring the abundance of mtDNA is to quantify the number of mtDNA copies, relative to the diploid nuclear genome (2 copies per cell) as a reference. By quantitative real-time PCR (qPCR), the differential amplification of a target mtDNA amplicon and of an autosomal or nuclear DNA (nDNA) amplicon yields a mtDNA:nDNA ratio. When multiplied by 2, the mtDNA:nDNA ratio gives the number of mtDNA copies per nucleated cell (Malik and Czajka, 2013). By next generation sequencing-based methods, where millions of small genomic fragments are indiscriminately "read", mtDNAcn can be derived from the ratio of the relative number of mtDNA and nDNA reads (Ding et al., 2015). A third method consists in counting the absolute number of mtDNA and nDNA copies in a given sample, using digital PCR (dPCR) (O'Hara et al., 2019). A fourth method consists in estimating mtDNAcn from genotyping microarray probe intensities, which again is reflective of the mtDNA:nDNA ratio (Hagg et al., 2020). Based on the strength of associations between mtDNAcn and various clinical and physiological parameters, sequencing-based estimations particularly from whole genome sequencing (WGS) appear to yield the most robust mtDNAcn estimates (Longchamps et al., 2020). Thus, not all mtDNA quantification methods are equivalent.

For valid qPCR measures, it is essential that the primer-probe set for the nuclear genome, which is used as reference for mtDNAcn, be present in only one copy per haploid human genome. As such, identifying unique single copy genes is a reasonable starting point for developing a nDNA assay. Some previous studies have opted to use multi-copy nDNA genes known as repeatable or repeat elements for normalization (e.g., (Meng et al., 2016)). However, the number of these nDNA repeat elements are in the thousands and vary from person to person (Feusier et al., 2017; Konkel et al., 2015), yielding unusual mtDNAcn values. Some genes also have recent evolutionary duplications that could contribute to the positive signal. Therefore, differences in the nDNA repeat element number, for example based on ethnic origin and ancestry (Konkel et al., 2015), can change the denominator in the mtDNA:nDNA ratio and therefore mtDNAcn – even where the number of mtDNA copies per cell are the same. Therefore, it is essential to use validated mtDNAcn assays using verified single-copy nDNA genes, such as those recommended in (Fazzini et al., 2018; Malik et al., 2011).

It should also be noted that the DNA extraction methodology, or the kit used for DNA isolation, also influences the absolute mtDNAcn. Alterations in mtDNA:nDNA ratio during extraction is likely due to differential retention of smaller or larger DNA molecules by the silica matrix in the elution columns (Guo et al., 2009) or by magnetic beads (Fazzini et al., 2018). These retention preferences also may be influenced by the exact chemistries employed. Depending on the kit used, mtDNAcn values on extracted DNA from the *same source tissue* (i.e., the same sample) can differ by up 100% (i.e., half or double) (Guo et al., 2009). Strikingly, in one study mtDNAcn measured from the same samples but with DNA extracted using different kits were shown to be only weakly correlated (Fazzini et al., 2018). Lysis-based methods that do not involve traditional DNA isolation may also yield greater reproducibility than column-based extraction (Longchamps et al., 2020). These results emphasize the importance of consistently using validated methodology for DNA extraction destined to mtDNAcn (Ajaz et al., 2015), and of systematically reporting the DNA extraction method when reporting mtDNAcn data. These results also emphasize the advantage of DNA precipitation (Ware et al., 2020) or lysis methods without isolation (Longchamps et al., 2020; Picard et al., 2012), which do not rely on column matrices with the potential to skew the mtDNA:nDNA ratio.

Essentially, mtDNAcn is not an absolute number of mtDNA copies, but a ratio of mtDNA to existing nuclei or nuclear genomes in the sample. The ratiometric nature of mtDNAcn also influences the interpretation of mtDNAcn data in samples containing cells with mitochondria but that lack a nucleus, such as blood platelets.

7. Do all cells have the same mtDNAcn?

Different cell types have widely diverging mtDNAcn. For example, tissues that have greater energy demands naturally have a higher mtDNAcn (i.e., more mtDNA copies per cell). mtDNAcn in the heart is ~2,000–5,000 copies per nucleus, ~1,000–3,000 in skeletal muscles, ~500–1,000 copies in liver, and ~ 150–600 copies in blood leukocytes (Kelly et al., 2012; Rausser et al., 2021). Therefore, different cells and tissues have up to an order of magnitude difference in mtDNAcn (Kelly et al., 2012; Wachsmuth et al., 2016). Even within specific organs like

the brain, mtDNAcn in different cortical and sub-cortical regions varies up to 2–3-fold (Brinckmann et al., 2010; Frahm et al., 2005; Fuke et al., 2011). Therefore, regional sampling variation can contribute to significant noise in tissue mtDNAcn measures.

