HIV Infection, Nucleoside Analogue Therapy and Somatic Mitochondrial DNA Mutation: Implications for Ageing?

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Dedication

I would like to dedicate this work to my wife, Sarah. Without her continuous support, it would not have been possible.

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Abstract

It has been hypothesised that patients with long-term treated HIV infection may exhibit features of accelerated physiological ageing. Given previously established links between, a) anti-retroviral therapy and mitochondrial DNA (mtDNA), and b) mtDNA and ageing, I hypothesised that anti-retroviral therapy may lead to the accumulation of mtDNA mutations, in an acceleration of the molecular process seen in normal human ageing.

Using a combination of single cell molecular analyses and ultra-deep sequencing (UDS), I demonstrated that HIV-infected patients with prior exposure to polymerase (pol) γ inhibiting NRTI (nucleoside analogue reverse transcriptase inhibitor) therapy show increased accumulation of somatic mtDNA mutations within cells, in a pattern similar to that seen much later in life due to normal ageing. Empirical data and *in silico* modelling suggested this is likely to be mediated by the accelerated clonal expansion of pre-existing (age-associated) mtDNA mutations, rather than by increased mutagenesis.

I went on to further develop the UDS methodology, to explore more fundamental questions about the characteristics of mtDNA mutations in ageing, health and disease. In so doing, I showed that low-level mtDNA heteroplasmic mutation appears to be universal, and that many ostensibly somatic mutations may in fact have been maternally transmitted at very low levels.

I explored the utility of serum FGF-21 (fibroblast growth factor 21) and phosphorus magnetic resonance spectroscopy (^{31}P-MRS) as non-invasive measures of muscle mitochondrial dysfunction in anti-retroviral treated patients. Both showed significant abnormalities, although neither proved sensitive or specific in my patient group.

Finally I explored the frequency and severity of fatigue in contemporary HIV-infected patients, showing that half of all patients have excessive fatigue despite good immune function and suppressed HIV viraemia. Patients with prior exposure to pol γ inhibiting NRTIs were almost universally fatigued, suggesting that persistent mitochondrial dysfunction due to accumulated mtDNA mutations may be important in driving fatigue in this patient group.

Contents

Doctoral Statement (Introduction)

1.1 HIV and ageing, an overview

It has recently been hypothesised that long-term HIV infection may cause a clinical syndrome resembling accelerated ageing (Casau, 2005; Effros *et al.*, 2008). Such an observation has arisen from multiple considerations. Firstly, clinical and epidemiological data describes an excess of comorbid diseases which resemble those of older age (for example, cardiovascular disease, osteoporosis, cognitive impairment) (Currier *et al.*, 2003; Thomas and Doherty, 2003; Valcour *et al.*, 2004; Brown and Qaqish, 2006; Heaton *et al.*, 2010). Secondly, there is accelerated physiological decline, such as an increase in the age-associated loss of aerobic capacity, and an increase in frailty, which is seen as a core phenotype of physiological ageing (Oursler *et al.*, 2006; Desquilbet *et al.*, 2007). Thirdly, and from a more pathophysiological standpoint, HIV-infected patients show an acceleration of immune senescence, despite virally suppressive anti-retroviral therapy, and despite reconstitution of absolute numbers of CD4 T lymphocytes (Effros *et al.*, 1996; Bestilny *et al.*, 2000; Appay *et al.*, 2007). However, an alternative approach to the consideration of the ageing is 'bottom-up', from the cellular level, a process often referred to as 'intrinsic ageing'. Intrinsic ageing describes the notion that there is a fundamental ageing process within humans, which is not simply the sum total of increasing chronic diseases acquired during life. Such a process starts with molecular damage, thence leading to cell, tissue and organ dysfunction, as succinctly reviewed by Kirkwood (Kirkwood, 2005; Kirkwood, 2008). Intrinsic ageing is proposed as the 'canvas' upon which the diseases of older age (for example, neurodegeneration) may develop (Campisi and d'Adda di Fagagna, 2007).

One of the best characterised tenants of intrinsic ageing in healthy persons is the phenomenon of acquired (somatic) mitochondrial DNA (mtDNA) mutation (Linnane *et al.*, 1989; Corral-Debrinski *et al.*, 1992a; Corral-Debrinski *et al.*, 1992b; Simonetti *et al.*, 1992; Brierley *et al.*, 1998; Michikawa *et al.*, 1999; Pesce *et al.*, 2001; Wang *et al.*, 2001; Lin *et al.*, 2002; Del Bo *et al.*, 2003). Given a wealth of data linking HIV infection, anti-retroviral therapy and mitochondrial dysfunction (as outlined below), it is plausible that an acceleration of intrinsic ageing might therefore occur in anti-retroviral treated HIV-infected patients through a mitochondrial mechanism.

1.2 Mitochondrial DNA replication and maintenance

Human mtDNA is a compact circular genome of 16.5kb, encoding 13 essential polyproteins of the respiratory chain, the principle intracellular source of energy (ATP) production (DiMauro and Schon, 2003). Unlike the nuclear genome which replicates only at cell division, mtDNA is continuously turned over throughout life, with a half-life estimated to be 1-10 days *in vivo* (Gross *et al.*, 1969). There is a sole mtDNA polymerase, pol γ, encoded by the nuclear gene, *POLG* (Kaguni, 2004; Hudson and Chinnery, 2006). Multiple additional nuclear genes encoding proteins with direct or indirect involvement in mtDNA replication and maintenance have recently been described (Copeland, 2008).

1.2.1 Somatic mtDNA mutations, their character and formation

It has been widely assumed that somatic mtDNA mutations arise largely as a consequence of oxidative damage to mtDNA. This notion arises from the fact that mitochondria are the principle source of intra-cellular energy production, and as such are also the principle source of ROS (reactive oxygen species), a natural by-product of oxphos (oxidative phosphorylation) reactions. Furthermore mtDNA is vulnerable to oxidative damage due to: its proximity to the inner mitochondrial membrane (the site of oxphos); a lack of protective histones; and more limited DNA repair mechanisms compared with nuclear DNA (nDNA). This has lead to the 'vicious cycle hypothesis' whereby mtDNA mutation causes a functional change in a respiratory chain subunit, leading to partial uncoupling of the respiratory chain, the result of which is increased ROS production, and further mtDNA mutation (Harman, 1956; Linnane *et al.*, 1989; Richter, 1995; Mecocci *et al.*, 1999).

However, much recent work has focussed on the role of mtDNA replication error in the generation of somatic mtDNA mutations. Replication error is of course an obvious candidate mechanism of mtDNA mutation formation in many of the inherited disorders of mtDNA maintenance (such as *POLG* defects) where secondary mtDNA defects (somatic mutations) are the molecular hallmark. In contrast, oxidative damage has been assumed to be the key player in driving the mtDNA damage associated with ageing and neurodegeneration. It is however equally plausible that natural mtDNA replication error

occurs even in the absence of a defective polymerase or other abnormal regulatory protein, and this process may also contribute to age-associated mtDNA mutation formation (Krishnan *et al.*, 2008).

There are two main types of mtDNA mutations: large-scale deletion mutations and point mutations. Large-scale deletion mutations are the predominant mutation type leading to cellular defects of mitochondrial function within post-mitotic (non-dividing) tissues, such as muscle and neurons (Corral-Debrinski *et al.*, 1992a; Bua *et al.*, 2006). As the mitochondrial genome is comprised almost entirely of coding sequence, large-scale deletion mutations typically delete several mitochondrial genes and thus have a high likelihood of a functional consequence within the cell. In contrast, large-scale deletion mutations are not readily observed in replicative tissues, a phenomenon which is assumed to arise because the resultant cellular oxphos defect would lead to a replicative disadvantage for the cell, and thus cells with such mutations would quickly be lost. This effect can be observed in primary cell culture, where myoblasts are derived from a muscle sample of a patient with an inherited single mtDNA deletion disorder. The deletion is detectable at significant levels in DNA extract from the biopsy itself, but disappears after only a few passages *in vitro.* In contrast in post-mitotic tissues, the non-dividing cells cannot, by definition, be subject to a replicative disadvantage, and the mutations can therefore persist within cells, despite their functional consequences. The means by which deletion mutations appear in the first place remains to be fully explained (Krishnan *et al.*, 2008). It is however increasingly clear that short homologous sequences within separate parts of the mitochondrial genome are almost certainly of key importance (Samuels *et al.*, 2004). Virtually all deletion mutations are located within the 'major arc' of the mitochondrial genome, between the origins of replication (Bua *et al.*, 2006). The prototype example of this is the 13bp repeat sequence found at each end of the 4977bp mtDNA 'common deletion' mutation (δmt.8483-13446). The 'common deletion' is so-called because it is the single most commonly observed large-scale deletion mutation. However, in absolute terms it is not in fact very common, comprising perhaps no more than 5-10% of all deletion mutations detected in patient samples. It furthermore appears that the vast majority of deletions have 5' and 3' ends which fall within hot-spots near to the common deletion loci (Samuels *et al.*, 2004). Elegant chemical modelling has shown that molecules arising from deletion

origins in these regions have inherent stability, which may facilitate their formation (Guo *et al.*, 2010).

With respect to the initial event in deletion formation, the prevailing theory has been that the primary problem arises from the 'strand asynchronous' method of mtDNA replication, which inherently temporarily creates portions of single-stranded DNA (ssDNA). This view has recently been challenged by a new theory which proposed that a double-strand break (DSB) in the mtDNA is the initiating event (Krishnan *et al.*, 2008). The response to a DSB will be 3' to 5' exonuclease activity. This will temporarily create ssDNA, which may well contain repeats which are able to undergo homologous annealing, leading to a deleted molecule. The advantage of this theory is that it relies on a process (DSB) that is already known to occur, both in health and disease. It implies a role for replication stalling, but also accommodates ROS or irradiation damage as a direct cause of DSBs.

1.2.2 MtDNA mutations, heteroplasmy and clonal expansion

Cells contain hundreds to tens of thousands of copies of the mitochondrial genome. Therefore if a somatic mtDNA mutation arises *de novo* within a cell it will initially be far outnumbered by wild-type mtDNA. The proportion of mutant mtDNA within a cell or tissue is termed the heteroplasmy level. Typically mutant mtDNA has to exceed a heteroplasmy level of ~65% to cause a functional defect within a cell (Durham *et al.*, 2005). Furthermore a cell will typically attempt to compensate for the presence of high proportional levels of mutant mtDNA by increasing the total cellular mtDNA content, a process known as mtDNA proliferation (Durham *et al.*, 2007). It is assumed that this process occurs as it is advantageous to increase the amount of wild-type mtDNA within the cell to try and restore normal oxphos function, but the cell is unable to discriminate between wild-type and mutant mtDNA and thus the only option is to proliferate total cellular mtDNA content. There is therefore some debate as to whether it is the percentage level of mutant mtDNA or the residual level of wild-type mtDNA within a cell that is important in determining whether there is a functional defect within that cell. Furthermore, the way in which cellular mtDNA copy number is regulated largely remains to be determined.

How do somatic mtDNA mutations therefore reach high levels within cells? Broadly there are two competing theories. Firstly, that mutant mtDNA has a competitive advantage over wild-type mtDNA and therefore preferentially expands within a cell. Or secondly, that there is no selective advantage to mutant mtDNA, but it can expand simply through the continued turnover of mtDNA throughout the human lifespan. The latter process arises from the consideration that mtDNA is constantly turned over, even in non-dividing cells, and has been termed 'relaxed replication'. The process has been modelled *in silico* based on empirically derived parameters, such as the *in vivo* half-life of mtDNA (Chinnery and Samuels, 1999; Elson *et al.*, 2001). Essentially the process leading to expansions of mutations within some cells is the chance play of random drift. Under this model, there are of course predicted to be far more cells where a mutation arises at a very low level and is rapidly lost. The additional factor acting within cells that do contain mutations is that of mtDNA proliferation as mentioned above, a process for which there is extensive empiric evidence. The net result of this compensatory response is that once a mutation reaches a high enough intracellular level to induce a proliferation response, the effect of this will be to also increase the copy number of mutant mtDNA within that cell, ultimately making it very difficult for the mutant mtDNA to ever then be lost from that cell by further natural drift. The relaxed replication model is attractive in that it relies only on parameters for which empirical evidence exists, and it can equally well explain the behaviour of all types of mtDNA mutations within cells: large-scale deletions, pathogenic point mutations, non-pathogenic point mutations. Conversely, a model based on a selective replicative advantage of mutant mtDNA would require empiric evidence for that phenomenon. Such a process is easiest to envisage for large-scale deletion mutations, where the smaller mutant molecule may be able to replicate more quickly (or have a higher probability of successfully completing replication) than the larger wild-type molecule. There is now a small amount of *in vitro* evidence for this process from trans-mitochondrial cybrid cell lines containing a heteroplasmic mix of wild-type mtDNA with a single large-scale deletion mutation (Moraes *et al.*, 2001). When such cells lines are subjected to a profound but transient depletion in mtDNA content (by ethidium bromide treatment), the repopulation of the smaller mutant mtDNA is significantly faster than that of the wild-type mtDNA (Diaz *et al.*, 2002). However, given time the repopulation of wild-type mtDNA does 'catch up' and the balance of wild-type to mutant mtDNA is then restored. Therefore the percentage heteroplasmy level eventually returns to baseline. Furthermore, upper estimates of the *in vivo* half-life of mtDNA are

in the range of 8-16 days, whereas the time taken to complete a single cycle of mtDNA replication is measured in tens of minutes. Thus it seems improbable that any slight differential in speed of replication of deleted vs. wild-type mtDNA during a single cycle of replication, would translate into a shift in the balance of heteroplasmy. Finally it is hard to envisage how a point mutation would have any replicative advantage.

These contrasting models have potential implications for the relative timing of mutation formation with respect to the functional cellular defect that ultimately results, with consequence differences in inference of the speed of the clonal expansion process in between (Khrapko, 2011). This concept is discussed further below.

1.2.3 The challenges of measuring mtDNA mutation

In various studies of acquired mtDNA mutation, including ageing and neurodegeneration, a variety of measures of mtDNA mutation have been employed, some qualitative (for example 'clamped' PCR to detect the presence or absence of a specific mtDNA somatic point mutation of interest), and some quantitative. There is no accepted 'gold standard': all current methods have technical challenges and the approach depends in part on the tissue under study (**Table 1**).

In post-mitotic tissue (such as muscle), as mutations will be physically constrained by lack of cell division, it is common to initially look for cells that are deficient in mitochondrial function, as such cells are likely to contain clonally expanded somatic mtDNA mutations. This is achieved through sequential COX/SDH (cytochrome *c* oxidase / succinate dehydrogenase) histochemistry (Muller-Hocker, 1989). The principle of this assay is that COX contains respiratory chain subunits which are encoded by mtDNA. Therefore mtDNA mutations (especially large-scale deletions) will usually cause loss of COX activity (loss of brown stain). In contrast, SDH is entirely encoded by nDNA, and therefore activity will be maintained in the face of an mtDNA defect, and the cell will counterstain blue. The specific mutation may then be identified by single cell laser micro-dissection of the COX-deficient cell, followed by single cell molecular analyses (Brierley *et al.*, 1998; Taylor *et al.*, 2001; He *et al.*, 2002; Taylor *et al.*, 2003; Bender *et al.*, 2006). This method therefore has the advantage of helping to find the 'needle in the haystack', as somatic mtDNA mutations will vary from cell-to-

cell, and thus will be individually rare in the tissue as a whole. Furthermore, although this method does not directly quantify the mutations themselves within the tissue, given that COX-deficient cells will contain clonally-expanded somatic mtDNA mutations, the percentage of cells showing a COX defect can be easily determined from examination of the histological images, and this count will give a surrogate for the 'functionally relevant' mutation burden.

In replicative tissues (such as peripheral blood mononuclear cells, PBMCs) or homogenised tissues, alternative approaches must be employed. For example, in the first paper to consider somatic mtDNA mutations in the context of HIV, Mallal and colleagues used SSCP (single strand conformational polymorphism) analysis to screen for differences in DNA sequence between pairs of samples (pre-treatment / on treatment in this case) (Martin *et al.*, 2003). Again this method helps to select for changes, but is not quantitative.

Deletion mutation load may be relatively easily quantified by real-time PCR (He *et al.*, 2002). If mtDNA point mutational burden (sometimes, less accurately, referred to as 'mutation rate') is to be formally quantified, three main methods have been compared: post-PCR cloning, single molecule PCR (smPCR) and random mutation capture (RMC) (Trifunovic *et al.*, 2004; Kraytsberg *et al.*, 2009). Their comparison has recently been evaluated by Greaves and colleagues (Greaves *et al.*, 2009). All are technically challenging and give conflicting *absolute* measures of mtDNA mutation burden; however all have proven useful in the *comparative* analysis of patient / experimental groups.