Importantly for blood studies, mtDNAcn also vary widely among circulating immune cells. In purified leukocytes, new evidence from a small cohort (n = 21) of healthy women and men spanning four decades of life show that mtDNAcn ranges from ~150 to 600 copies depending on the cell subtype analyzed (e.g., monocytes, neutrophils, T or B lymphocytes, etc.) (see Fig. 1**C**). Whereas B cells and memory T lymphocytes have high mtDNAcn (450–600 copies per cell), mtDNAcn in neutrophils is 100–200 (Rausser et al., 2021). *In vitro* studies using single-cell approaches to measure mtDNAcn have also revealed that mtDNAcn show substantial cell-to-cell differences reaching up to an order of magnitude, even for clonally-derived cultured cells (O'Hara et al., 2019). Such variation is likely influenced by cell size and metabolic characteristics. Therefore, the absolute mtDNAcn is not a global feature of the organism, but is rather highly dependent on the tissue and cell type analyzed.

One general assumption for mtDNAcn measurements is that mtDNAcn in one tissue reflects a true property of the whole individual – in other words, that mtDNAcn measured in one tissue would reflect the donor equally well as another tissue. In different immune cell subtypes from the same person, this may be generally true, with most, although not all, leukocytes sharing 20–60% (average = 26%) of their variance in mtDNAcn (Rausser et al., 2021). Another study also found moderate correlations (r = 0.37–0.42, up to 18% of shared variance) between muscle and brain tissues (Frahm et al., 2005). However, a study using different methods to examine mtDNAcn variation between 12 different organs of the same individuals showed that their mtDNAcn in different tissues was largely uncorrelated (Wachsmuth et al., 2016). The outlier tissue pairs exhibiting the highest inter-correlations were skeletal muscle with liver (r = 0.37), and skeletal muscle with small intestine (r = 0.37), which represent a relatively small 14% of shared variance in their mtDNAcn. Blood was not correlated with any of the other tissues, some tissues showed negative correlations (e.g., skin and small intestine), and different regions of the same brain and person exhibited little to no correlation in their mtDNAcn (Wachsmuth et al., 2016). So finding high mtDNAcn in someone's blood says little about this person's brain or muscle mtDNAcn. This suggests that mtDNAcn is not a rigidly shared property of different tissues within an individual, nor is it shared between different regions of a given organ like the brain.

In fact, of the natural variation in mtDNAcn observed between individuals, the best estimates put the genetic inheritance of mtDNA at 8% (Hagg et al., 2020). This means either that current methods have failed to capture the source of variance in mtDNAcn, or that the majority of variance in mtDNAcn is not genetically determined but rather determined by the interactions of cellular, metabolic, dietary, behavioral, psychosocial, and other factors – in an organ-specific manner.

Recent evidence from repeated weekly measures in purified cell subtypes in a single individual also suggested that mtDNAcn and RC chain function may dynamically change from week-to-week (Rausser et al., 2021), opening the possibility that mtDNAcn is substantially more dynamic that previously imagined. As it is the case for other biological measures that exhibit high within-person variation (e.g., cortisol, see (Segerstrom et al., 2017)), perhaps multiple time points will be required to capture an accurate estimate of mtDNAcn for each individual, although more work is needed to establish the (in)stability of mtDNAcn in human cells.

Together, the inconsistent correlation between tissues and the low estimated genetic heritability of mtDNAcn in a given tissue implies at least two main scenarios. One scenario is that mtDNAcn within a given tissue is somewhat stochastically determined, which is possible given the excess mtDNA copies in most cell types. An alternative scenario is that in different individuals, certain tissues require more or less mtDNAcn relative to other tissues to sustain health, so the ratio of mtDNAcn across organs is different in different people. In other words, different individuals may maintain states of health via different combinations of mtDNAcn and mitochondrial function across tissues. Thus, blood mtDNAcn is likely not a generalizable property of the whole body or individual, but may provide some quantitative information specifically about the blood.

8. What influences mtDNAcn in whole blood?

In healthy individuals, each milliliter of blood contains on average 4.5–11 $\times$ 10^9 cells that include different subtypes of leukocytes, such as $CD4^+$ and $CD8^+$ T lymphocytes, antibody-producing B lymphocytes, neutrophils, monocytes, and others; and 150–450 $\times$ 10^9 platelets (Chernecky and Berger, 2013). On average, this represents ~ 40 platelets for each leukocyte (range ~ 20–80) (Shim et al., 2020) (see Fig. 1**D**). In terms of mtDNA content, each platelet has on average 5 mitochondria with mtDNA genomes, but *no nucleus* (Melchinger et al. 2019). Here we consider three key questions related to the determinants of whole blood mtDNAcn:

Do platelets influence mtDNAcn? Because mtDNAcn calculations (mtDNA:nDNA) use the nuclear genome as normalizer (i.e., denominator), the lack of nucleus in platelets makes platelet mtDNAcn infinitely large: *number of mtDNA copies / zero [no nuclear genome] = infinity*. As a result, each additional platelet contributes to the numerator (mtDNA) but add nothing to the denominator, thus inflating the product. Consequently, individuals with more platelets have significantly higher whole blood mtDNAcn, irrespective of actual mtDNAcn in immune cells (Knez et al., 2016; Kumar et al., 2018). The influence of platelets on mtDNAcn has been directly quantified by either platelet supplementation (Hurtado-Roca et al., 2016) and platelet depletion experiments (Urata et al., 2008; Rausser et al., 2021), demonstrating that manipulating platelet abundance significantly influences mtDNAcn not only in whole blood, but even in isolated PBMCs. In some studies where platelet abundance is measured on the same sample used for mtDNAcn, it may be possible, at least in part, to statistically correct mtDNAcn for platelet abundance.

Does leukocyte composition influence mtDNAcn? Different individuals have different proportions of white blood cells in circulation (Patin et al., 2018). Some individuals have many neutrophils (which have the lowest mtDNAcn, ~150 copies/cell), whereas other individuals have high levels of circulating B lymphocytes (highest mtDNAcn, ~450–600 copies/cell) (Rausser et al., 2021). Because these cells have dramatically different mtDNAcn, a significant fraction of the variance in mtDNAcn in mixed white blood cells or whole blood is driven by cell type proportions, rather than by intrinsic mtDNAcn within each cell (Fig. 1**E**). Because neutrophils have lower mtDNAcn than lymphocytes, a higher neutrophil:lymphocyte ratio is, as expected, associated with lower mtDNAcn (Shim et al., 2020). In fact, cell type distribution also varies widely by age (Patin et al., 2018; Rausser et al., 2021), in response to stress (Dhabhar et al., 2012), and across the day-night diurnal cycle (Dhabhar et al., 1994; Lange et al., 2010). This kind of dynamic within-person variation in peripheral blood cell composition adds to the potential leukocyte confound. Thus, even in the absence of difference in mtDNAcn among any of the immune cells, changes in proportions of circulating cells is sufficient to significantly influence mtDNAcn in whole blood and cell mixtures (e.g., PBMCs).

Do platelets or lymphocytes matter most? Both platelet and leukocyte abundances independently contribute to whole blood mtDNAcn, but the platelet:leukocyte ratio may be particularly significant. Consistent with the infinitely large mtDNA levels per nucleus in platelets (mtDNA copies divided by zero nucleus), individuals with lower platelet:leukocyte ratio (rather than the absolute platelet count) have lower mtDNAcn (Hurtado-Roca et al., 2016; Knez et al., 2016; Shim et al., 2020). Experimentally manipulating the platelet:leukocyte ratio showed that a 3-fold deviation in the platelet:leukocyte ratio increases mtDNAcn by up to 50% (Shim et al., 2020), confirming that platelet count is major driver of whole mtDNAcn in blood. Even in "platelet depleted" PBMCs processed with two platelet depletion steps (low-speed centrifugations), PBMCs still

contain contaminating platelets that inflate mtDNAcn by at least 10% (Rausser et al., 2021). Physiologically, both platelet and leukocyte production or mobilization in part reflect hematopoiesis in the bone marrow.

Fig. 3 summarizes the current state of knowledge about the relative influence of cellular factors on mtDNAcn measured in whole blood, buffy coat, PBMCs, PBMCs with platelet depletion, and sorted cells (e.g., by flow cytometry). Purified cell populations provide the highest degree of biological specificity with the least confounds.

Note that the same discussion may also apply to saliva. Saliva contains a large number of cells from which bulk material (DNA) is frequently isolated in epidemiological and other studies where blood is difficult to access. In saliva, half of the cells are leukocytes (monocytes, granulocytes, and lymphocytes) and the other half are large, flat buccal epithelial cells (Theda et al., 2018) (see Fig. 1**F**). Cell type proportions in saliva differ between children and adults, are widely variable between individuals, and are also strongly influenced by oral health (Theda et al., 2018). Thus, although relatively less is known about cell type composition and other factors that may influence salivary mtDNAcn, similar to blood, if total genomic DNA is extracted and used for mtDNA:nDNA quantification, the proportions of different cell types are expected to influence total mtDNAcn.