1.2.4 'Next-generation' re-sequencing for mtDNA

Most recently there has been great interest in the application of massively parallel ('next generation') re-sequencing (NGS) to the question of mtDNA mutations. Three main NGS platforms have been used to date: Illumina (previously Solexa) GA, Applied Biosystems SOLiD, and Roche 454 FLX GS. A small handful of studies (including two of my studies, presented herein) have now looked at the use of NGS to deep sequence mtDNA in search of low-frequency heteroplasmic variants (He *et al.*, 2010; Payne *et al.*, 2011; Payne *et al.*, 2013b). A larger number of studies have used NGS technology

to simply obtain a consensus mtDNA sequence (Hudson *et al.*, 2012). Although the practical approach is essentially the same in these two scenarios, the questions being asked are different, and the challenges, particularly in analysis, are far more substantial in the case of deep sequencing. Firstly the question is one of coverage per base position. In order to obtain a good consensus sequence, coverage of at least ~30-fold per base position is typically planned in the experimental design. Inevitably, with all NGS platforms, there may be considerable variation in coverage between base positions. The reasons for this are not fully defined, but are likely to relate, at least in part, to local sequence context (for example G-C content). Thus by extension, if one wishes to deep sequence then this degree of over-coverage ideally needs to be built in to the experimental design with respect to the 'minor allele frequency' of interest. For example, if one is interested in examining heteroplasmic variants at the 1% level, then 30-50 fold coverage would ideally be designed for that variant, therefore a total of 3000-5000 fold over-coverage per base position. The standard NGS approach for large genomes is fragment sequencing. Thus total DNA extract (for example from human blood, or from a bacterial culture), is fragmented (ultrasonically or enzymatically) into small fragments (typically ~200bp) to which 'adapter' DNA oligomers are then ligated. These fragments are typically then PCR enriched before sequencing. The issue of variable coverage presumably reflects the fact that fragments from some parts of a genome participate in these reactions less efficiently than others. NGS of mtDNA creates the additional challenge of isolating the mtDNA from nDNA. Although mtDNA is present at hundreds to tens of thousands of copies per cell, owing to the small size of the mitochondrial genome, mtDNA amounts to only 1-2% of the total DNA content (mass) within a cell. Thus if total cellular DNA extract is subjected to fragment NGS, the signal from mtDNA will be negligible. Furthermore, in this scenario there is a substantial potential for confounding by nuclear mitochondrial sequences (NumtS, or pseudogenes) (Simone *et al.*, 2011). Currently, three potential solutions exist. All were compared in the original mtDNA deep sequencing by NGS paper (He *et al.*, 2010). Firstly there is physical enrichment of mtDNA by methods based on ultracentrifugation, sometimes termed a 'mito-prep'. Technically such methods are considered easiest to perform on cultured cells; however methods do exist for fresh muscle biopsy samples, and in diagnostic practice may be used to obtain mitochondrial fractions for biochemical analysis. Evidence suggests that these methods are very variable in the extent to which they enrich the mtDNA fraction. An optimal result of enrichment might be considered a DNA extract that comprises 75% mtDNA. This represents a ~40-fold enrichment. In

contrast however, a 10-fold enrichment, which might be considered adequate for other applications, would preclude the reliable deep-sequencing of mtDNA (as it achieves an mtDNA content of only ~10-20%). Furthermore, if there is any nDNA carry-over (which is essentially inevitable), then the issue of NumtS remains. However, the key theoretical advantage of a 'mito-prep' is that it is not PCR-dependent, which potentially reduces PCR error, and thus noise, as will be discussed further below. The second method of mtDNA enrichment is long-range PCR. Typically the mitochondrial genome is first PCR amplified by a small number of over-lapping long-range PCR amplicons. This is currently probably the preferred approach, as it is relatively quick to perform, and it may be possible to avoid NumtS, by designing long-PCR primer pairs to avoid their amplification. It will generally be easier to avoid amplification of NumtS with long rather than short amplicons. The long-PCR products are then purified, fragmented and sequenced in essentially the same way as total DNA. The disadvantage of this approach is the additional long-PCR step which has the potential to introduce PCR error. Polymerases with high fidelity and 3'-5' exonuclease activity should be used in an attempt to reduce this issue. A harder to define issue is that of amplification bias, that is, the potential of some molecules to be preferentially amplified within the PCR reactions. Experience suggests that long-PCR may be particularly prone to this phenomenon, with the possibility that only a minority of DNA molecules actually participate in the amplification reaction. Finally, long-PCR enrichment will not significantly improve the issue of variable coverage, as fragmentation is still required. In fact the presence of long-PCR amplicon ends, and areas of overlap, may actually compound this issue. We have used long-PCR enrichment to perform NGS (using the Roche 454 FLX GS platform) of mtDNA where the aim was a consensus read (Hudson *et al.*, 2012). I have also used this approach for the deep sequencing of mtDNA using the Applied Biosystems SOLiD platform (unpublished observations). The third approach to mtDNA enrichment is by making short PCR amplicons. The advantage of this approach is that it negates the need for fragmentation, as amplicons can be designed that are already of suitable size for sequencing (with the relevant adapter sequences incorporated into the amplicon generation primers). This approach is best suited to the 454 FLX platform, as this allows relatively long amplicons (typically 250-400bp, although even longer amplicon protocols are now available). This technique has been the method of choice for deep sequencing small genomes, such as regions of viral genomes. Over-coverage can be very accurately planned as variable coverage will not be an issue. Furthermore, bioinformatic alignment is usually technically easier as the locus of each amplicon is

pre-defined. Conversely, the disadvantage of short amplicon re-sequencing for mtDNA is that a large number of amplicons would need to be generated to cover the entire mtDNA genome, therefore the preparation would be time-consuming. Furthermore it will be impossible to avoid NumtS in many parts of the mitochondrial genome with short amplicons. I have extensively explored the use of short amplicon deep sequencing of mtDNA using the 454 FLX platform, as described in the work presented herein (Payne *et al.*, 2011; Payne *et al.*, 2013b). I elected to use two amplicons in areas of the mitochondrial genome predicted to have contrasting rates of base substitutions (based on population polymorphism data) (Pereira *et al.*, 2009). Both these amplicons were specifically designed to avoid NumtS, and this was confirmed empirically by demonstrating failure to amplify a product from rho_0 DNA (DNA that has been entirely depleted of mtDNA, as detailed in my papers).

As discussed above, in order to use NGS for deep sequencing, a high degree of over-coverage must be used to ensure adequate coverage of low level heteroplasmic variants. In general, sufficient coverage can be relatively easily achieved, in particular with Illumina GA where coverage of mtDNA typically attains tens or hundreds of thousands of fold. Thus the depth to which low level heteroplasmic variants can be resolved is not often limited in practical terms by coverage. However, coverage is not the only determinant of resolution. For example, 500,000-fold coverage could be achieved on the Illumina GA, thus theoretically giving 50-fold over-coverage of a heteroplasmy present at 0.01%. However, pragmatically such a variant could not be resolved owing to noise limiting the sensitivity of the assay. Noise is likely to be multi-factorial owing to the multi-step nature of the assay: primary PCR error (short amplicon or long-PCR), enrichment PCR (in the case of fragment sequencing), sequencing reaction PCR (e.g. cluster generation for Illumina GA, or emulsion PCR (emPCR) for 454), and base-calling. Technically therefore, in comparison with other methods of mtDNA mutation detection, NGS is most akin to post-PCR cloning (as it involves a primary PCR step). However, it has the significant advantage of vastly higher throughput.

The exact error rate of NGS for mtDNA, and thus the achievable lower limit of resolution is a subject of on-going debate. It seems likely that the reading of the sequence (base-calling) is the step (of the several outlined above) that contributes the

vast majority of error. In the case of the PCR-dependent steps, if suitably high fidelity polymerases with proof-reading activity are used (such as pfu or KOD), error rates as low as 10^{-6} per bp should be achievable for the PCR steps. In contrast the base-calling error rate has been estimated to lie in the range of ~0.1-2%. Unfortunately there have been no direct head-to-head studies between platforms, which would be a very welcome addition to the field. With the kind of base-calling error rates suggested above, combined with adequate coverage, a lower limit of heteroplasmy resolution of ~2% has been suggested from theoretical modelling (Li and Stoneking, 2012). Previous studies have generally chosen to adopt a heteroplasmy threshold above which variants are considered to be 'real'. Thus the prototype study adopted a threshold of 1.5%, whereas the threshold in some subsequent studies has been as high as 10% (although coverage was rather inadequate in that paper) (He *et al.*, 2010; Li *et al.*, 2010).

Although heteroplasmy resolution of ~2% represents a significant advance on Sanger sequencing (limit of heteroplasmy detection ~20%), from first principles we may expect that *de novo* mtDNA mutations will initially be present at extremely low levels, and thus would be below the limit of detection of such an NGS assay. Thus the assay is geared only to detect higher level heteroplasmic variants, which are expected to have either been transmitted (maternally inherited) at low levels, or represent hot-spots for recurrent mutations (for example some mtDNA non-coding control region loci). Ideally however we would like to develop ways to go below this relatively modest threshold in the hope of examining rarer variants, which are more likely to truly represent new somatic mutations. The solutions to this potentially lie in a combination of technical and bioinformatic advances. Firstly there are simple bioinformatics tools that can be used to remove substantial amounts of noise, for example by the use of bidirectional read data. Thus only a variant that is present in both read directions is accepted. I have used this method in one publication herein (Payne *et al.*, 2013b) as well as in the analysis of SOLiD data (unpublished). The justification is that base-calling errors are likely to be unidirectional (or at last markedly asymmetric) as they will be sequence context specific. This argument probably applies most strongly to the 454 FLX platform, where pyro-sequencing error is well known to be very largely dependent on long homopolymeric tracts. We thus also arrive at the consideration that the base-calling error rate of a platform may in some cases (notably 454 FLX pyro-sequencing) be rather non-random, whereas in others (Illumina GA) it may be more truly random.

Paradoxically therefore, non-random error may actually be advantageous in attempting to improve depth of resolution as analytical approaches can be used which simply filter out positions known to be noisy, as I have demonstrated in the work presented herein. The disadvantage of this approach is that true variants within these positions will also not be called, and thus a complete sequence (including heteroplasmies) for the entire amplicon cannot be achieved. However, in my work I have favoured optimising specificity at the expense of sensitivity, especially when my concern was to determine whether such low level heteroplasmic variants were 'universal' (i.e. present in all individuals). Finally one can run suitable technical negative controls to empirically determine the validity of the bioinformatic approach and the putative lower limit of resolution. Usually this will involve running a cloned DNA sample, where it is assumed that all detected variants represent noise. (In fact, this will not be quite true as even the biological polymerase of the cloning vector will have a small error rate.) Not all studies have included such a control, and some have used the commercially available controls such as a DNA phage (Li *et al.*, 2010). The disadvantage of this approach is that it is not sensitive to any context specific error rate changes, and I have thus used clones of the actual mtDNA amplicons. In this way I have shown that my approach has a lower limit of resolution of ~0.2%, a significant improvement on prior work.

Finally, since my work, further technical advances have been published, based on the principle of unique molecular tagging. This was first applied with NGS (454 FLX) for HIV RNA deep resequencing, but related methods have now been published for mtDNA (Jabara *et al.*, 2011). The essence of the method is that, at an additional initial step, individual molecules of nucleic acid are given unique molecular tags (through the use of oligomers with multiple degenerate base positions). Thus all duplicate molecular tags are excluded in the analysis (they are assumed to be PCR duplicates), and if multiple reads with the same molecular tag show different variants, then only the consensus of those reads is taken, on the assumption that the less frequent variants are PCR errors. In order to calculate the variant frequency, one adds up the number of unique molecular tags that carry the same variant.

In conclusion, although deep sequencing of mtDNA by NGS does not offer the extremely high depths of resolution cited for smPCR or RMC, it has the great

advantages of massively higher throughput, and very broad coverage. The recent methodological advances should allow NGS to address some of the fundamental questions in mtDNA biology, for example to what extent low level variants are transmitted, and how variants accumulate with age. These questions have fundamental implications for our understanding of inheritance, population evolution, and ageing.

1.3 Inherited disorders of mtDNA maintenance

Primary mtDNA disorders arise due to primary mutations with the mitochondrial genome, as single base substitution mutations (for example MELAS, m.3243A>G) or single large-scale deletion mutations. In contrast, the disorders of mtDNA maintenance comprise mutations of nuclear genes which directly or indirectly affect mtDNA replication and maintenance. The prototype example is defects in *POLG*, encoding the mtDNA pol γ, and dozens of mutations have now been described within this gene (Hudson and Chinnery, 2006). More recently pathogenic mutations have been reported in other nuclear genes such as *TWINKLE* and *ANT1* (Copeland, 2008). These disorders are both genotypically and phenotypically diverse, but in all cases the disease is felt to be caused by the secondary effects on mtDNA and consequent disruption of cellular oxphos function. In some cases they cause severe infantile onset disease. In these cases the principle secondary mtDNA defect is profound depletion of mtDNA (reduction of cellular mtDNA content), presumably due to profound difficulty in adequately synthesising mtDNA. However, in many cases, disease is typically late onset (in middle age). In these cases the secondary mtDNA defect takes the form of somatic (acquired) mutations in mtDNA. These disorders predominantly affect neuromuscular tissues, which have a high energy (ATP) requirement, and are comprised of post-mitotic (non-dividing) cells. In these tissues the predominant mtDNA mutations are large-scale deletions, and in diagnostic practice, such disorders are often screened for by detecting the presence of multiple large-scale deletion mutations within DNA extracted from skeletal muscle biopsy.

1.4 Acquired mtDNA mutations, neurodegeneration and ageing

MtDNA has a mutation rate estimated to be ~5-15 times that of the nuclear genome, and somatic mtDNA mutations are well described in normal human ageing (Linnane *et al.*, 1989; Corral-Debrinski *et al.*, 1992a; Richter, 1995; Michikawa *et al.*, 1999; Fayet *et*

al., 2002; Taylor and Turnbull, 2005; Bua *et al.*, 2006). Ageing individuals have been shown to have gradual accumulation of COX-deficient cells in multiple tissues (including skeletal muscle, cardiac muscle, neurons, and colonic crypts) (Muller-Hocker *et al.*, 1992; Brierley *et al.*, 1996; Brierley *et al.*, 1998; Taylor *et al.*, 2003; Bender *et al.*, 2006). Such cells contain high proportional levels of somatic mtDNA mutations which are unique within each cell. The degree to which such mtDNA damage is causal in the ageing process remains a subject of much research, however recent supporting evidence has come from elegant mouse models, where a proof-reading deficient pol γ leads to accelerated accumulation of mtDNA mutations and a prematurely aged phenotype (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). Finally somatic mtDNA mutations have been linked with a range of common neurodegenerative diseases of ageing. For example brain tissue shows accumulation of somatic mtDNA mutations with normal ageing, but these are further increased in Alzheimer Disease (Corral-Debrinski *et al.*, 1994; de la Monte *et al.*, 2000; Lin *et al.*, 2002; Coskun *et al.*, 2004; Wang *et al.*, 2005).

1.4.1 The early mutation hypothesis and ageing

Critical to our understanding of the role of somatic mtDNA mutations in ageing and in disease states is a consideration of the role of heteroplasmy. As described above, a pathogenic mtDNA mutation arising within a cell can only cause a functional defect within that cell if it manages to exceed a critical threshold. Given that such a mutation will arise as a single event among potentially thousands of wild-type mtDNA molecules, how does this occur? The 'relaxed replication' hypothesis of clonal expansion, as outlined above, predicts that although mtDNA mutation events may occur throughout life, the mutation events that ultimately lead to cellular COX defects probably need to occur very early in life (perhaps during childhood or adolescence). Modelling experiments suggest that, based on a non-selective process of clonal expansion, continuous mtDNA replication over a period of decades is required for some mutant mtDNA species by chance to achieve the required levels within cells to cause a functional (COX) defect (Elson *et al.*, 2001). Thus a fundamental implication of this 'relaxed replication' model is that it requires a great deal of time for these seeding mutations to clonally expand, and has thus been termed the 'early mutation' hypothesis (Khrapko, 2011). Support for this model comes from recent observations, including some I present herein, that although the proportion of COX deficient cells (which

contain these clonally amplified somatic mtDNA mutations) progressively increases throughout older age, the overall number of mutations ('mutation burden') in the tissue homogenate, does not significantly increase (Greaves *et al.*, 2009; Payne *et al.*, 2013b). This recent observation would be consistent with a notion that mutations are present from early in life and only a tiny proportion ever become clonally expanded.

Finally, the 'earliest point' in childhood for a 'somatic' mtDNA mutation to appear, would be if it were in fact inherited. Very low level inherited variants could be present at birth, below the limit of detection, but over time these variants could occasionally clonally expand within cells. They would thus appear as ostensibly somatic mutations. If this were true, this would tend to give further weight to the 'early mutation' hypothesis, and would also suggest that strategies to target mitochondrial ageing may be better targeted at clonal expansion, rather than mutagenesis. I explore this very fundamental issue in mtDNA biology in a paper presented herein (Payne *et al.*, 2013b).

1.5 HIV infection and anti-retroviral therapy

In many ways anti-retroviral therapy is one of the great success stories of modern medicine. Since 1996, the standard of care has been combination anti-retroviral therapy (cART, often also referred to as highly-active anti-retroviral therapy, HAART) (Collier *et al.*, 1996). This therapy uses at least 3 drugs from at least two classes, and leads to durable suppression of viraemia without the emergence of viral resistance in most cases. This in turn allows the restoration of immune function, and prevents opportunistic infections.

The earliest anti-retroviral drug class were the nucleoside analogue reverse transcriptase inhibitors (NRTIs). The first of these, zidovudine (AZT) has been used since 1987 (Fischl *et al.*, 1987). The clinical action of the drug arises as it lacks a 3' hydroxyl group compared with the natural nucleoside (thymidine). It therefore causes chain termination if incorporated into an elongating HIV cDNA molecule. There are now more than twenty licenced anti-retroviral drugs spread across multiple classes. Most target viral enzymes: reverse transcriptase inhibitors, protease inhibitors (PIs), integrase inhibitors, and fusion inhibitors. One drug, maraviroc, an entry inhibitor, binds to a human target, the CCR5 co-receptor, which most HIV strains use. However, NRTIs still remain the

mainstay of HAART, and the vast majority of regimens comprise two NRTIs, combined with a third agent (usually a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a PI). The initial NRTIs to be developed following AZT were didanosine (ddI), zalcitabine (ddC), stavudine (d4T), and lamivudine (3TC). More recently abacavir (ABC), tenofovir (TDF, a *nucleotide* RTI) and emtricitabine (FTC) have been licenced. Of these various options, in industrialised countries combinations of TDF/FTC, and ABC/3TC are overwhelmingly used. These choices are principally due to favourable toxicity profiles compared with older NRTIs. In contrast in the developing world, d4T and AZT remain in common use. ddC is no longer licenced. However, in industrialised country cohorts there are significant numbers of patients who have extensive prior exposure to the older NRTIs (AZT, ddI, ddC, d4T). The significance of this lifetime burden of NRTI exposure is central to the arguments outlined in my publications herein.

1.6 Anti-retroviral therapy and the polymerase γ hypothesis

Early in the anti-retroviral era it was recognised that a significant minority of patients experienced serious acute or sub-acute treatment toxicity such as symptomatic lactic acidosis and hepatic steatosis, which resembled severe inherited mitochondrial disease (Bissuel *et al.*, 1994). Such acute effects were generally reversible with cessation of the causative drug (Helbert *et al.*, 1988). Later it was proposed that more common and insidious treatment complications such as peripheral neuropathy and lipodystrophy may also be mitochondrially-mediated (Carr *et al.*, 2000; Dalakas *et al.*, 2001). Mechanistically, it became apparent from *in vitro* data that many of the older NRTIs cause inhibition of pol γ, and thus inhibition of mtDNA replication, manifesting *in vitro* and *in vivo* as a reduction in cellular mtDNA content (depletion) (Arnaudo *et al.*, 1991; Casademont *et al.*, 1996; Dalakas *et al.*, 2001; Lewis *et al.*, 2001; Lim and Copeland, 2001; Cherry *et al.*, 2002; Cote *et al.*, 2002; Miro *et al.*, 2003; Nolan *et al.*, 2003; Walker *et al.*, 2004; Buffet *et al.*, 2005; Haugaard *et al.*, 2005; Cherry *et al.*, 2006). This biochemical effect arises in the same manner as the therapeutic effect of NRTIs on HIV reverse transcriptase (HIV-RT), namely chain termination during DNA synthesis. The affinity of NRTIs for mtDNA pol γ has been estimated to be ~500 fold less than that for HIV-RT; however this varies between specific drugs. The hierarchy of pol γ *in vitro* inhibition is described as: ddC>ddI>d4T>AZT=3TC>ABC, a notion which fits reasonably well with *in vivo* data on observed severity of mtDNA depletion, and with the frequency of observed treatment complications (Lim and Copeland, 2001). Of the

N(t)RTIs in current common clinical usage, both ABC and TDF are reported to have minimal effect on mtDNA replication (Johnson *et al.*, 2001; Birkus *et al.*, 2002; McComsey *et al.*, 2005b; Venhoff *et al.*, 2007).