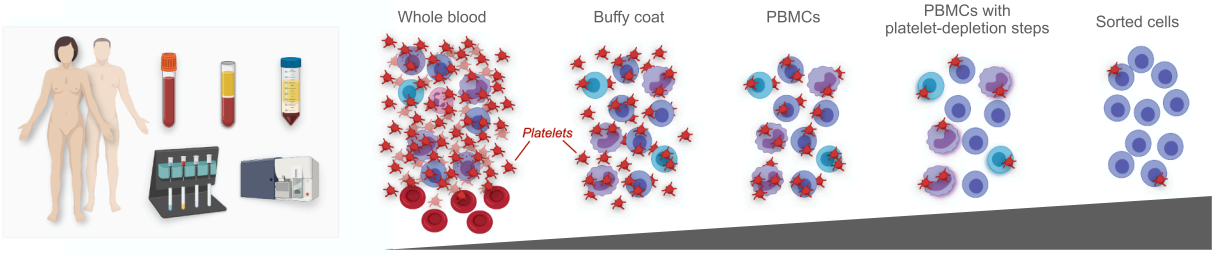
9. Are whole blood mtDNAcn measurements simply noisy, or artifactual?

One potential research area where platelet abundance may have introduced confusion is in studies of aging. All mtDNAcn studies from whole blood or platelet-contaminated cell mixtures have reported a negative correlation of mtDNAcn with age (e.g., Mengel-From et al., 2014; Moore et al., 2018; Verhoeven et al., 2018; Zhang et al., 2017). Two studies demonstrated that the age-related decline in mtDNAcn occurs preferentially after the age of ~ 50 years (Knez et al., 2016; Mengel-From et al., 2014). However, age is also associated with a robust decline in platelet abundance among both women and men (Biino et al., 2013; Rausser et al., 2021; Zhang et al., 2015), declining by up to 1–6% per decade of life. Women also consistently have a greater abundance of circulating platelets than men (Biino et al., 2013; Rausser et al., 2021; Zhang et al., 2015). This sex difference in platelet abundance could explain why women consistently have higher mtDNAcn than men when measured in whole blood, buffy coats, or PBMCs.

Because of the age-related decline in circulating platelets, DNA samples from older individuals likely contain quantifiably less mtDNA from platelets, which should effectively *decrease* mtDNAcn; even if mtDNAcn was unchanged in every circulating leukocyte. Consistent with this point, a study of whole blood mtDNAcn and age-related biomarkers in a cohort of 672 individuals also showed as expected that whole blood mtDNAcn was negatively associated with age, and positively associated with the inflammatory marker interleukin-6 (IL-6) (Moore et al., 2018). However, after mtDNAcn was statistically adjusted for platelet abundance and leukocyte counts measured via complete blood count (CBC), mtDNAcn was no longer associated with neither age nor IL-6 (Moore et al., 2018).

Some evidence from studies with high biological specificity indicate that mtDNAcn may in fact increase with age. In a small study where specific immune cell subtypes from individuals between ages 20–60 were purified by flow cytometry (i.e., as platelet-free as possible), mtDNAcn did not decline with age but was positively correlated, exhibiting a ~10% increase per decade in most cell subtypes analyzed (Rausser et al., 2021). This effect was uniquely detectable in sorted cells, not observed in PBMCs. In other studies in human skeletal muscle, liver, heart and brain, mtDNAcn was either not correlated with age (Frahm et al., 2005; Wachsmuth et al., 2016), or was even strongly positively correlated with age in a subset of individuals (Wachsmuth et al., 2016). The age-related increase in mtDNAcn could, as in cases of mitochondrial disorders, be the result of compensatory upregulation of mtDNAcn in response to accumulating age-related defects. Together, these results demonstrate that whole blood mtDNAcn is confounded by platelet abundance and by the mixture of different cell types that may preclude the straightforward interpretation of mtDNAcn and its true association with age, and potentially with other variables.

Although the precise mitochondrial properties of different circulating human immune cell subtypes have only recently begun to be described, the effect of platelet contamination on mtDNAcn in whole



Factors contributing to mtDNA:nDNA ratio	Whole blood	Buffy coat	PBMCs	PBMCs with platelet-depletion steps	Sorted cells
Hematopoiesis: Platelet production (thrombopoiesis)	+++	+++	++	+	-
Hematopoiesis: Total leukocyte production	+++	+++	+	+	-
Hematopoiesis: Neutrophil:Lymphocyte ratio	+++	+++	-	-	-
Hematopoiesis: Leukocyte subtypes distribution	++	++	++	+++	-
cf-mtDNA	+	+	-	-	-
Leukocyte mtDNAcn	+	+	+	++	+++