1.6.1 Mitochondrial DNA mutations and anti-retroviral therapy

It follows from the polymerase γ hypothesis that if the causative NRTI is removed, then mtDNA levels will recover, and indeed there is *in vivo* data to support this (McComsey *et al.*, 2005b). Although most patients in industrialised countries are now treated with ABC or TDF based HAART, there are large numbers of patients who have had many years of exposure to the pol γ inhibiting NRTIs (ddC, ddI, d4T, AZT) in the past. These patients will not be expected to show persistent mtDNA depletion, and indeed I have now demonstrated this (Payne *et al.*, 2011). If there is any persistent effect on mtDNA in such patients it is therefore likely to be a qualitative defect: somatic mutations. Based upon our knowledge of the action of NRTIs, coupled with our understanding of mtDNA mutation formation, there are several hypothetical means by which NRTI therapy could promote mtDNA mutations (as discussed further below). The first evidence that NRTIs may indeed lead to somatic mtDNA mutation came from a longitudinal study of patients commencing d4T therapy. Surprisingly, 5 of 16 subjects developed new mtDNA mutations in PBMCs (peripheral blood mononuclear cells) after starting therapy (Martin *et al.*, 2003). Shortly thereafter, another group described a similar phenomenon, again in blood (McComsey *et al.*, 2005a). These observations have been revisited only recently when a small series of papers have sought to detect anti-retroviral associated acquired mtDNA mutations in a variety of settings (Ortiz *et al.*, 2011; Jitratkosol *et al.*, 2012). In my work I demonstrate that patients with a history of exposure to pol γ inhibiting NRTIs show an excess of skeletal muscle fibres containing high levels of somatic mtDNA mutations (both point mutations and large-scale deletion mutations) (Payne *et al.*, 2011). Importantly the relevant drug exposures were often in the remote past, suggesting that the mutations are indeed irreversible. Studies examining mtDNA mutations in the setting of HIV / anti-retroviral therapy are presented in **Table 2**.

1.6.2 What is driving NRTI induced mtDNA mutation?

The intuitive deduction when considering mechanisms whereby anti-retroviral therapy may increase somatic mtDNA mutations is accelerated *de novo* mutagenesis. This is

essentially the correlate of an inherited *POLG* defect, whereby there will be continuous production of higher levels of mtDNA mutations throughout a treatment period. There are plausible biological mechanisms whereby this might occur, for example impairment of the limited exonuclease function of pol γ by NRTIs would lead to poor proof-reading. This biochemical effect has been reported at least for AZT (Lim and Copeland, 2001). Furthermore it is suggested that, in addition to any effects on pol γ, AZT may inhibit TK2 (mitochondrial thymidine kinase) plausibly leading to purine / pyrimidine imbalance and thus mtDNA mutations (Rylova *et al.*, 2005; Akman *et al.*, 2008). The difficulty with this *de novo* mutagenesis hypothesis however, is that such mutations will need to undergo clonal expansion before they achieve high enough levels within cells to cause a functional (COX) defect. Under a simple 'relaxed replication' model (as described above), these mutations will be predicted to take many years or decades to reach high levels within cells. It would therefore be predicted that a typical period of NRTI exposure during early adult life would lead to a COX defect only in late middle age, if ever (**Figure 1**). Furthermore modelling data suggests that the relative increase in mutation rate would need to be very substantial indeed to cause a significant increase in COX defects (Elson *et al.*, 2001).

Therefore, I have proposed an alternative hypothesis of accelerated clonal expansion (Payne *et al.*, 2011). This hypothesis requires no additional *de novo* mutagenesis, as clonal expansion can act on the pre-existing somatic mtDNA mutations which arose early in life, as described previously. Modelling this scenario suggests that a finite period of mtDNA depletion associated with NRTI therapy, creates an intracellular 'bottle-neck', leading to accelerated molecular segregation and thus accelerated clonal expansion of pre-existing (age-associated) mtDNA mutations. The severity of mtDNA depletion will predict the rapidity of clonal expansion, and this is in keeping with my empirical data whereby potent pol γ inhibitors (ddC, ddI) which cause profound mtDNA depletion, caused a much higher number of COX deficient fibres (i.e. more rapid clonal expansion) than weaker inhibitors (d4T, AZT). Importantly this model acts rapidly during a period of NRTI treatment (as the 'seeding' mutations are already present), and furthermore the mutations are 'locked in' at the end of the period of NRTI exposure as they have already reached high levels in some cells, a 'point of no return'. In conclusion, the resultant COX defect is predicted to appear rapidly during the causative NRTI therapy, and be persistent thereafter.

Finally, a variation on a non-selective clonal expansion model is the idea that NRTI therapy may select for mutant mtDNA. This is certainly plausible in the case of large-scale deletion mutations, where we may suggest that deleted (smaller) mtDNA molecules would replicate more readily in the face of NRTI-induced pol γ inhibition than full-size molecules. There is analogous data to support this notion from the non-HIV setting, as previously described but NRTI-specific data is awaited (Diaz *et al.*, 2002). Such a process would ultimately serve to further accelerate clonal expansion.

1.7 The functional consequences of somatic mtDNA damage

The evidence that somatic mutation in mtDNA and associated COX defects are *temporally* related to the normal human ageing process is increasingly robust, however the key question remains to what extent they are *causally* related? The *POLG* 'mutator' mouse would seem to provide evidence for a causal relationship in that, as far as we know, the only difference between this mouse and the wild-type is that it accumulates mtDNA mutations at a significantly increased rate, leading to a progeroid phenotype (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). However, in the case of human *POLG* defects, or other inherited disorders of mtDNA maintenance, there is also more rapid accumulation of secondary mtDNA defects, but the phenotype is usually one of late-onset neurodegenerative disease, but not premature ageing. Clearly therefore, the situation is more complex. Khrapko has recently discussed important differences between the premature ageing in the *POLG* mouse and normal human ageing (Khrapko and Vijg, 2007). It turns out that levels of mtDNA mutations in the homozygous *POLG* mouse are vastly (several orders of magnitude) increased compared with the wild-type mouse, even in the case of a very elderly wild-type animal. Furthermore, the heterozygous *POLG* mouse shows an intermediate level of mtDNA mutation, but an apparently normal phenotype. It appears therefore that there must be a very large increase in mtDNA mutation rate in the mouse, above a rather high threshold, in order to cause a progeroid phenotype. If we then consider the mtDNA mutation rate in an elderly human, this appears to be greater than that in an elderly wild-type mouse, but less than that in the *POLG* heterozygous mouse. This observation has led some writers to suggest that, because the mtDNA mutation rate in the elderly human clearly falls well below the threshold for a functional effect in the mouse, then this implies that mtDNA

mutations are not causally related to normal ageing in humans. The flaw in this argument is potentially however the very great difference in normal lifespan in humans (>80 years) and mice (<3 years). It appears that the rate of mtDNA turnover (mtDNA 'half-life') does not alter significantly between species. Therefore the elderly human has experienced far more cycles of mtDNA replication than the mouse. As described earlier, mtDNA turnover is thought to be the 'engine' driving clonal expansion of mtDNA mutations. Thus in a long-lived mammal such as the human, a low mutation rate may well still be entirely compatible with a functional role in ageing given the long period of time for those mutations to clonally expand within individual cells and lead to defects of oxidative function within those cells.

Moving away from animal models, is there evidence that humans with healthy mitochondria are less 'biologically aged' than humans of equivalent chronological age who have greater somatic mitochondrial defects? Early work from Doug Turnbull's group suggested that elderly patients with increased physiological performance (for example grip strength); tended to have lower proportional COX defects on lower limb skeletal muscle biopsy (Brierley *et al.*, 1996). Of course, this observation could either be compatible with the notion that slower accumulation of mtDNA defects results in preserved function, or that preserved muscular function (for example through better general health, and higher levels of exertion), results in the preservation of mitochondrial function. Further longitudinal studies are desperately needed in this area to better address such questions. However, such studies are difficult to do owing to the vast timescales involved; as described earlier, a COX defect in an individual in their seventh decade might have resulted from a new mutation event several decades earlier.

Is there other indirect evidence to support a causal role for mtDNA mutation in ageing? If certain mtDNA genotypes were more or less susceptible to somatic mtDNA mutation, then we might expect to see differences in the rate of ageing between such populations. Work by David Samuels suggests that this notion may hold true when comparing animal species. As described earlier, short mtDNA sequence repeats, are strongly associated with the formation of large-scale mtDNA deletion mutations. Samuels demonstrated that species with lower numbers of such homologous repeats in their mtDNA genome show increased longevity (Samuels, 2004). Within the human species,

mtDNA is also highly polymorphic, and one mtDNA haplogroup (D4a) contains SNPs (single nucleotide polymorphisms) that disrupt that 13bp repeat associated with the δ4977bp 'common deletion'. The D4a haplogroup contains unexpectedly large numbers of centenarians (Bilal *et al.*, 2008). Finally another polymorphism in the non-coding mtDNA control region, results in a change in the origin of mtDNA replication, with predicted consequences for mtDNA turnover. This group also shows increased longevity (Zhang *et al.*, 2003).

1.7.1 Clinical implications of mitochondrial ageing in HIV-infected patients

The increasing evidence for accelerated mtDNA somatic mutation accumulation in the setting of certain NRTI therapy may currently be considered principally as 'accelerated intrinsic ageing'. The field of biogerontology, although rapidly evolving, is still in its relative infancy, and the downstream effects of intrinsic ageing at the tissue and organism level are becoming increasingly well defined. Of particular note are the recent links made between mitochondrial and telomere function in ageing, thus bringing together two fundamental tenants of biogerontology (Sahin *et al.*, 2011). On this note, it is interesting to observe that certain NRTIs (including TDF) are suggested to have some affinity for TERT (telomerase reverse transcriptase), suggesting links between anti-retovirals and telomere biology (Hukezalie *et al.*, 2012). Further research is warranted.

The downstream consequences of mtDNA somatic mutations at the tissue level are shown in **Figure 2**. It seems likely that 'mitochondrial ageing' in NRTI-treated HIV-infected patients might have a role in driving frailty and perhaps sarcopenia in this patient group. Both these gerontological markers have been shown to be prevalent in HIV-infected patients and in the non-HIV setting are predictive of adverse clinical outcomes (Chabi *et al.*, 2008; Waters *et al.*, 2009; Cruz-Jentoft *et al.*, 2010; Erlandson *et al.*, 2013). Simple clinical measures of 'ageing' are lacking, and extensive long-term follow-up may be required to establish a clear causal link between markers of intrinsic ageing in HIV and clinical outcomes. These findings are likely to be of most relevance to those patients with extensive past exposure to pol γ inhibiting NRTIs who are entering older age. This specific subgroup of patients should be considered for particular close study within clinical cohorts for evidence of accelerated frailty, and appropriate multi-disciplinary measures implemented. Finally, millions of patients in

the developing world have been exposed to AZT and d4T in recent years as part of anti-retroviral roll-out programmes and these data are likely to reinforce the WHO position that such patients should be switched to TDF based therapy as soon as is feasible (WHO, 2006).

Finally, we may turn the question on its head and ask, 'what can the NRTI story tell us about the role of mtDNA in normal human ageing?' It has been argued that although there are animal models causally linking mtDNA mutations and ageing the association in humans is less clear cut (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Trifunovic *et al.*, 2005; Khrapko and Vijg, 2007). Progeroid diseases in humans may be considered 'extremes' in which the ageing process is qualitatively as well as quantitatively altered. In contrast, in the setting of NRTI exposure, crudely we may have acceleration of one aspect of ageing at the molecular level in genetically normal persons, and a patient group in whom we have possible clinical evidence of premature ageing.

1.8 The need for biomarkers of mitochondrial dysfunction

In the study of mitochondrial dysfunction in inherited mitochondrial disorders and in normal ageing, skeletal muscle biopsy remains the 'gold standard' investigation. This arises from the fact that it is a relatively accessible tissue, contains large numbers of mitochondria, and the natural history of mtDNA mutations in this tissue is relatively well understood. However in studies related to ageing or when a therapeutic intervention is planned (which might include an anti-retroviral switch), methods more amenable to serial measurement would be required.

A possible candidate as a non-invasive measure is phosphorus magnetic resonance spectroscopy (^{31}P-MRS) of skeletal muscle. This technique uses MR imaging to obtain spectra from ATP metabolites. The subject performs a repeated exercise paradigm in the scanner, and metabolites are then tracked during recovery. Of principle interest is the rate of re-synthesis of ATP following its depletion during the exercise. Patients with mitochondrial dysfunction will be expected to show delayed ATP re-synthesis. In contrast a highly trained athlete may show more rapid ATP re-synthesis. Such abnormalities have previously been shown in a variety of inherited mitochondrial disorders, and the technique has been used in serial monitoring studies (Penn *et al.*,

1992; Chinnery *et al.*, 2001). In the context of HIV, a study from early in the epidemic used ^{31}P-MRS to evaluate patients with myopathy due to high-dose AZT monotherapy, but did not show clear differences compared to controls (Miller *et al.*, 1991). More recently, d4T has been studied in healthy controls, and acute defects on ^{31}P-MRS were observed (Fleischman *et al.*, 2007). The relevant question in the modern era though is whether patients hypothesised to have mitochondrial damage consequent on *prior* mitochondrially-toxic NRTI therapy, will have persistent defects of oxidative function detectable on ^{31}P-MRS which we address herein.

At one time measurement of mtDNA depletion in PBMCs (peripheral blood mononuclear cells) was proposed as a biomarker of clinical NRTI-induced mitochondrial toxicity. Although some studies have demonstrated significant correlations between mtDNA depletion and clinical abnormalities, such as lactic acidosis, a number of limitations to the use of PBMC mtDNA content have been described (Cote *et al.*, 2002; Montaner *et al.*, 2003). Firstly, PBMCs are not a tissue that demonstrates meaningful clinical mitochondrial toxicity, and in many studies mtDNA levels in PBMCs show poor correlation with those in the clinically relevant tissues (e.g. muscle, fat) (Cherry *et al.*, 2006; Maagaard *et al.*, 2006). Secondly, untreated HIV infection itself may also be associated with mtDNA depletion in PBMCs compared with healthy, HIV-uninfected, individuals (Miura *et al.*, 2003; Miro *et al.*, 2004). Although not conclusively proven, the most likely explanation for this observation is that cellular mtDNA depletion is a consequence of HIV-induced T-cell dysfunction. The significance of these observations is uncertain and there is no good data to suggest that cellular mtDNA content in other tissues is low in untreated HIV infection. Furthermore, with respect to the scenario in current developed world practice, the vast majority of patients are no longer taking the pol γ inhibiting NRTIs (ddC, ddI, d4T, AZT). Data suggests that mtDNA copy number in blood recovers on switching away from these agents (McComsey *et al.*, 2005b). This effect has been shown for switch to ABC, and would be predicted to also apply to TDF, where *in vitro* data suggests a lack of mtDNA depletion. Thus mtDNA copy number blood is predicted to reflect only current NRTI exposure and not mitochondrial damage from prior exposures.

Recently serum FGF-21 (fibroblast growth factor 21) levels have been proposed as a promising biomarker for inherited mitochondrial disease (Suomalainen *et al.*, 2011). FGF-21 is thought to increase mitochondrial oxidative function in a PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) dependent manner. Levels showed a very high predictive value for biopsy COX defect (area under ROC (receiver operated curve) = 0.95). Correlation was highest in children with early onset (therefore clinically severe) mitochondrial disorders. Such children may have very high level COX defects on biopsy (up to 60% of fibres). In contrast in adults with late-onset disease, which is more typical of the inherited disorders of mtDNA maintenance, phenotypes are less severe, and COX defects are less pronounced (typically <10%). Furthermore elevated serum FGF-21 levels have been reported in other metabolic disorders, including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus, and obesity (Moyers *et al.*, 2007; Chen *et al.*, 2008; Zhang *et al.*, 2008; Dushay *et al.*, 2010). This is likely to be because a major target for FGF-21 and PGC-1α activity is adipose tissue. Many patients with longstanding HAART-treated HIV infection will be expected to have increased frequency of the metabolic syndrome and insulin resistance (Carr *et al.*, 1998; Gan *et al.*, 2002). Although the aetiology of some of this pathology may overlap with that of acquired mitochondrial damage (for example d4T may cause both insulin resistance and pol γ inhibition), in many cases it will not (for example PIs may promote central obesity but not pol γ mediated mtDNA injury). Thus there may theoretically be heterogeneous reasons why FGF-21 levels may be expected to be elevated in HIV infection. My paper presented herein explores the determinants of elevated serum FGF-21 levels in HIV-infected patients on contemporary HAART (Payne *et al.*, 2013c).

1.9 Preventing somatic mtDNA mutation: therapeutic avenues

A rational approach to ameliorate the effects of age-associated mtDNA mutations depends to a large extent on how and when those mutations arise, and what their natural history may be in terms of the progression from a new mutation to a clonally expanded mutation within a single cell, leading to a functional defect. As described above, many of these fundamental aspects of mtDNA biology remain the subject of on-going research, and as such, discussion of how best to prevent these changes is currently largely a matter of speculation.

If one takes the view that *de novo* mtDNA mutations arise at significant levels throughout the normal human lifespan, and that these may clonally expand relatively rapidly (perhaps through positive selection), then prevention of new mutation formation may be of therapeutic benefit. In the case of mutations arising from ROS damage, anti-oxidant treatment might be proposed. Anti-oxidant compounds frequently show beneficial effects *in vitro* but almost none of these benefits have so far been translated *in vivo.* There are a few animal models which have suggested a beneficial effect. For example a mouse model has been developed which expresses a mitochondrially targeted antioxidant. These mice show reduced levels of ROS, reduced mtDNA mutations, and increased life span (Schriner *et al.*, 2005). Furthermore, a recent study in LHON (Leber's hereditary optic neuropathy) has shown benefit of idebenone in improving visual function in the setting of a randomised controlled trial (Klopstock *et al.*, 2011). The cause of visual failure in LHON is thought to be mitochondrial dysfunction, and idebenone is thought to act as an anti-oxidant. This may therefore represent the first beneficial effect of an anti-oxidant compound in a mitochondrial disorder. Clearly, more work needs to be done to elucidate the precise pathophysiological mechanisms affected, but these observations at the least suggest that this approach may be worthy of further study, including in related mtDNA disorders.

Alternatively it may be that natural replication errors are of primary importance. Thus one would wish to either increase the fidelity of pol γ, or increase mtDNA repair mechanisms. Currently there are no clear means to achieve either of these aims.

Conversely, we may take the view that clonal expansion is the more important process. As discussed above, there is some recent evidence to suggest the relative importance of clonal expansion in normal human ageing, and this is borne out in my own work in ageing, and NRTI therapy. If *de novo* mutations are therefore very early events, and clonal expansion is a slow process, as proposed in the 'relaxed replication' model, then targeting clonal expansion would seem attractive. Indeed, clonal expansion is a necessary step, no matter how slowly or rapidly it occurs. Currently, the key putative means to change the rate of clonal expansion is to modify mitochondrial biomass or mtDNA copy number (the two appear to be intrinsically linked). How may we therefore

increase cellular mtDNA content? In the case of *in vitro* studies this effect has principally been achieved by uridine supplementation (Walker *et al.*, 2006). However there is no good data to suggest that uridine supplementation is beneficial *in vivo,* at least in otherwise healthy animals or humans (McComsey *et al.*, 2010). It is likely that *in vitro* uridine prevents mtDNA content in cells from being constrained by the rigorous demands of continuous cellular replication. In a rodent model, uridine supplementation has however prevented the detrimental effects of NRTI treatment on the brain, presumably by preventing profound mtDNA depletion (Venhoff *et al.*, 2010). Another technique that has proved successful *in vitro* is supplementation with dNMPs. As we have shown, interestingly these supplements increase mtDNA content even in control cell lines, but have a greater effect in cell lines that have an mtDNA replication defect or are NRTI treated (Bulst *et al.*, 2012). However, the best evidence for such an approach probably comes from exercise studies. It has long been established that endurance exercise increases cellular mtDNA content. Frail elderly subjects show reduced cellular mtDNA content compared with active elderly and younger subjects. This reduction in copy number may plausibly accelerate the clonal expansion of mtDNA mutations within cells in these subjects, leading to a more rapid increase in COX defects. Exercise studies have been attempted in patients with inherited mtDNA defects (Murphy *et al.*, 2008). On serial biopsy, subjects with single deletion mtDNA disorders, showed a decrease in the proportion of COX deficient fibres, and an increase in COX intermediate fibres following a period of endurance exercise training. COX intermediate cells are suggestive of cells where wild-type mtDNA content is just about sufficient to maintain cellular oxphos function (Murphy *et al.*, 2012). The exercise-treated patients also have increased cellular mtDNA content. Finally, the most compelling evidence perhaps comes from a recent elegant paper using the pol γ 'mutator' mouse as described previously. When subjected to endurance exercise the homozygous mutant mouse appears phenotypically as the wild-type mouse, rather than developing the progeroid state. MtDNA content is significantly increased in the mouse subjected to endurance exercise. However, the mutant mtDNA proportion remains comparable with that observed in the homozygous mutant mouse that did not undergo endurance exercise (Safdar *et al.*, 2011). This suggests that by increasing mitochondrial biomass, endurance exercise cannot shift the proportional balance of wild-type and mutant mtDNA, but it can prevent the functional consequences of mutant mtDNA on the cell. These theoretical concepts, as applied to HIV infection and anti-retroviral therapy are shown in **Table 3**.

1.10 Fatigue and dysautomia

Fatigue is a highly prevalent symptom but its physiological basis remains poorly understood. By definition, patients with Chronic Fatigue Syndrome (CFS, also known as Myalgic Encephalitis / Encephalomyelitis, ME) have disabling fatigue in the absence of a known physical or psychiatric illness (Fukuda *et al.*, 1994). This case definition might imply that the aetiology of fatigue found in association with physical and psychiatric diseases is therefore different to that in CFS. In fact however, recent work suggests that symptomatology is very similar (Jones *et al.*, 2009). Furthermore, fatigue in chronic physical illness tends to correlate poorly, if at all, with traditional markers of disease severity, at least until that disease is very severe. For example in Primary Biliary Cirrhosis (PBC) which has been well studied as a prototype disease where fatigue is highly prevalent, fatigue does not associate with liver function (Newton *et al.*, 2006b; Pells *et al.*, 2013).

The extent to which physiological, as opposed to psychological factors contribute to the aetiology of CFS is a subject of on-going debate. Recently there has been increased interest in physiological factors. Orthostatic intolerance (dysautonomia) is highly prevalent in CFS as well as fatigue-associated chronic diseases (including PBC, and multiple sclerosis, MS), but not in chronic diseases in which fatigue is uncommon (for example primary sclerosing cholangitis, PSC) (Freeman and Komaroff, 1997; Rowe and Calkins, 1998; Schondorf *et al.*, 1999; Schondorf and Freeman, 1999; Flachenecker *et al.*, 2003; Chaudhuri and Behan, 2004; Newton *et al.*, 2006a; Newton *et al.*, 2007a; Newton *et al.*, 2007b; Newton *et al.*, 2007c). Furthermore the prevalence of fatigue has been shown to be high in patients with vasovagal syncope (VVS), a form of primary orthostatic intolerance (Legge *et al.*, 2008). The association between fatigue and orthostatic intolerance is co-linear in terms of the severity of both symptoms, and this observation holds equally well for CFS as for other chronic diseases. This is therefore further evidence that common physiological pathways may underpin fatigue in diverse chronic diseases as well as CFS. The therapeutic approaches that have been developed for CFS may therefore also be of some benefit to patients experiencing fatigue in association with chronic illnesses (NICE, 2007).

What may be the pathophysiological mechanism linking dysautonomia and fatigue? Preliminary observations have suggested that oxygen delivery to muscles and the brain may be dysregulated in patients with fatigue and dysautonomia, but much further work is needed to fully explore this hypothesis (Jones *et al.*, 2010). This observation does not tell us about the nature of the relationship in terms of whether or not it is causal, and in which direction the causality may lie. In the case of CFS, it perhaps seems more plausible that fatigue predates dysautonomia, perhaps initially as a response to an illness (for example viral infection). During a period of significant rest, and perhaps under the influence of neuroendocrine factors, autonomic function may become dysregulated. This may then drive further fatigue (for example through the pathways suggested above), and a vicious cycle may ensue. In the case of various fatigue-associated chronic illnesses, it seems equally plausible that either fatigue or dysautonomia came first, but in either case the end result is similar.

Should therapeutic approaches to CFS or fatigue in the context of chronic illness therefore target dysautonomia? It is possible to gain objective improvement in autonomic function through either physical measures (such as compression stockings), or pharmacological measures (such as fludrocortisone which will increase retention of salt and water, or midrodrine which increases vascular tone). To date no high-quality studies have suggested any benefit of these approaches on fatigue itself. This may be because studies did not stratify CFS patients into those with and without dysautonomia at enrolment. Alternatively, it may be that although peripheral vascular tone can be improved, there is not a corresponding beneficial effect on the important 'target tissues', for example brain and muscle.

What do we know about the role of mitochondria in fatigue? Preliminary magnetic resonance spectroscopy studies have suggested that muscle mitochondrial function is abnormal in the context of CFS and PBC. The principle abnormality seems to be delayed acid clearance following repeated exercise (Jones *et al.*, 2010). There may be a number of potential explanations for this observation. Firstly it may be that fatigue-induced deconditioning has caused a reduction in the oxidative capacity of the muscle. Secondly it may be that dysautonomia associated with the fatigue has adversely affected vascular supply, and thus oxygen delivery to the muscle, or the ability to appropriately

adapt this supply to the exercise challenge. Finally, in the case of PBC, it may be that the mitochondrion is a direct pathological target as the auto-antibodies seen in PBC (AMA, anti-mitochondrial antibody) are directed against a component of the respiratory chain.

1.10.1 Fatigue and HIV

Fatigue was a highly prevalent symptom in the pre-HAART era (Darko *et al.*, 1992). At this time, there was a correlation between fatigue and clinical disease state (i.e. the CDC stage, AIDS-defining illnesses etc.). Many such patients were profoundly immunosuppressed and had high levels of HIV replication, both in the periphery, and in the brain. It is therefore unclear to what extent fatigue should be expected to remain an issue in the current era where patients are generally well treated on HAART, with fully suppressed plasma HIV viral loads, and good immune reconstitution.

My work on fatigue in HIV presented herein, shows some interesting novel insights (Payne *et al.*, 2013a). Firstly fatigue was very common (affecting ~50% of patients) despite good immune function, and near universal HAART treatment. In keeping with CFS and other fatigue-associated medical disorders, there was a strong correlation of fatigue with dysautonomia symptoms. Again, the direction of a potential causal relationship is a subject for debate, but it seems at least likely that dysautonomia is an aggravating or perpetuating factor in this patient group. What may have been the initial precipitant of fatigue or dysautonomia? This may be heterogeneous between patients, as clearly not all are affected, despite seemingly similar courses of HIV infection. We may speculate that in some subjects the initial untreated HIV infection may have adversely affected the immune or neuroendocrine response, as has been hypothesised for CFS following a viral infection. Furthermore in the case of HIV infection, we know that in most cases, immune function remains qualitatively abnormal, with increased immune activation even in 'successfully' treated HIV (Jiang *et al.*, 2009). Alternatively, or additionally, some HIV infected patients may experience an adverse psychological reaction to their diagnosis which may contribute to the onset of fatigue.

In addition, I identified a sub-group of HIV-infected patients where fatigue was near universal. This group was characterised by longstanding HIV infection, long history of

anti-retroviral treatment, previous exposure to mitochondrially toxic anti-retroviral drugs (pol γ inhibiting NRTIs), and the presence of clinical side effects of such drugs, such as the lipodystrophy syndrome. It is certainly plausible that residual mitochondrial dysfunction contributes to on-going fatigue in this group.

1.11 Concluding remarks

There are numerous ways in which the older NRTI anti-retroviral drugs may increase somatic mutations in mtDNA, and I show empiric evidence of this herein. It is plausible that the mechanism is via an acceleration of the clonal expansion of pre-existing (age-associated) mtDNA mutations. Fuller understanding of the exact mechanism involved has potential implications for predicting the natural history of such mutations as patients continue to age.

Conversely, NRTI exposure arguably presents a rather unique scenario in which aspects of normal mtDNA maintenance are iatrogenically altered. Observations from NRTI treatment may further the debate on the fundamental biology of mtDNA mutations in normal human ageing.

Through my work I have defined a sub-group of mainly highly treatment experienced HIV-infected patients, with prior exposure to pol γ inhibiting NRTIs who show evidence of increased somatic mtDNA mutation and residual cellular mitochondrial dysfunction, long after such drugs have been switched to cleaner agents. This group should be the focus of close attention as they continue to age in order to determine the extent to which they will demonstrate accelerated frailty or increased comorbidity. I have already shown that increased fatigue may be one clinical consequence of this damage. Finally, given that many millions of patients in sub-Saharan Africa have been exposed to d4T in recent years as part of HAART rollout programmes, it remains to be seen what the extent of complications will be in this group in the future.

Figure 1. Early mutation hypothesis, clonal expansion, and putative role of nucleoside analogue reverse trancriptase inhibitors.

Diagrams show an individual COX (cyctochrome *c* oxidase) positive cell (brown) in a young individual eventually becoming COX deficient (blue) in later life, over a representative timescale. Multiple copies of wild-type mitochondrial DNA (mtDNA) are initially present within the cell, shown as green circular molecules (in reality '00's to '0,000's per cell). A mutation occurs (red molecule) which is initially at a low percentage heteroplasmy level within the cell, but over time may clonally expand to reach a high heteroplasmy level causing the COX defect. (a) Under an 'early mutation' hypothesis, new mtDNA somatic mutations that lead to COX deficient cells late in life, arise in early life and clonally expand very slowly, by a non-selective process of drift ('relaxed replication'). (b) Under an alternative model, mutant mtDNA clonally expands relatively rapidly within cells, and therefore the observed COX defect in late life is due to a relatively recent mutation. Such a process of rapid clonal expansion is likely to require a selective replicative advantage for mutant mtDNA. A hypothesis of accelerated clonal expansion of mtDNA mutations due to NRTI (nucleoside analogue reverse transcriptase inhibitor) exposure is most coherent with an 'early mutation' hypothesis (a) where the somatic mutations have already occurred by early adult life, and therefore expand to result in COX deficient cells many years earlier than expected.

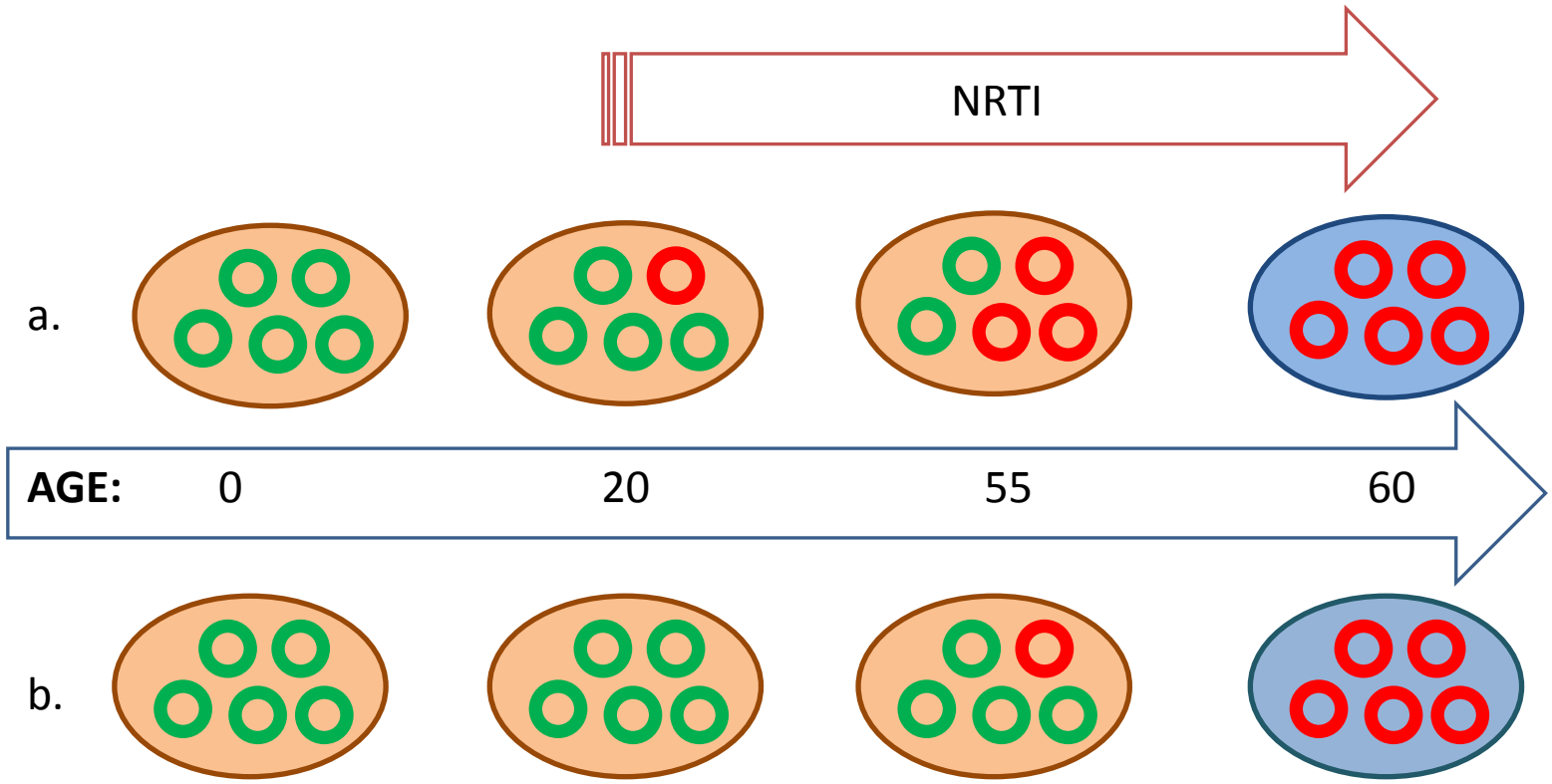
NRTI
a.
AGE:
0
20
55
60
b.

Figure 2. Hypothesised model of how age-associated somatic mitochondrial DNA mutations may lead to a functional defect at the tissue level.

Somatic mutations may arise either through oxidative damage to mitochondrial DNA (mtDNA), for example, due to reactive oxygen species (ROS), or through natural replication errors. MtDNA mutations may cause synthesis of abnormal respiratory chain proteins, leading to partial uncoupling of the mitochondrial respiratory chain. This may lead to a vicious cycle of increased ROS. In order for somatic mtDNA mutations to cause a functional mitochondrial (COX, cytochrome *c* oxidase) defect at the cellular level, the mutation must clonally expand to reach a high percentage level within the cell. Frequent COX deficient cells will decrease function of the tissue, and may also undergo apoptosis. Boxes shown in red may plausibly be adversely affected by HIV or NRTI (nucleoside analogue reverse transcriptase inhibitor) anti-retroviral therapy (ART) exposure.