Most direct measure of cellular mtDNAcn

Fig. 3. Summary of known factors contributing to mtDNAcn in human blood. Preparations from left to right have increasing biological specificity, and consequently have more directly interpretable mtDNAcn. *Whole blood* is the complete (anti-coagulated) material collected, which contains all cellular constituents in variable proportions. *Buffy coat* is the complex cell mixture at the red blood cell-plasma interface after centrifugation of anti-coagulated blood. *PBMCs*, peripheral blood mononuclear cells, are typically isolated using Ficoll 1077-based separation with centrifugation and platelet-depletion steps, which depletes most granulocytes and a substantial portion of platelets. PBMCs can also be actively immuno-depleted of contaminating platelets using magnetic-activated cell sorting (MACS) with antibodies directed at platelet cell surface markers (e.g., CD41). *Sorted cells* are isolated using a combination of positive and negative selection using MACS, or by flow cytometric cell sorting. Sorted cells represent the purest preparation with the highest level of biological specificity, and therefore allow the most directly interpretable measurement of cellular mtDNAcn. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

blood and PBMCs have been known since the early 2000's (Banas et al., 2004; Urata et al., 2008). Taking into account these biological considerations, which strongly influence whole blood mtDNAcn, can improve the design and interpretation of future studies.

10. Is mtDNAcn a marker of mitochondrial content or mitochondrial biogenesis?

Few studies have directly examined this question. *Mitochondrial biogenesis* is the orchestrated synthesis of mitochondrial components, which effectively increases the cellular mitochondrial content, also referred to as mitochondrial mass or mitochondrial content. One study in human skeletal muscle biopsies ($n = 16$) directly measured the actual mitochondrial content (i.e., the volume of muscle cells occupied by mitochondria) by electron microscopy, and quantified mtDNAcn by qPCR. In skeletal muscle, the correlation of EM-based mitochondrial content and citrate synthase (CS) enzymatic activity, an established marker of mitochondrial content, was strong ($r = 0.84$, $p < 0.001$) (Larsen et al., 2012). In comparison, the correlation of mitochondrial content and mtDNAcn was substantially smaller ($r = 0.35$, $p = 0.23$) (Larsen et al., 2012). Another study in human muscle and brain measuring mtDNAcn and a housekeeping mitochondrial protein (Porin, or VDAC1) found no correlation between mtDNA abundance and protein-based mitochondrial content (Brinckmann et al., 2010), suggesting that in human tissues, mtDNAcn is not a valid marker of mitochondrial content.

Experimentally, mtDNAcn and mitochondrial content can also change relatively independently from each other. This is unlike what would be predicted if mtDNAcn reflected mitochondrial biogenesis. For example, it is possible to completely deplete the mtDNA (by inhibiting mtDNA replication) in cultured human cells, and to observe abundant (albeit respiratory deficient) mitochondria populating the cytoplasm (e.g., (Schubert et al., 2015)). On the other hand, by over-expressing proteins that positively regulate mtDNA replication, mtDNAcn can be artificially increased, but without concomitant biogenesis (Ylikallio et al., 2010). In response to pharmacological treatments in mice, a 80–90% elevation of mtDNAcn can occur out of proportion from a 30% elevation in mitochondrial biogenesis and volume density (Vettor et al., 2014). And in the heart, transgenic overexpression of the mtDNA replication machinery can also double mtDNAcn without changing RC enzymatic activities (Ikeda et al., 2015), convincingly demonstrating the uncoupling of mtDNAcn and biogenesis *in vivo*.

Certain exposures have the opposite effects. For example, 72 h of cigarette smoke exposure in fibroblasts decreases mtDNAcn by 25–30% despite induction of mitochondrial biogenesis and increases in protein abundance (Giordano et al., 2015). There is also evidence that exercise, a physiological challenge that increases energy demand, can acutely decrease skeletal muscle mtDNAcn while mitochondrial content or respiratory chain function is preserved (Egan et al., 2013; Puente-Maestu et al., 2011). Such an acute decrease in mtDNAcn appears to occur despite the induction of canonical mitochondrial biogenesis pathways (AMPK, PGC-1α) induced by exercise (Neufer et al., 2015), again demonstrating the uncoupling of mtDNAcn from mitochondrial biogenesis and bioenergetics in human tissues.

Finally, mtDNAcn can also be uncoupled from mitochondrial content in human disease. For instance, in patients with high levels of mtDNA mutations that severely impairs respiratory chain function, mtDNAcn is elevated by 3–7-fold without equivalent increase in cellular mitochondrial content (Brinckmann et al., 2010). At the single-cell level, affected muscle cells with mtDNA mutations can also exhibit 2–4-fold higher mtDNAcn (Yu-Wai-Man et al., 2010), and intracellular foci of mutant mitochondria can exhibit 1-2-fold higher mtDNA abundance (Vincent et al., 2018), illustrating compensatory upregulation of mtDNA replication. And in blood leukocytes of patients with mtDNA defects, elevated mtDNAcn can also indicate compensatory upregulation and an attempt at preserving normal bioenergetic capacity (Giordano et al., 2014), indicating an anti-correlation between mitochondrial RC function and mtDNAcn. A summary of biological states that can lead to either reduced or elevated mtDNAcn is provided in Fig. 4.