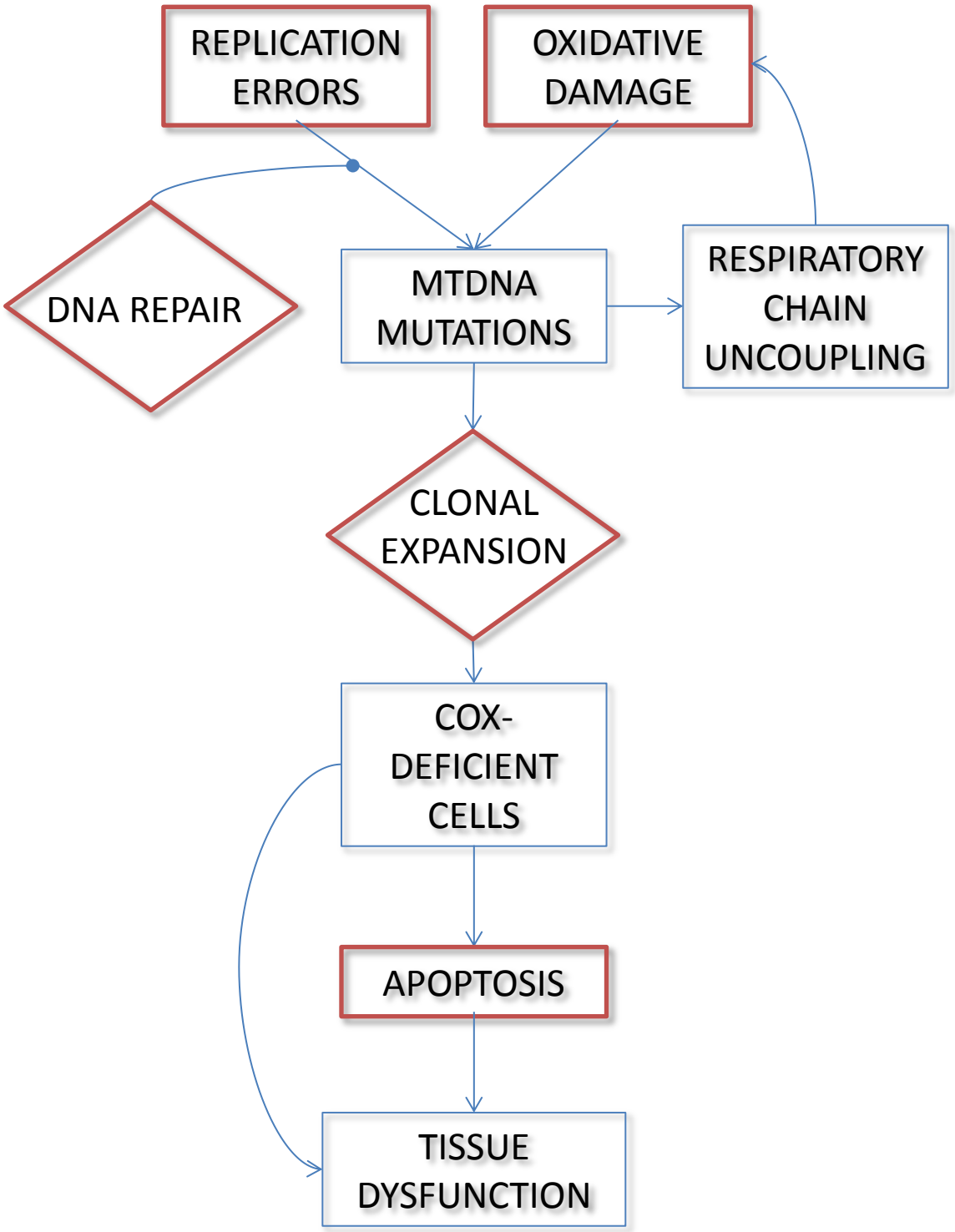
REPLICATION ERRORS
OXIDATIVE DAMAGE
DNA REPAIR
MTDNA MUTATIONS
RESPIRATORY CHAIN UNCOUPLING
CLONAL EXPANSION
COX-DEFICIENT CELLS
APOPTOSIS
TISSUE DYSFUNCTION

Table 1. Overview of current methods for detecting or quantifying mitochondrial DNA mutations.

Method	Point mutations or deletions	Detection or quantification	Advantages	Limitations
Post-PCR cloning	Point mutations	Detection and quantification	Well-established method	Resolution limited by PCR-induced noise and achievable depth of coverage
Single molecule PCR (smPCR)	Point and deletion mutations	Detection and quantification	Low rate of noise. Ability to amplify whole mtDNA genome	Time-consuming and relatively technically demanding method; low depth of coverage, therefore samples only a tiny fraction of all mtDNA molecules
Random mutation capture (RMC)	Point mutations	Quantification	Very low rate of noise	Detects only one specific mutation type, therefore mutation rate may not reflect that of other mutation types
Next-generation resequencing (NGS)	Point mutations	Detection and quantification	Very high throughput, very high depth of coverage, can quantify each mutation detected	Resolution good, but limited by noise (mainly base-calling error rate)
Real-time PCR (generic major arc assay, e.g. ND1:ND4)	Deletion mutations	Quantification	Quick method of quantifying deletion burden	Can only accurately quantify relatively high heteroplasmy levels of mutations (>~25%), therefore better suited to single cell analyses
Real-time PCR (specific assay, e.g. 'common deletion')	Deletion mutations	Detection and quantification	Quick method of detecting and quantifying very low heteroplasmy levels (e.g. <0.1%) of a specific deletion mutation	Limited to quantifying a single deletion mutation, therefore may not reflect total mutation burden
Allele-specific PCR (e.g. clamped PCR)	Point mutations	Detection	Quick method of detecting relatively low levels of a specific point mutation (e.g. mt.414T>G)	Limited to detecting a single point mutation. Not quantitative
COX histochemistry, single cell analysis	Point and deletion mutations	Quantification (COX histochemistry), and detection (single cell)	COX histochemistry quantifies functionally abnormal cells, and localises somatic mutations	Will not routinely detect low level mutations that have not become clonally amplified within cells

Table 2. Summary of papers examining mitochondrial DNA mutations in the setting of HIV or anti-retroviral therapy. mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism analysis; TA, thymidine analogue; SSCP, single-strand conformation polymorphism analysis; NRTI, nucleoside analogue reverse transcriptase inhibitor; TTGE, temporal temperature gradient gel electrophoresis; AZT, zidovudine; DGGE, denaturing gradient gel electrophoresis; HIV-SN, HIV-associated sensory neuropathy; ddC, zalcitabine; d4T, stavudine; BER, base excision repair; DRG, dorsal root ganglion.

Citation	Species / Subjects	Tissue	Findings	Methods	Comments
Bartley *et al.* (2001). AIDS 15(3):419-420	Human (single case report of fatal lactic acidosis)	Blood, skeletal muscle, liver	Large-scale deletion mutations in hepatic mtDNA	PCR, RFLP, Sanger sequencing	Possibility this was unmasking of underlying mitochondrial disease
Martin *et al.* (2003). *Am J Hum Genet* 72(3):549-560	Human (subjects starting TA based therapy)	Blood	5 of 16 patients at one year developed new heteroplasmic mtDNA point mutations	SSCP, cloning, Sanger sequencing	Mutations seen in mtDNA non-coding and protein coding regions
Walker *et al.* (2004). *Cardiovasc Toxicol* 4(2):133-153	Mouse (adult females, exposed *in utero* to NRTIs)	Cardiac muscle	Increased mutations in mtDNA tRNA genes following *in utero* AZT exposure	PCR, DGGE, Sanger sequencing	No increase in mtDNA deletion mutations seen. Some lesions may have been strain-specific rather than acquired.
McComsey *et al.* (2005). *J Acquir Immune Defic Syndr* 39(2):181-188	Human (serial samples from NRTI treated patients)	Blood	2 of 54 NRTI treated patients developed mtDNA point mutations whilst on therapy	PCR, TTGE, Sanger sequencing	Mutations were in mtDNA non-coding region only.
Chan *et al.* (2007). *Environ Mol Mutag* 48(3-4):190-200	Mouse (young adult mice, exposed *in utero* and in early post-natal period to NRTIs)	Cardiac muscle	Increased mutations in mtDNA tRNA genes following *in utero* and early post-natal AZT exposure	PCR, DGGE, Sanger sequencing	No increase in mtDNA deletion mutations seen. Some lesions may have been strain-specific rather than acquired.
Torres *et al.* (2009). *Environ Mol Mutag* 50(1):10-26	Human (infants exposed *in utero*)	Umbilical cord (vascular endothelium and smooth muscle)	Increased mutations in mtDNA tRNA genes following *in utero* AZT exposure.	PCR, DGGE, Sanger sequencing	Many mutations were at known polymorphic sites, some were novel. No comparison was made with maternal mtDNA sequence.
Wu *et al.* (2009). *Mutat Res* 664(1-2):28-38	HuH-7 human hepatoblast cell line	*In vitro*	Increased frequency of mtDNA d-loop mutations with d4T exposure	PCR, Sanger sequencing	Hypothesised to be due to decreased mtDNA BER

Balcarek *et al.* (2010). *J Acquir Immune Defic Syndr* 55(5):550-557	Mouse (young adult mice exposed to NRTIs)	Cardiac muscle	AZT and ddC treated mice showed mtDNA common deletion mutation which was absent or at very low levels in controls	PCR, semi-quantitative	Uridine supplementation somewhat lessened the increase in the mtDNA common deletion mutation levels seen with NRTI treatment
Lehmann *et al.* (2011). *Ann Neurol* 69(1):100-110	Human (post-mortem frozen tissue)	Peripheral nerves	Increased levels of mtDNA common deletion mutation in HIV-SN	Real-time PCR	Common deletion more common in distal nerves than DRG (these are 'older' mitochondria)
Ortiz *et al.* (2011). *J Infect Dis* 203(5):620-624	Human (patients starting TA based therapy)	Blood	None of 29 patients developed new mtDNA mutations on therapy	PCR, Sanger sequencing	Could have missed low-level heteroplasmic mtDNA mutations
Payne *et al.* (2011). *Nat Genet* 43(8):726-727	Human (cross sectional analysis of currently and previously NRTI treated subjects)	Skeletal muscle	NRTI treated subjects showed mtDNA mutations which were clonally expanded within single cells. Also increase in common deletion mutation. Pattern of mutations similar to that seen later in life due to normal ageing	Single cell real-time PCR and Sanger sequencing; next-generation sequencing; *in silico* modelling	Data consistent with accelerated expansion of pre-existing (ageing-associated) mtDNA mutations, without increased *de novo* mutagenesis
Jitratkosol *et al.* (2012). *AIDS* 26(6):675-683	Human (NRTI treated mothers and infants exposed *in utero*)	Blood	AC/TG mutations only were increased in mothers and trend towards increase in infants	Post-PCR cloning and Sanger sequencing of mtDNA d-loop	Reported significant issues with assay noise

Table 3. Putative strategies to slow mitochondrial ageing in HIV infection or anti-retroviral therapy.

Strategy	Process targeted	Comments
Reduce oxidative stress	Mutagenesis – oxidative damage	Identify and use anti-retrovirals with minimal effect on oxidative stress, identify and treat disease-associated oxidative stress
Mitochondrially targeted anti-oxidant	Mutagenesis – oxidative damage	In general, human studies of anti-oxidant supplements in other diseases have been disappointing
Increase polymerase fidelity	Mutagenesis – replication errors	Use anti-retrovirals with minimal effect on pol γ base misincorporation
Increase excision repair	Mutagenesis – replication errors	Use anti-retrovirals with minimal effect on pol γ exonuclease function
Increase mitochondrial biomass	Clonal expansion	Identify treatments or processes (such as endurance exercise) which may increase mitochondrial biomass, and therefore total and wild-type mtDNA content within cells, reducing the effect of clonally-amplified mutant mtDNA
Reduce amplification of mutant mtDNA	Clonal expansion	Avoid the use of anti-retrovirals which inhibit pol γ polymerase function, and thus potentially confer replicative advantage to deleted mtDNA

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Mitochondrial aging is accelerated by anti-retroviral therapy through the clonal expansion of mtDNA mutations

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Abstract

There is emerging evidence that people with successfully treated HIV infection age prematurely, leading to progressive multi-organ disease (Effros *et al.*, 2008), but the reasons for this are not known. Here we show that patients treated with commonly used nucleoside analog anti-retroviral drugs progressively accumulate somatic mitochondrial DNA (mtDNA) mutations, mirroring those seen much later in life caused by normal aging (Trifunovic *et al.*, 2004; Bua *et al.*, 2006). Ultra-deep re-sequencing by synthesis, combined with single-cell analyses, suggests that the increase in somatic mutation is not caused by increased mutagenesis but might instead be caused by accelerated mtDNA turnover. This leads to the clonal expansion of preexisting age-related somatic mtDNA mutations and a biochemical defect that can affect up to 10% of cells. These observations add weight to the role of somatic mtDNA mutations in the aging process and raise the specter of progressive iatrogenic mitochondrial genetic disease emerging over the next decade.

Main Text

Somatic mtDNA mutations accumulate in individual cells during normal human aging, leading to cellular bio-energetic defects of oxidative phosphorylation (Brierley *et al.*, 1996; Bua *et al.*, 2006). Transgenic mice with a defective mtDNA polymerase (pol γ) accumulate secondary mtDNA mutations and a prematurely aged phenotype (Trifunovic *et al.*, 2004), but it is still not clear whether the mtDNA mutations are a cause or a consequence of aging in humans. Accelerated senescence has recently been described in humans with successfully treated HIV infection (Effros *et al.*, 2008). These patients become frail at an early age, decline physiologically (Oursler *et al.*, 2006; Desquilbet *et al.*, 2007) and acquire age-associated degenerative disorders affecting the cardiovascular system and the brain leading to dementia (Valcour *et al.*, 2004; Guaraldi *et al.*, 2009). Several nucleoside analog reverse transcriptase inhibitor anti-retroviral drugs (NRTIs) used in the treatment of HIV inhibit the function of pol γ (Lim and Copeland, 2001), raising the possibility that drug treatment contributes to the accelerated aging phenotype through mtDNA damage. NRTIs are well known to cause an acute, temporary and reversible reduction in the amount of mtDNA (mtDNA depletion), and one previous study detected mtDNA deletions in patients being actively treated with NRTIs (Cote *et al.*, 2002; McComsey *et al.*, 2005; Maagaard *et al.*, 2006). However, no previous studies have looked at the possibility of irreversible long-term effects of the drugs on mtDNA mutations after NRTI treatment has ceased.

We studied skeletal muscle from 33 HIV-infected adults, all aged 50 years or under, stratified by lifetime exposure to NRTIs previously shown to affect pol γ *in vitro* (Lim and Copeland, 2001) (Online Methods and Supplementary Table 1), and 10 HIV-uninfected healthy controls (HIV−) of comparable age. We initially looked for a defect of mitochondrial oxidative phosphorylation within individual cells using cytochrome *c* oxidase–succinate dehydrogenase (COX-SDH) histochemistry. Cellular COX defects would not be expected in this younger subject group (<0.5%) (Brierley *et al.*, 1996). The frequency of COX-deficient muscle fibers in HIV-infected non-treated (treatment-naïve, HIV+/NRTI−) subjects (n = 12) was indistinguishable from that observed in HIV− controls, with the majority having no COX-deficient fibers. By contrast, NRTI-exposed (HIV+/NRTI+) subjects (n = 21) had an increased frequency of COX-deficient muscle fibers (maximum 9.8%, P = 0.047), reaching or exceeding levels expected in healthy elderly individuals (Brierley *et al.*, 1996) (Fig. 1). The severity of

the COX defect was strongly predicted by cumulative lifetime NRTI exposure, rather than therapy at the time of study, implicating a persistent and cumulative mitochondrial defect (r^2 = 87%, $P < 0.001$; Supplementary Fig. 1).

We then defined the molecular basis for the COX deficiency observed in NRTI-exposed subjects. We first excluded persistent mtDNA depletion. The mtDNA content in homogenized skeletal muscle did not differ between HIV+/NRTI+ and HIV+/NRTI− patients (Supplementary Fig. 2). In keeping with this, the analysis of individual laser-captured single muscle fibers (n = 128) showed that only a small minority of COX-deficient fibers (6 out of 70, or 9%) from NRTI-treated patients had mtDNA depletion compared to adjacent fibers with normal COX activity. By contrast, the vast majority of the isolated COX-deficient fibers contained markedly increased amounts of mtDNA (geometric mean of 2.1-fold proliferation, maximum 21.3-fold; $P < 0.001$ for difference in mean mtDNA content between COX-deficient and normal fibers) (Fig. 2a). Focal mtDNA proliferation is often seen in association with pathogenic mtDNA mutations. In keeping with this, the majority of the COX-deficient fibers analyzed (40 out of 70 fibers from 12 HIV+/NRTI+ patients) showed high percentage levels of mtDNA molecules containing large-scale deletion mutations, exceeding the percentage level of mutation required to cause a COX defect (~60% (Hayashi *et al.*, 1991)). We detected no deletion mutations in adjacent skeletal muscle fibers (n = 58) with normal COX activity. Analysis of the mtDNA deletion break points (n = 15 fibers from four HIV+/NRTI+ patients) revealed different deletions in different fibers, all of which were clonal within individual fibers. Most of the clonally expanded deletions were unique; the only deletion observed more than once was the mt.δ4977 'common deletion', the commonest age-associated somatic mtDNA mutation (Corral-Debrinski *et al.*, 1992; Brierley *et al.*, 1998) (Fig. 2b,c and Supplementary Table 2).

Although less common than large-scale deletion mutations, mtDNA point mutations are also found in COX-deficient fibers from healthy aged subjects (Fayet *et al.*, 2002). In keeping with this, in the NRTI-treated patients, we found COX-deficient fibers not containing a deletion to harbor non-synonymous somatic mtDNA point mutations (5 out of 29 fibers). These mutations are predicted to alter a highly conserved amino acid and have not previously been described as inherited polymorphic variants in 5,140 humans (Table 1) (one variant, 12797T>C, had been observed as a somatic variant in a single

human sequence) (Pereira *et al.*, 2009) and thus provide an explanation for the associated cellular COX defect. Other fibers contained high levels of noncoding control-region (nt 16,024 to nt 576) variants, which were previously described in healthy aged humans.

We then estimated the total burden of mtDNA deletion mutations at the whole-tissue level. The proportion of mtDNA molecules containing the mt.δ4977 'common deletion' was significantly higher in NRTI-treated patients compared with untreated patients (HIV+/NRTI+ (mean ± s.e.m.), −3.45 ± 0.25 $\log_{10}$(/mtDNA); HIV+/NRTI−, −4.56 ± 0.31 $\log_{10}$(/mtDNA); $P = 0.012$) (Fig. 3) and were comparable with those previously reported in very elderly healthy subjects (Lee *et al.*, 1994). Furthermore, the proportion of COX-deficient muscle fibers from NRTI-treated subjects which contained mt.δ4977 was very similar to that reported in healthy aged individuals (Brierley *et al.*, 1998). Pathogenic mutations within single fibers (of which the majority were deletions) were accompanied by proliferation of mtDNA, which occurs in an attempt to maintain adequate levels of wild-type mtDNA, as shown previously (Chinnery and Samuels, 1999). As a result, mutated mtDNA also proliferates within the fiber. Over time, this will lead to a detectable increase in the level of deletions at the whole-tissue level.

To estimate the relative burden of mtDNA point mutations between treatment groups in homogenized skeletal muscle, we designed an ultra-deep re-sequencing by synthesis (UDS) assay using FLX GS technology (Roche 454). First, we carried out a series of control experiments to show the sensitivity of UDS to detect mtDNA point variants. We initially established that UDS of an mtDNA template did not generate an intrinsically different signal when compared to a nuclear DNA template by sequencing amplicons of cloned autosomal and mitochondrial DNA fragments as well as an autosomal DNA amplicon from genomic DNA (Supplementary Table 3). By this approach, we confirmed a very low background noise level for the UDS assay (Online Methods and Supplementary Fig. 3). As a positive control, we then compared two mtDNA amplicons from skeletal muscle DNA of *POLG* patients ($n = 4$), individuals known to harbor high levels of somatic mtDNA point mutations (Del Bo *et al.*, 2003; Wanrooij *et al.*, 2004). One mtDNA amplicon was in the hypervariable noncoding control region (*MT-HV2*) predicted from 5,140 population-level sequences (Pereira *et al.*, 2009) to have a high mutation rate, and one was in a highly conserved mtDNA coding region (*MT-CO3*).