Thus, collectively: i) the limited correlation of mtDNA with mitochondrial content in human and animal models, and ii) the decrease in mtDNAcn despite elevations in mitochondrial biogenesis markers in human cells, iii) the uncoupling of mtDNA and mitochondrial RC capacity in cellular and animal studies, and iv) the increase mtDNAcn with RC dysfunction in human mitochondrial disease lead to two conclusions. First, mtDNA is an imperfect marker of mitochondrial content. And second, mtDNAcn is not a marker of mitochondrial biogenesis.

11. So what does whole blood mtDNAcn represent?

Whole blood mtDNAcn may indirectly reflect *hematopoiesis biology*. Hematopoiesis within the adult bone marrow is responsible for the production of all myeloid and lymphoid immune cell types, including granulocytes (e.g., neutrophils, eosinophils) and mononuclear cells (e.g., monocytes, lymphocytes), as well as platelet and platelet precursors. In addition to hematopoiesis, the *mobilization* of different immune cells from the vascular wall of secondary lymphoid tissues (i.e., demarginalization) where a portion of immune cells reside, also contribute to rapid changes in the composition of circulating immune cells (Ince et al., 2018). Four main lines of evidence support the idea that whole blood mtDNAcn reflects hematopoiesis.

First, as discussed above, by far the strongest correlate of whole blood mtDNAcn is the abundance of white blood cells and platelets, all of which are produced through hematopoiesis in the bone marrow. *Second*, a large fraction of inter-individual differences in mtDNAcn in PBMC mixtures may be explained by the proportion of specific cell types. For example, people with the highest PBMCs mtDNAcn are those with higher B cell abundance (Rausser et al., 2021). *Third*, analyses of genetic variants associated with whole blood mtDNAcn in 295,150 participants indicate an enrichment for pathways directly related to bone marrow biology including immune activation, cell–cell adhesion, haematopoiesis, apoptosis, and platelet production; and to a lesser extent mitochondrial biogenesis and plasma lipoprotein assembly (Hagg et al., 2020). And *fourth*, phenome-wide association studies of mtDNAcn indicate that high mtDNAcn is associated with greater prevalence of hematological disorders, such a myeloproliferative disease (a bone marrow disorder), lymphoid leukemia (hyperproliferation of lymphocytes), and other malignant neoplasms and cancer, as well as diseases of the spleen (a secondary lymphoid tissue) (Hagg et al., 2020). Factors that alter hematopoiesis and therefore circulating immune cell composition, alter mtDNAcn.

Thus, even though blood mtDNAcn does not reflect tissue mtDNAcn or mitochondrial energy production capacity in other tissues, the reason why whole blood mtDNAcn consistently exhibits associations with a broad range of age-related disease states may not be because it reflects mitochondrial function or energy production capacity per se, but possibly because it reflects complex hematopoiesis and immunological processes. In other words, mtDNAcn may be a proxy measure of hematological and hematopoiesis biology. Therefore, mtDNAcn may serve as a quantitative index of the physiological resources available and deployed within the bone marrow. In turn, immune regulation and inflammatory signaling can directly impact a number of physiological processes (Furman et al., 2019), so it is sound to expect that an integrative measure of immune regulation like mtDNAcn could predict future health outcomes.

It is also possible that some systemic element of mitochondrial health directly impacts the hematopoiesis – as it is the case in severe genetic mitochondrial disorders caused by deletions within the mtDNA (Rotig et al., 1995). Consistent with this idea, a large-scale population study with the UK Biobank identified mtDNA genetic variants associated with white blood cell counts and other hematological parameters (Yonova-Doing et al., 2021). And severe genetically-inherited RC defects may also