Mean coverage was 5,892 sequence reads per amplicon in each direction. Consistent with an error-prone pol γ, these subjects showed an increase in mtDNA point variants detectable at >0.2% frequency in the *MT-HV2* amplicon (OR = 2.33, $P = 0.002$) (Fig. 4) when compared to healthy controls ($n = 4$). We detected no increase in variants in the *MT-CO3* amplicon. These findings were confirmed on replicate samples (Supplementary Fig. 4). When we studied skeletal muscle mtDNA from the HIV+/NRTI+ subjects ($n = 8$), the overall burden of point variants within each amplicon was indistinguishable from HIV+/NRTI− subjects ($n = 4$) and healthy HIV− controls ($n = 4$), all of comparable age (OR = 1.08, $P = 0.79$ for comparison of HIV+/NRTI+ and HIV− for *MT-HV2*). Furthermore, there was no correlation between COX defect in HIV+/NRTI+ subjects (range up to 10%) and mutation burden on the UDS assay.

Given that NRTI-treated subjects showed high-level COX defects (up to 10% of fibers) which contained clonal mutated mtDNA species, one explanation for our findings is accelerated segregation of pre-existing (age-associated) mtDNA mutations caused by NRTI treatment rather than *de novo* somatic mutation. In contrast, the *POLG* subjects showed a significant increase in point mutation burden in the UDS assay (although only in *MT-HV2*, $P = 0.002$) but a low proportion of COX-deficient fibers. Although the UDS data does not exclude the possibility of a slight increase in mutagenesis in NRTI-exposed subjects, it would not be of the level predicted to be required (>100-fold increase (Elson *et al.*, 2001)) to cause the observed COX defects.

To determine whether accelerated clonal expansion was a plausible explanation for our findings, we used an established computational model based solely on experimentally derived parameters (Elson *et al.*, 2001) and simulated the effects of NRTI-induced chain-termination during mtDNA replication (Lim and Copeland, 2001). The *de novo* mutation rate was not altered from the original model of aging muscle. A finite NRTI exposure predicted a period of temporary mtDNA depletion which was concordant with reported mtDNA levels (Cherry *et al.*, 2006; Maagaard *et al.*, 2006) and the COX defects observed (Maagaard *et al.*, 2006) in acutely treated HIV patients. This resulted in accelerated clonal expansion of pre-existing mtDNA mutations and led to an irreversible increase in the frequency of COX-deficient muscle fibers (Fig. 5a,b). The severity of predicted COX defect was dependent on the degree of replication failure and the duration of exposure (Fig. 5b,c), which is in keeping with our observations in patient

muscle that had suggested a strong dependence on these factors (Supplementary Fig. 1). *In silico* modeling is thus consistent with the hypothesis that accelerated clonal expansion of pre-existing (age-associated) mtDNA somatic mutations is sufficient to explain our observations in NRTI-treated subjects. Having established the model, we explored the effect of timing of NRTI exposure and showed that later periods of therapy predicted a higher frequency of COX deficiency (Fig. 5d). This is because of older subjects harboring a greater number of age-related somatic mtDNA mutations than younger subjects, which rapidly clonally segregate during NRTI therapy. This is in keeping with the observation that mitochondrially mediated clinical complications of NRTI therapy appear to be more common in older individuals (Smyth *et al.*, 2007). Finally we modeled the longer-term effects of treatment. Using this approach, an HIV-infected individual treated with NRTIs during their third decade is predicted to develop ~5% COX-deficient cells by age 60 (Fig. 5b–d). This is similar to or exceeds that seen in the healthy very old (Brierley *et al.*, 1996).

Although the UDS data for mtDNA point mutations support the hypothesis of accelerated clonal expansion of pre-existing age-related mutations rather than increased mutagenesis, it is possible that additional mechanisms may be involved for mtDNA large-scale deletions, including a replicative advantage favoring deleted molecules (Diaz *et al.*, 2002). Furthermore, although UDS provides great depth of mutational analysis, it is analogous to the PCR-cloning method of mutation rate determination and as such will tend to exaggerate an estimate of the mutation rate (Kollberg *et al.*, 2005; Greaves *et al.*, 2009).

The rapid clonal expansion of somatic mtDNA mutations we observed in NRTI-treated HIV-infected patients provides a plausible mechanism for accelerated aging in treated HIV infection. This is potentially of great importance for the millions of HIV-infected patients in the developing world where these drugs remain the mainstay of therapy (WHO, 2006) and adds weight to a causal role for somatic mtDNA mutations in human aging.

Methods

Ethics

This study was approved by the Newcastle and North Tyneside Local Research Ethics Committee. Informed consent was obtained from all subjects.

Clinical details

Clinical details are described in the Supplementary Note.

Histochemistry

We obtained 20 μm frozen sections from fresh-frozen lower limb skeletal muscle biopsies and placed them on polyethylene naphthalate (PEN) membrane slides (Leica) for subsequent laser microdissection. COX (cytochrome *c* oxidase) contains subunits encoded by the mitochondrial genome and stains brown (positive) in the presence of preserved respiratory chain activity. SDH (succinate dehydrogenase) provides an effective counter stain (blue), as this respiratory chain complex is entirely encoded by the nuclear genome and will be preserved in the presence of an mtDNA defect. Thus, COX-deficient fibers are predicted to contain somatic mtDNA mutations. ATPase histochemistry was performed on adjacent frozen sections in order to determine fiber type (oxidative or glycolytic).

Molecular analyses

All primers used are listed (Supplementary Table 4). All nucleotide positions refer to the revised Cambridge Reference Sequence (rCRS, NC_012920).

Individual skeletal muscle fibers were captured by laser microdissection (Leica) and digested in 30 μl of lysis buffer (50 mM Tris-HCl pH 8.5, 0.5% Tween-20 and 200 μg/ml proteinase K). Real-time PCR was performed as previously described (Durham *et al.*, 2007). Briefly, mtDNA content was determined using a target template in *MT-ND1*. When comparing COX-deficient and normal fibers, these were matched for fiber type and adjusted for fiber size. We estimated the proportion of mtDNA molecules containing large-scale deletions using a target template in *MT-ND4*. For determination

of relative mtDNA content at the whole-tissue level, we performed real-time PCR as above with the inclusion of the nuclear template, *B2M*. The proportion of mtDNA molecules in muscle homogenate containing the mt.δ4977 'common deletion' were estimated by real-time PCR comparing *MT-ND1* and a product (CD) specifically amplified only in the presence of the common deletion. CD-*ND1* real-time PCR was performed in a 20 µl reaction comprising 1× Evagreen supermix (Bio-Rad), 0.625 µM primers and 50 ng DNA. PCR protocol comprised 98 °C for 2 min, followed by 40 cycles of 98 °C for 5 s and 60 °C for 20 s. In addition to a PCR negative, DNA extracted from whole blood of a 25-year-old healthy control subject was used to define the lower limit of sensitivity for this assay, as negligible mt.δ4977 is expected to be detectable in blood by these methods (Lee *et al.*, 1994; Shieh *et al.*, 2007).

Long-range PCR to detect mtDNA deletions in individual fibers was performed using nested PCR as previously described (Bender *et al.*, 2006). Deletion break points were then characterized by amplification of a ~500-bp fragment across the deletion break point. Break-point PCR reactions were performed in a 25 µl reaction containing 1× ImmoBuffer (Bioline), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Immolase (Bioline) and 1 µl of long-range PCR product, diluted 1:50 with PCR-grade water. PCR conditions were 95 °C for 10 min and 25 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s. Cycle sequencing was performed using BigDye Terminator v3.1 kit (Applied Biosystems) and visualized through a 3130× Genetic Analyzer (Applied Biosystems).

Whole-genome sequencing from individual fibers was performed based on our previous methods (Durham *et al.*, 2006). A nested PCR comprising a primary PCR with nine overlapping primer pairs was followed by 36 overlapping secondary PCR primer pairs. Primary PCR was performed in a 50 µl volume containing 1× PCR buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 0.001% w/v gelatin), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 µM primers, 1.75 U AmpliTaq Gold (Applied Biosystems) and 1 µl lysate. PCR conditions were 94 °C for 10 min and 38 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 2 min. Final extension was 8 min. Secondary PCR was performed in a 25 µl volume containing 1× PCR buffer (as above), 0.2 mM dNTPs, 0.8 µM primers, 0.65 U AmpliTaq Gold and 1 µl of primary PCR product. PCR conditions were as above except for 1 min extension and 30 cycles. Cycle sequencing was performed as above.

Ultra-deep re-sequencing by synthesis (UDS; Roche 454 GS FLX) was performed by PCR amplification of two mtDNA amplicons: one in the noncoding (control region) hypervariable segment 2 (*MT-HV2*) (amplicon position, nt 162 to nt 455, 294 bp) and one in the coding region, COX subunit 3 (*MT-CO3*) (amplicon position, nt 9,307 to nt 9,591, 285 bp). Primer specificity and lack of amplification of nuclear pseudogenes was predicted by BLAST (Altschul *et al.*, 1990) and confirmed by failure of amplification of any product from rho_0 cellular DNA. In addition we generated a nuclear DNA amplicon (*BRCA2*, NC_000013.10, 32,907,099–32,907,295). Amplicon generation was performed in a 50 μl volume containing 1× buffer for KOD Hot Start DNA Polymerase (Novagen), 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.3 μM primers, 1 U KOD Hot Start DNA Polymerase (Novagen) and 100 ng DNA. Cycling conditions were 95 °C for 2 min followed by 30 cycles of 95 °C for 20 s, 60 °C for 10 s and 70 °C for 4 s. Emulsion PCR and sequencing were performed according to manufacturer's instructions (Roche 454). Confirmatory experiments were performed by amplicon sequencing a larger amplicon in the same regions using Roche 454 GS FLX Titanium system. Amplicon positions for Titanium assay were: *MT-HV2*, nt 109 to nt 483; *MT-CO3*, nt 9,304–9,653; *BRCA2*, 32,907,060–32,907,350. Amplicon generation PCR was as for the initial FLX assay with the exception of a 5 s extension per cycle. Repeat assays were performed for HIV−, HIV+/NRTI+ and *POLG* subjects (n = 4 each). Amplicons were additionally generated from cloned DNA fragments (*MT-HV2* clone, nt 16,548 to nt 771; *MT-CO3* clone, nt 9,127 to nt 9,661; *BRCA2* clone, 32,906,828–32,907,480; cloned in pGEM-T-Easy vector, Promega). An analysis pipeline of PyroBayes and Mosaik (Quinlan *et al.*, 2008) was used to call and align bases from the 454 flowgram output. Subsequent analysis of variants was done in R using the custom made R library flowgram (available from the authors I.W. and M.S.K.). For comparison of samples with varying coverage depths, 5,000 sub-sampled sequences were used for all samples in all analyses. Recent studies of low-level variance in mtDNA using next-generation sequencing by synthesis technology have employed the Illumina GA platforms (He *et al.*, 2010). Experience to date suggests that this approach appears limited to a resolution of ~1–1.5% variant frequency or higher, below which true variance cannot be distinguished from noise, despite very high theoretical read depths. In order to improve on this depth of resolution, we filtered the raw FLX flowgram output for sites predicted to give poor resolution. As FLX resequencing employs pyrosequencing technology, it is

prone to sequencing errors associated with mononucleotide tracts. Analysis of our outputs from cloned DNA confirmed this observation, and such sites were excluded from further analysis. Such an approach enabled resolution to variants with measured frequency ≥0.2%, whereby there was negligible variance detected in any cloned DNA amplicon or the nuclear (*BRCA2*) amplicon from genomic DNA at this level (~0.5% of base positions). Comparison with mtDNA amplicon sequence variants from patient samples thus indicated that almost all low frequency variants (>0.2%) reflect true sequence variation rather than noise. Power calculations indicate that this assay will have 80% power to detect an absolute increase in mutation burden of 2.7% at $P < 0.05$.

Modeling of NRTI effects on mtDNA replication

Modeling was performed by development of a validated simulation model of mtDNA replication and age-associated clonal expansion of mtDNA mutations based solely on experimentally derived parameters (Elson *et al.*, 2001). The effect of NRTIs on mtDNA replication was modeled by including a probability of failure for each replication event. In the case of a replication failure, the mtDNA molecule being copied was assumed to be destroyed. With this assumption, any failure rate of 50% or greater results in the complete loss of the mtDNA from the simulation. *De novo* mutations were modeled by including a probability of mutation formation at each replication event, with a probability of 5×10^{-5} per replication, which was kept constant across all simulated exposure groups. The *de novo* mutation rate was set at this value in order to keep the probability of forming clonal expansions below 1% before age 70 in the control case. Other relevant parameter values were the optimal mtDNA copy number (N_{opt} = 5,000), the mtDNA half life (10 days) and the maximum proliferation factor (α = 15). Two-thousand cells were simulated to measure the probability of developing clonal expansions of mtDNA mutations. Simulated cells which fixed on the mutant (a very rare occurrence) were removed from the model. The simulation was written in FORTRAN and is available from the author (DCS).

Statistical analyses

Percentage levels of COX defect were compared between groups (≥500 fibers per subject) by Mann-Whitney test. Comparison of proportions of COX-deficient and normal fibers showing mtDNA deletions was made by χ^2 test. Comparison of mean

$\log_{10}$(mtDNA) content and $\log_{10}$(CD/mtDNA) levels in skeletal muscle homogenates was made using a *t* test. Statistical comparisons were performed using R. The multiple regression models were run in Origin 7 (OriginLab).

Figure 1: COX (cytochrome *c* oxidase) deficiency in single skeletal muscle fibers.

(a) COX histochemistry from a representative healthy control subject (HIV−) showing normal COX activity, whereas a nucleoside analog treated HIV-infected patient (HIV+/NRTI+) shows multiple COX-deficient fibers (counterstained blue by residual SDH (succinate dehydrogenase) activity). Scale bars, 100 μm. (b) COX defects observed in each subject group (HIV+/NRTI−, HIV-infected treatment-naïve subjects; each dot represents an individual patient biopsy; ≥500 fibers sampled per biopsy).

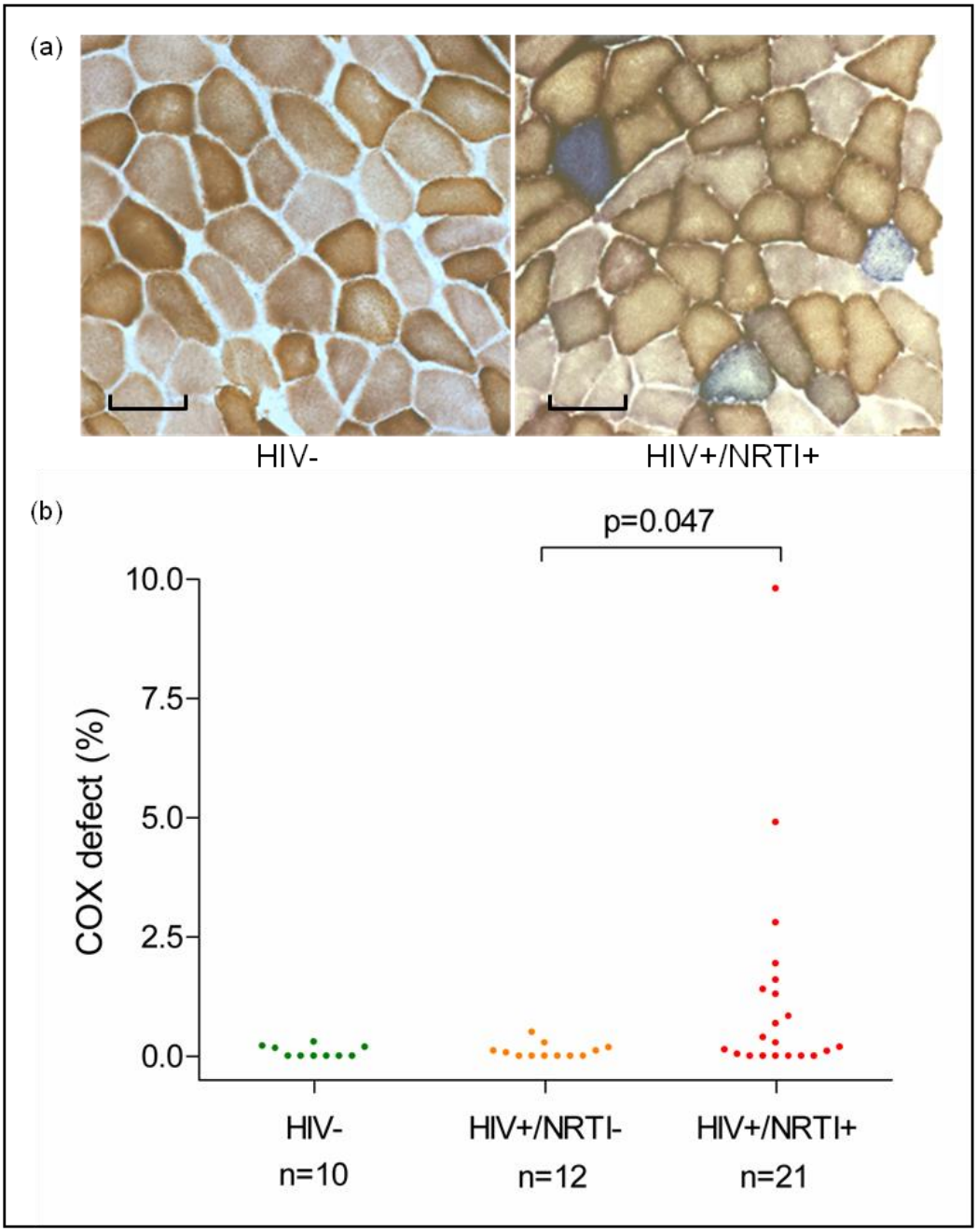

(a)
HIV-
HIV+/NRTI+
(b)
p=0.047
10.0
7.5
5.0
2.5
0.0
COX defect (%)
HIV-
n=10
HIV+/NRTI-
n=12
HIV+/NRTI+
n=21

Figure 2: Mitochondrial DNA analysis of single skeletal muscle fibers.