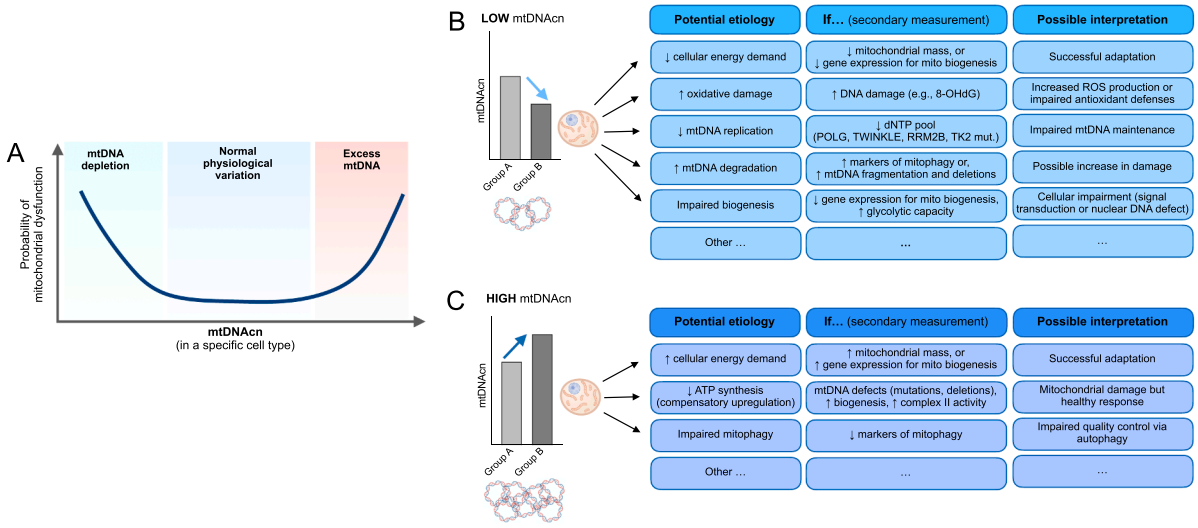


Fig. 4. Theoretical scenarios for changes in mtDNAcn in purified cell type preparations or in human tissues. (**A**) Both mtDNA depletion and excess mtDNA can indicate abnormal mitochondrial health, or mitochondrial dysfunction, as seen in rare cases of human disease (see text for details). mtDNAcn within specific cell subtypes exhibit substantial variation between individuals, and potentially even within individuals over time. (**B**) Potential etiology, mechanisms, and interpretation for reduced and (**C**) elevated mitochondrial mtDNAcn when measured in a purified cell population, after confounds related to cell composition have been ruled out. Note that this list is not exhaustive and other etiologies are possible. This also illustrates how parallel measurements of other mitochondrial features can contribute to the interpretation of mtDNAcn. *Abbreviations*: *dNTP*, deoxynucleotide triphosphate; *ROS*, reactive oxygen species; *8-OHdG*, 8-Oxo-2′-deoxyguanosine [a marker of oxidative DNA damage]; *POLG*, polymerase gamma [mtDNA polymerase]; *TWINKLE*, mtDNA helicase necessary for normal replication; *RRM2B*, ribonucleotide reductase regulatory TP53 inducible subunit M2B [a subunit of an enzyme involved in DNA synthesis]; *TK2*, thymidine kinase 2 [enzyme involved in DNA synthesis].

alter circulating immune cell composition and function (Tarasenko et al., 2017). Supposing that the naturally-occurring variation in mitochondrial health within the population was sufficient to impact hematopoiesis and the abundance of circulating cells, then mitochondrial (dys)function could indirectly manifest in changes of blood mtDNAcn *via* changes cell type composition. But here again, the direction of change may not be straightforward.

Depending on the scientific objective of interdisciplinary scientists, understanding *why* mtDNAcn is associated to health outcomes may be essential to gain insight into conserved disease or resilience mechanisms or pathways. However, if the objective of measuring mtDNAcn is to develop biomarkers, a mechanistic understanding of its underpinning may not necessary. Understanding the biology underlying the association of mtDNAcn and health outcomes may nevertheless contribute to: i) refine and improve the measurements specificity for mtDNAcn as a biomarker, and ii) do reverse inference to understand what disturbances in mtDNAcn are telling us about the state of the organism.

12. How can we increase the value and specificity of blood mtDNAcn?

If investigators are technically limited to previously collected whole blood, one approach to address the hematopoiesis hypothesis would consists in obtaining precise data about the circulating abundance of molecularly-defined specific cell subtypes. This can be achieved by flow cytometry or single-cell RNA sequencing, in parallel with mtDNAcn. Multivariate models (i.e., deconvolution methods) could then be developed to understand the proportion of variance in mtDNAcn attributable not just to general immune cell categories (e.g., granulocytes and lymphocytes) but to specific cell subtypes (naïve and memory CD4 and CD8 T cells, B cells, subtypes of monocytes, etc). Similar approaches using data from complete blood counts with differential (CBC with diff.) have been informative (Hagg et al., 2020; Moore et al., 2018), and can be enhanced using flow cytometry methods that quantify immunologically-defined cell subpopulations (Patin et al., 2018). However, existing large-scale datasets generally do not include detailed blood cell counts, and these measures also cannot be obtained post-hoc from frozen whole blood. In research contexts where investigators would be limited to design new studies with DNA extracted from whole blood only, the evidence outlined above justifies the budgeting and allocation of resources to perform detailed immune blood population phenotyping to enable the interpretation of mtDNAcn.

To further enhance the sensitivity and interpretability of mtDNAcn in relation to health-related phenotypes, the ideal approach consists in increasing the biological specificity of the primary measure. This is achieved by quantifying mtDNAcn directly in molecularly-defined subtypes of immune cells. For example, $CD4^{+}$ Naive T cells, monocyte subtypes, or other specific immune subtypes exist in sufficient abundance in circulation to be isolated by either flow cytometric cell sorting (also known as fluorescence-activated cell sorting, FACS) or by negative/positive selection by magnetic activated cell sorting (MACS) (Kramer et al., 2014). Flow cytometric cell sorting generally requires sophisticated equipment or core facilities, whereas MACS can be performed in most laboratories with the infrastructure required to isolated PBMCs. Compared to cell mixtures, cell-specific mtDNAcn quantification add biological specificity to detect meaningful mitochondrial associations related to exposures, other biomarkers, and possibly age and sex-related differences (Rausser et al., 2021).

In addition, measuring other markers of mitochondrial content and/or function in parallel with mtDNAcn can contribute to formulating biologically valid interpretations (see Fig. 4). As noted above, live assays of mitochondrial functions are generally low throughput and may require specific storage conditions (-170 °C liquid nitrogen storage to preserve enzymatic activities and respiratory capacity, *vs* −80 °C for DNA and other inert molecular markers). It is beyond the scope of this review to describe the range of existing mitochondrial function assays, but it is worth pointing out some that can be synergistic with mtDNAcn.

They include but are not limited to: i) citrate synthase (CS) activity, cardiolipin, or mitochondrial protein abundance to estimate *mitochondrial content* (McLaughlin et al., 2020); ii) *mtDNA integrity*, such as DNA damage, point mutations, or deletions (Lujan et al., 2020; Ye et al., 2014); or iii) *mitochondrial respiratory capacity*, such as oxygen

consumption by respirometry (Gumpp et al., 2020; Osto et al., 2020), which can and should be performed in specific cell types (Kramer et al., 2014), as well as *respiratory chain enzymatic activities* that reflect energy production capacity on either a per-cell or per-mitochondrion basis (Picard et al., 2018; Rausser et al., 2021). In the context of such direct measurements of respiratory chain function, mtDNAcn becomes a more biologically interpretable feature of mitochondrial health. The spectrum of mitochondrial approaches available to interdisciplinary scientists is also briefly discussed in (Picard et al., 2019).

Moreover, circulating markers of mitochondrial stress accessible in plasma or other biofluids, including GDF15 (Sharma et al., 2021), cf-mtDNA (Trumpff et al., 2021), or other emerging *mitokines* may also prove useful markers of mitochondrial health. However, they may lack specificity. GDF15, cf-mtDNA, and other circulating markers can be induced by a number of stressors not necessarily reflecting mitochondrial RC capacity or stress. Developing scalable, specific, and biological robust biomarkers of mitochondrial health in accessible human tissues remains an active area of research.

13. Conclusions

At a time when pressing questions around the basis of human health need to be addressed, unprecedented cohorts and new research tools converge with our evolving understanding of mitochondrial biology. As scientists investigate novel health pathways and disease mechanisms, incorporating the most promising biomarkers of mitochondrial health into existing studies can fuel the much-needed interdisciplinary work necessary to understand what influences health trajectories across the lifespan.

The statistically robust associations between mtDNAcn and various physiological, demographic, behavioral, psychiatric, environmental, and clinical variables suggest the existence of a true biological connection between mtDNAcn and human health. Mitochondria dysfunction has emerged as an important cause of disease, but a major challenge remains the biological specificity of tissues used (whole blood and cell mixtures *vs* specific cell types), and how the measured parameters, such as mtDNAcn, actually reflects the functional capacity of the organelle. Improving the specificity of our mitochondrial measures and their biological interpretability is critical to research progress.

To maximize the scientific value of mtDNAcn, and to understand the underlying mechanisms, purified cell populations should be systematically used. When not possible, investigators should collect precise data on the abundance of the major cellular constituents, such as subtypes of white blood cells and platelets, to adjust or rule out the potential confounds highlighted above. When no information on cell type abundance and platelets is available, data should be interpreted while considering the caveats discussed above.

In conclusion, although the biological interpretation of differences in mtDNAcn is tenuous, resolving the factors that drive differences in mtDNAcn between groups of individuals and across the lifespan is likely to yield important insights. These include but are not limited to hematological processes within the bone marrow, genetic factors, dietary and behavioral factors, and humoral factors that stimulate either the replication or removal of mtDNA. Thus, in combination with relevant markers assessed in homogenous or well-defined cell populations, continuing to add mtDNAcn to existing interdisciplinary studies with rich sets of outcomes is likely to contribute valuable insights into the role of mitochondria in human health, aging, and resilience.

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