(a) Mitochondrial DNA (mtDNA) content in individual COX (cytochrome *c* oxidase)-deficient muscle fibers from nucleoside analog treated HIV-infected (HIV+/NRTI+) subjects, expressed relative to mtDNA content in adjacent fibers of normal COX activity from the same subject. A few fibers show reduced mtDNA content, whereas the majority show increased content (geometric mean of 2.1-fold proliferation, maximum 21.3-fold; $P < 0.001$ for difference in mean mtDNA content between COX-deficient and normal fibers). (b) The majority of COX-deficient fibers (COX−) contained high percentage levels of mtDNA containing a large-scale deletion of the major arc, causing the COX defect; whereas no deleted mtDNA was detected in adjacent COX positive fibers (COX+) ($P < 0.001$). (c) Schematic representation of mtDNA large-scale deletion breakpoints in COX-deficient fibers from HIV+/NRTI+ patients relative to the mtDNA gene positions (transfer RNA and ribosomal RNA not shown). Each line represents an individual deleted region. O_L, origin of light chain replication; O_H, origin of heavy chain replication. ($n = 15$ fibers from four patients).

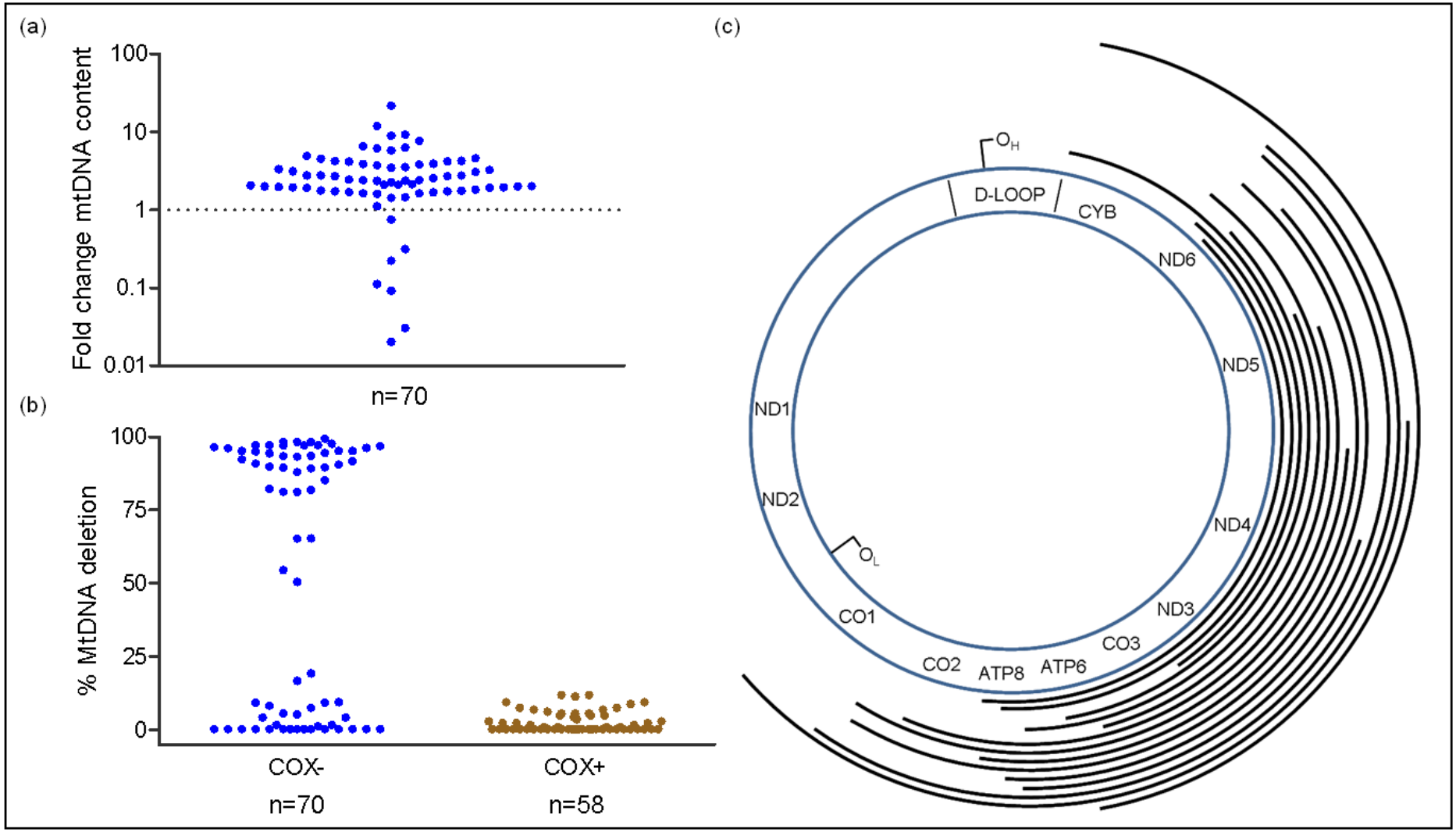

(a)
Fold change mtDNA content
100
10
1
0.1
0.01
n=70
(b)
% MtDNA deletion
100
75
50
25
0
COX-
n=70
COX+
n=58
(c)
O_H
D-LOOP
CYB
ND6
ND5
ND4
ND3
CO3
ATP6
ATP8
CO2
CO1
O_L
ND2
ND1

Figure 3: Proportional level of mt.δ4977 'common deletion' (CD) in homogenized skeletal muscle from HIV-infected subjects.

HIV+/NRTI+, HIV-infected, nucleoside analog exposed; HIV+/NRTI−, HIV-infected, treatment-naïve. The dashed line represents the lower threshold of the assay. NRTI-treated subjects showed significantly higher mean levels of common deletion than untreated subjects (HIV+/NRTI+ (mean ± s.e.m.), −3.45 ± 0.25 $\log_{10}$(/mtDNA); HIV+/NRTI−, −4.56 ± 0.31 $\log_{10}$(/mtDNA); P = 0.012). Box and whisker plot.

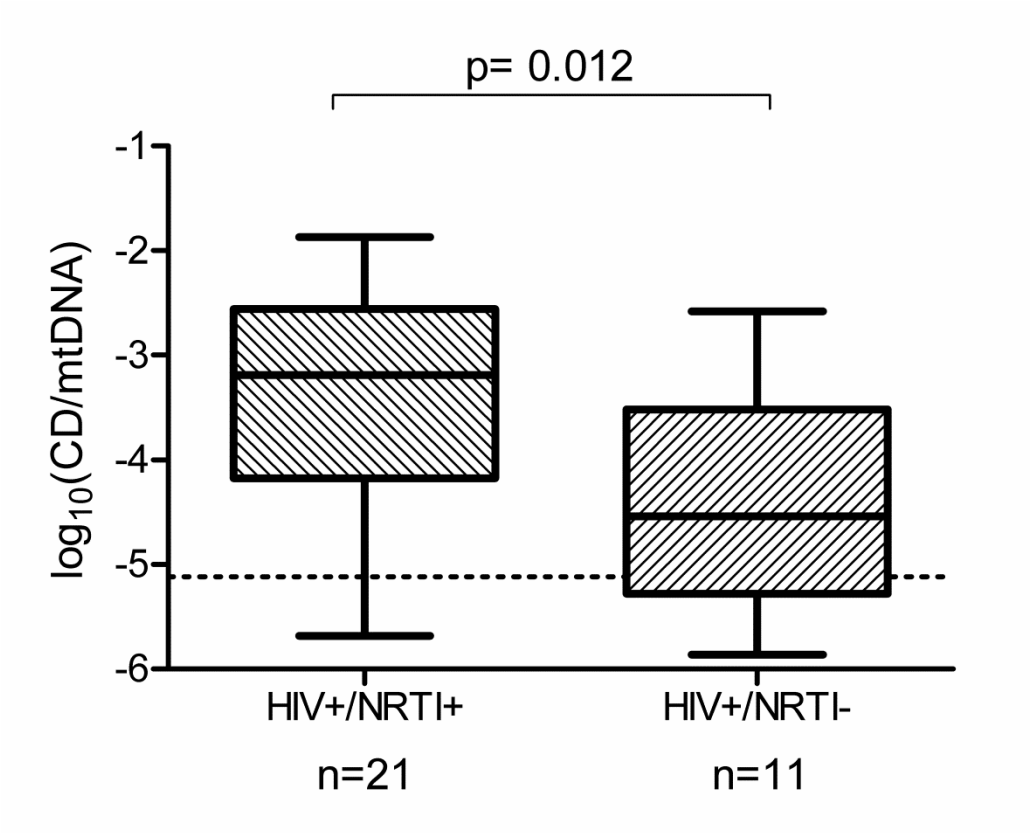


Figure 4: Ultra-deep re-sequencing by synthesis (UDS) of skeletal muscle mtDNA.

UDS (Roche 454 FLX GS) shows no difference in burden of low-level mtDNA point variants (exceeding 0.2% frequency) between HIV-infected nucleoside analog treated (HIV+/NRTI+, $n = 8$), HIV-infected treatment-naïve (HIV+/NRTI−, $n = 4$) and control (HIV−, $n = 4$) subjects in two amplicons located in mtDNA hypervariable segment 2 (*MT-HV2*) and mtDNA COX subunit 3 (*MT-CO3*). In contrast, positive control subjects with inherited *POLG* defects (POLG, $n = 4$) show an increased burden of low-level mutations compared with healthy controls in *MT-HV2* (OR = 2.33, $P = 0.002$).

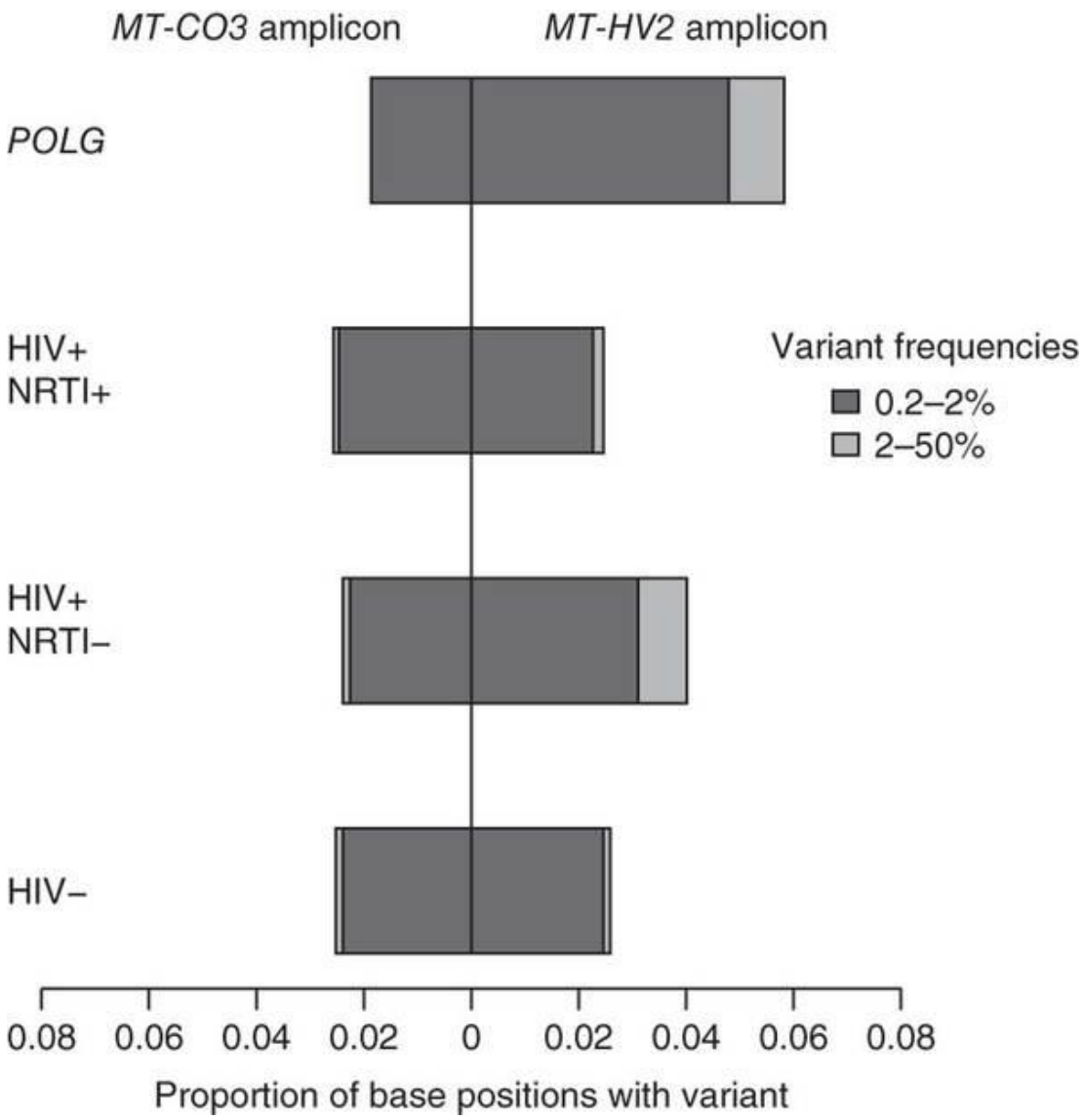
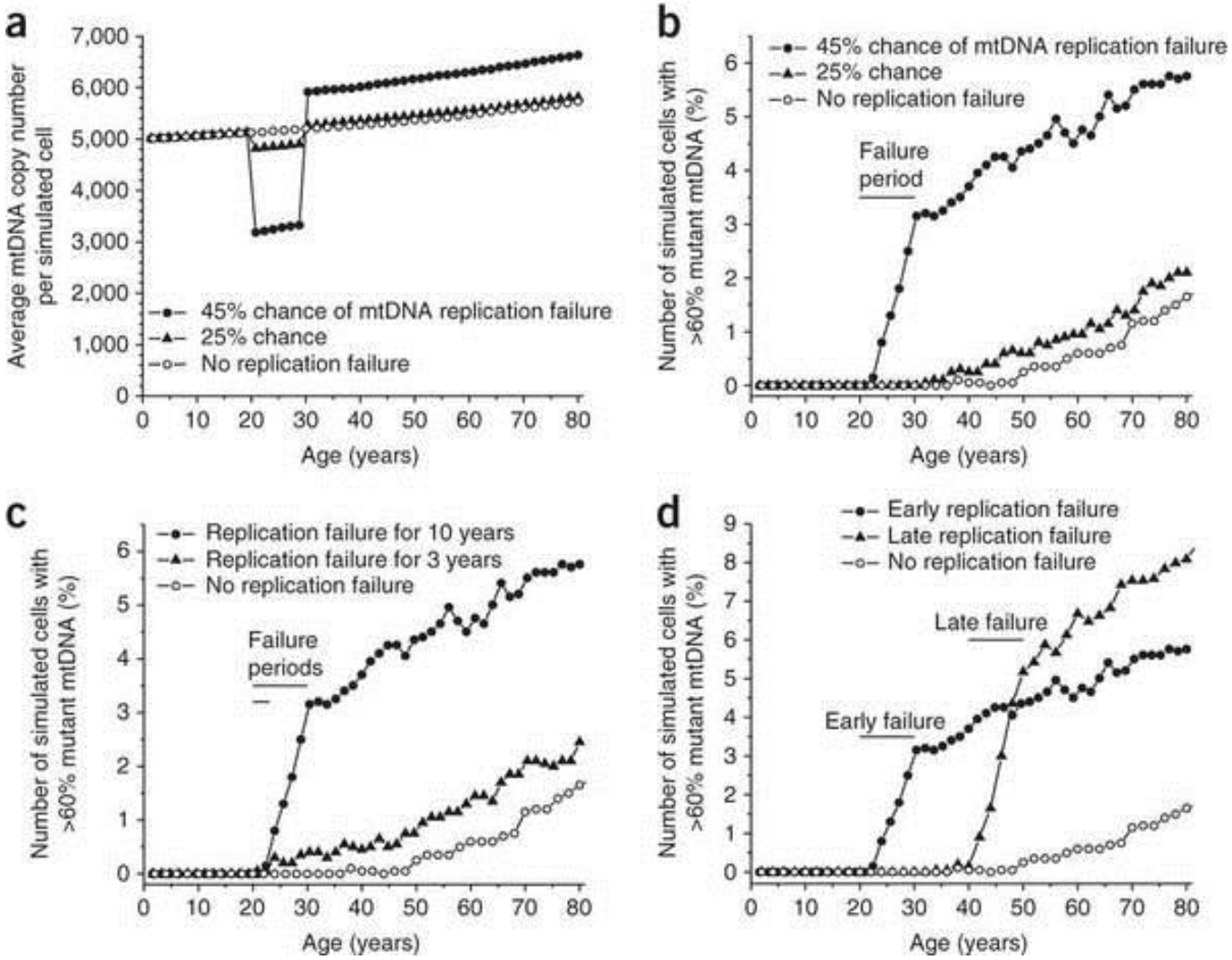

MT-CO3 amplicon
MT-HV2 amplicon
POLG
HIV+
NRTI+
HIV+
NRTI–
HIV–
Variant frequencies
0.2–2%
2–50%
0.08
0.06
0.04
0.02
0
0.02
0.04
0.06
0.08
Proportion of base positions with variant

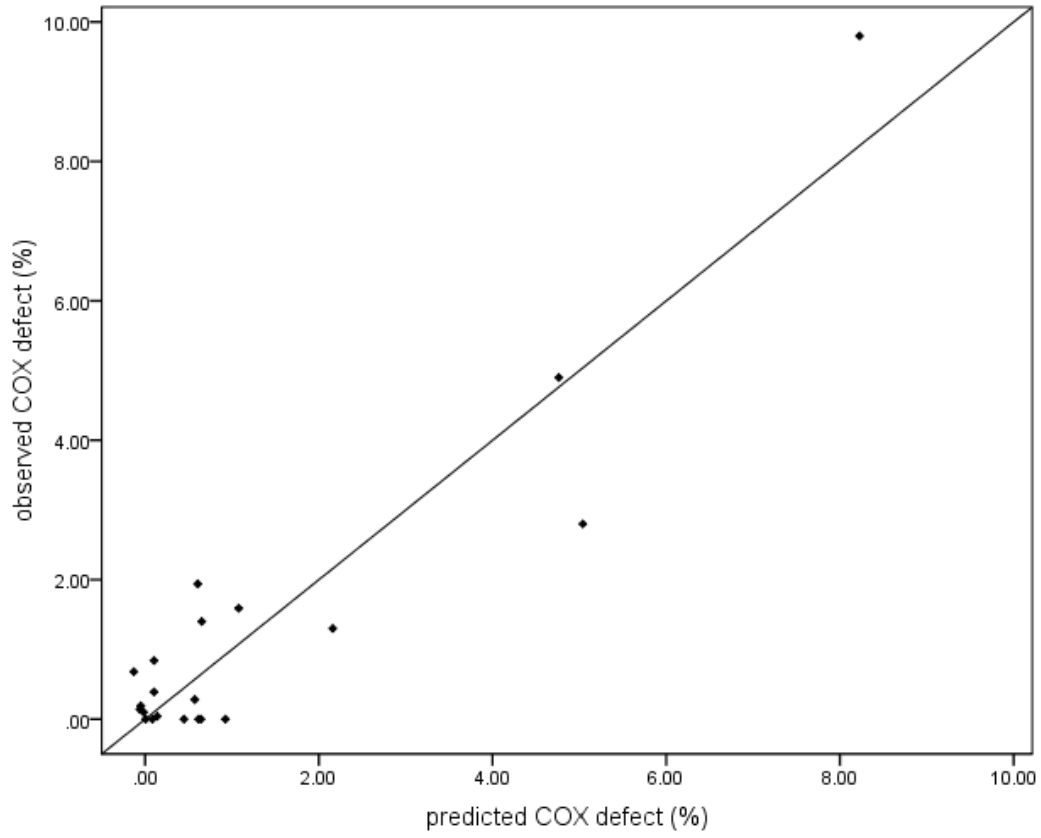
Figure 5: Simulations of the effects of partial mitochondrial DNA (mtDNA) replication failure caused by nucleoside analog (NRTI) exposure.

Using a validated computer model of mtDNA replication based solely on experimentally derived parameters[22], we incorporated a finite period of partial replication failure caused by the mtDNA chain-terminating effects of NRTI exposure[9], assigning a probability of failure per mtDNA replication event. All other parameters remained constant, including the *de novo* mutation rate[22]. We simulated 2,000 cells for 80 years. (a) The amount of mtDNA depletion during the NRTI exposure period caused by 25% and 45% probability of replication failure between 20 and 30 years of age. (>50% failure led to the complete loss of mtDNA.) The range of mtDNA depletion predicted is in keeping with published *in vivo* data[12, 23]. (b) This led to a persistent increase in the frequency of COX (cytochrome *c* oxidase)-deficient cells through the accelerated clonal expansion of preexisting somatic mtDNA mutations. (c) Direct simulation of the effects of NRTI exposure within our study population (two different periods, 10 and 3 years, starting at age 20, of replication failure with 45% probability). The range of COX defects predicted closely fits our empiric data. (d) Late exposure (40–50 years) had a more pronounced effect than early exposure (20–30 years) (with 45% probability of replication failure) caused by the higher number of preexisting (age-related) somatic mtDNA mutations at the time of exposure.

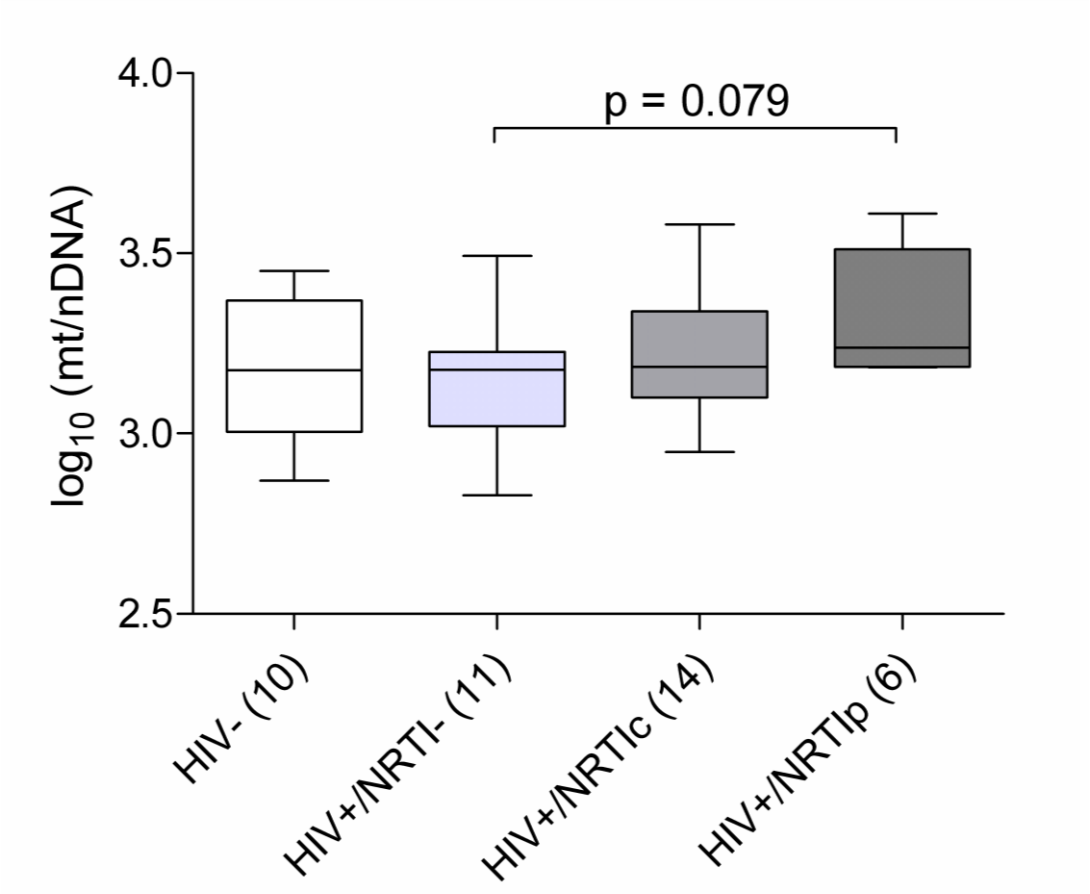

a
Average mtDNA copy number per simulated cell
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
45% chance of mtDNA replication failure
25% chance
No replication failure
0 10 20 30 40 50 60 70 80
Age (years)
b
Number of simulated cells with >60% mutant mtDNA (%)
45% chance of mtDNA replication failure
25% chance
No replication failure
Failure period
0 1 2 3 4 5 6
0 10 20 30 40 50 60 70 80
Age (years)
c
Number of simulated cells with >60% mutant mtDNA (%)
Replication failure for 10 years
Replication failure for 3 years
No replication failure
Failure periods
0 1 2 3 4 5 6
0 10 20 30 40 50 60 70 80
Age (years)
d
Number of simulated cells with >60% mutant mtDNA (%)
Early replication failure
Late replication failure
No replication failure
Late failure
Early failure
0 1 2 3 4 5 6 7 8 9
0 10 20 30 40 50 60 70 80
Age (years)

Supplementary Information

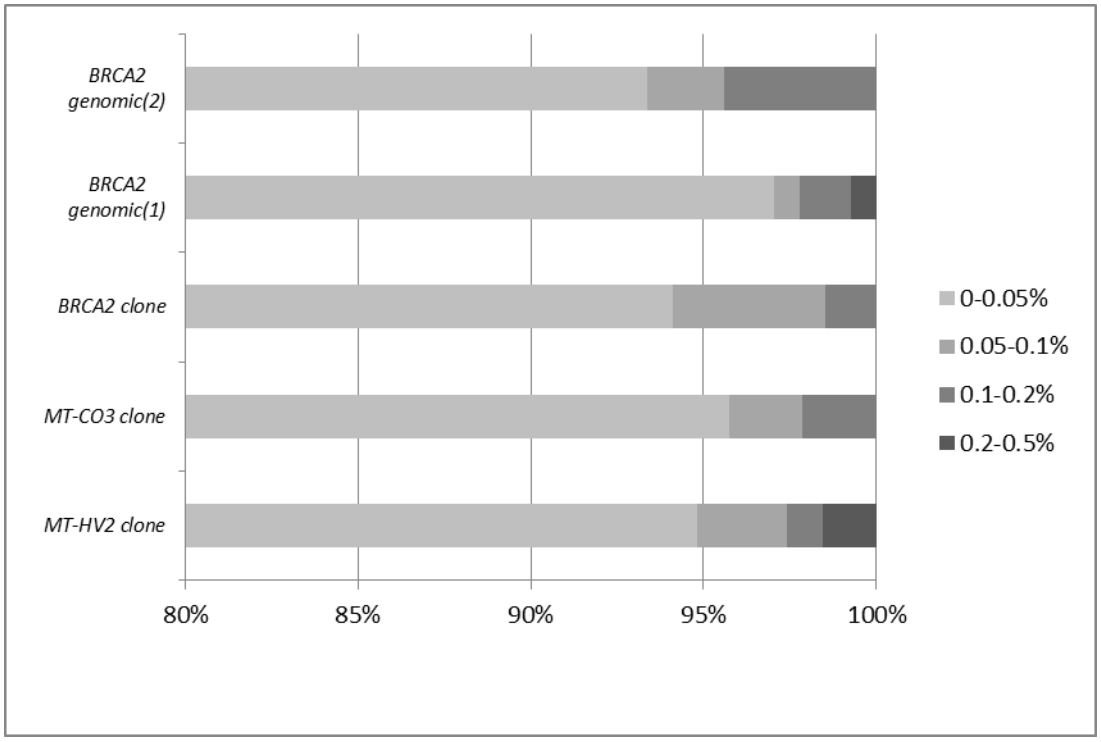
Supplementary Figure 1. Predicted COX defect according to cumulative NRTI exposure. Multivariate linear regression model of predicted percentage COX (cytochrome c oxidase) defects in skeletal muscle fibers from HIV-infected subjects, according to cumulative exposure to specific nucleoside analog anti-retroviral drugs (NRTIs). The inclusion in the model of cumulative (lifetime) exposure to those NRTIs implicated in perturbation of mtDNA replication (ddI, didanosine; ddC, zalcitabine; AZT, zidovudine; d4T, stavudine) was sufficient to explain 87% of the observed variation in COX defects (R = 0.93). Equation of regression line = $A + T_{ddI}\,B_{ddI} + T_{ddC}\,B_{ddC} + T_{AZT}\,B_{AZT} + T_{d4T}\,B_{d4T}$. T, duration of exposure (months). Coefficients: constant (A) = -0.459 ± 0.530; B_{ddI} = 0.107 ± 0.017; B_{ddC} = 0.093 ± 0.023; B_{AZT} = 0.011 ± 0.007; B_{d4T} = 0.017 ± 0.013. ddI ($p < 0.001$) and ddC ($p = 0.001$) were independently significantly associated with the proportion of COX deficient fibers. The p value for the model fit to the data is < 0.001.



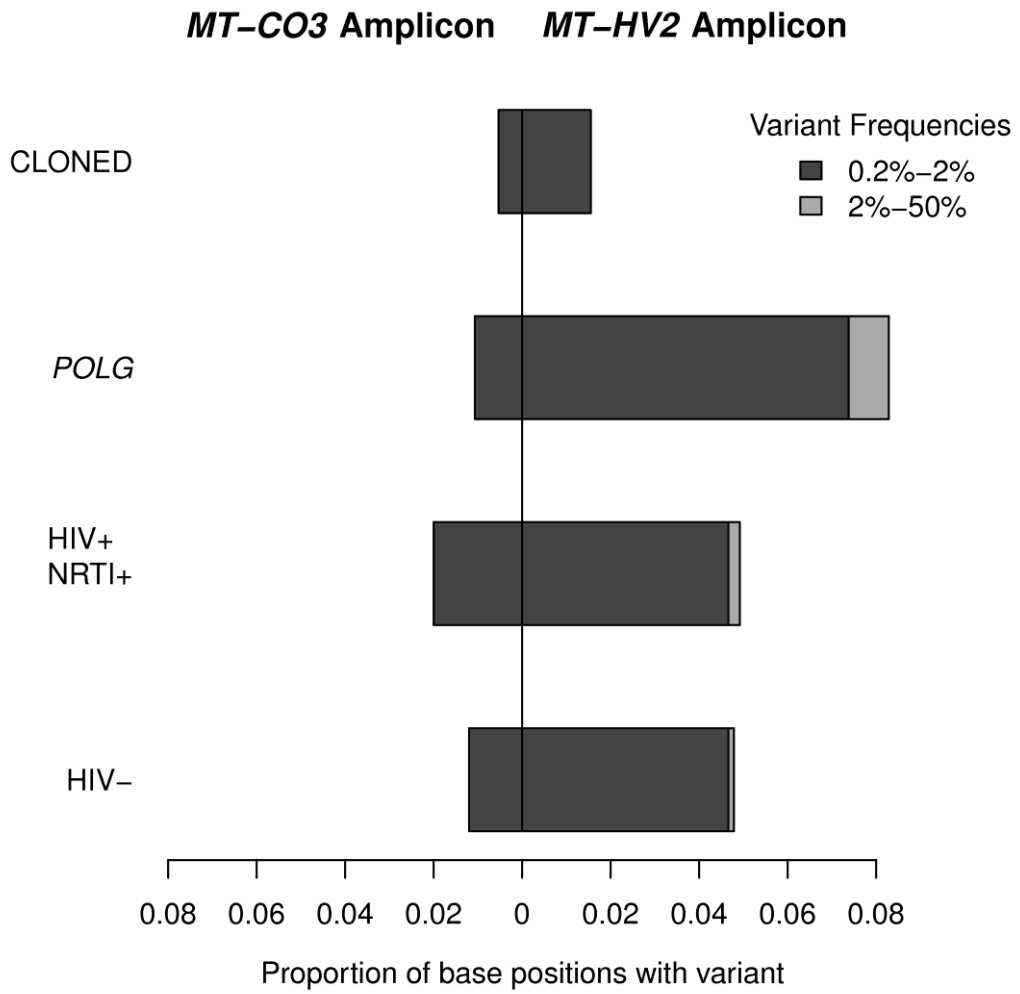
Supplementary Figure 2. MtDNA content in homogenized skeletal muscle. Relative mitochondrial DNA (mean $\log_{10}$ (mt/nDNA) ±SEM) content in homogenized skeletal muscle. HIV-, uninfected controls (n=10; 3.17 ±0.06); HIV+/NRTI-, treatment-naïve (n=11; 3.15 ±0.05); HIV+/NRTIc, currently treated with relevant nucleoside analog drug (n=14, all with AZT (zidovudine) exposure only; 3.22 ±0.05); HIV+/NRTIp, *prior* (but not current) exposure to relevant NRTIs (n=6, with prior AZT, d4T (stavudine), ddI (didanosine) and / or ddC (zalcitabine) exposure; 3.31 ±0.07). There was therefore no evidence of persistent mtDNA depletion in HIV+/NRTIp subjects, in fact previously treated patients showed a trend towards higher mean mtDNA content compared with HIV+/NRTI- (p = 0.079).



Supplementary Figure 3. Determination of experimental noise in UDS assay. Demonstration of low level of background noise in ultra-deep re-sequencing-by-synthesis (UDS, Roche 454 GS FLX) assay by study of autosomal genomic DNA (*BRCA2* amplicon) and cloned DNA (mtDNA hypervariable segment 2, *MT-HV2*; mtDNA COX subunit 3, *MT-CO3*; and *BRCA2*). Very few base positions (0.5% of total) demonstrate >0.2% variance frequency on UDS assay, confirming low intrinsic background noise, as well as no systematic difference in noise between autosomal and mtDNA amplicons.



Supplementary Figure 4. UDS replication experiment. Ultra-deep re-sequencing-by-synthesis (UDS) replication experiment (Roche 454 GS FLX Titanium). Comparison of point mutation burden (>0.2% variant frequency) in skeletal muscle DNA extract from HIV-uninfected controls (HIV-), NRTI-treated HIV-infected (HIV+/NRTI+) subjects and patients with inherited defects of *POLG* (n=4 each) as well as cloned DNA. Two amplicons were located in mtDNA hypervariable segment 2 (*MT-HV2*) and mtDNA COX subunit 3 (*MT-CO3*). Subjects with inherited *POLG* defects show increased burden of low-level mutations compared with healthy controls in *MT-HV2* (OR 2.00, p =0.001), whereas HIV+/NRTI+ subjects do not.



Supplementary Table 1. Subject demographic and treatment details.

NRTI, history of nucleoside analogue reverse transcriptase inhibitor exposure; ART, anti-retroviral therapy; LDS, anti-retroviral-associated lipodystrophy syndrome. Polymerase γ inhibiting NRTIs: AZT, zidovudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine. Other ART: 3TC, lamivudine; FTC, emtricitabine; ABC, abacavir; TDF, tenofovir; NVP, nevirapine; EFV, efavirenz; SQV, saquinavir; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir (therapeutic dosing); /r, ritonavir (pharmacokinetic boosting dosing); ATV, atazanavir; LPV, lopinavir; RAL, raltegravir. COX defect, proportion of cytochrome *c* oxidase deficient skeletal muscle fibers.

subject	HIV	NRTI	gender	age (years)	time since diagnosis (months)	current CD4 lymphocyte count (cells/μl)	nadir CD4 lymphocyte count (cells/μl)	current HIV viral load (copies/ml)	duration of ART (months)	poi y inhibitor NRTI (months)	current ART	lifetime ART	clinical LDS	clinical morbidity	COX defect
1	+	+	F	31	44	1048	218	<40	42	42	AZT 3TC NVP	AZT 3TC NVP	+		0.00%
2	+	+	F	46	38	566	8	<40	37	37	AZT 3TC EFV	AZT 3TC EFV	-		0.19%
3	+	+	M	38	138	682	150	<40	100	100	AZT 3TC NVP	AZT 3TC NVP	-		0.00%
4	+	+	F	31	49	630	168	<40	36	36	AZT 3TC EFV	AZT 3TC EFV	-		0.14%
5	+	+	M	32	95	553	197	<40	94	94	AZT 3TC EFV	AZT 3TC EFV	+		0.28%
6	+	+	F	30	51	532	83	<40	49	49	AZT 3TC EFV	AZT 3TC EFV	-		0.00%
7	+	+	M	44	52	619	259	<40	51	51	AZT 3TC EFV	AZT 3TC EFV	+		0.39%
8	+	+	F	48	32	253	17	<40	30	30	AZT 3TC EFV	AZT 3TC EFV	+		0.68%
9	+	+	M	35	83	585	4	<40	82	82	AZT 3TC EFV	AZT 3TC EFV	+		0.00%
10	+	+	F	33	54	397	114	<40	54	54	AZT 3TC NVP	AZT 3TC NVP	-		0.04%
11	+	+	M	49	98	588	107	<40	97	97	AZT 3TC EFV	AZT 3TC IDV EFV	+		1.94%
12	+	+	M	36	140	764	N/A	<40	140	140	AZT 3TC IDV	AZT 3TC IDV	-		1.6%
13	+	+	M	33	98	579	18	<40	98	98	AZT 3TC EFV	AZT 3TC EFV	-		0.0%
14	+	+	M	42	127	539	176	<40	126	126	AZT 3TC NVP	AZT 3TC IDV NVP	-		0.0%
15	+	+	M	43	119	1239	250	<40	118	101	TDF FTC EFV	AZT 3TC IDV EFV ABC TDF FTC	-		1.4%
16	+	+	M	45	165	592	305	<40	146	128	RAL ABC ATV/r	d4T 3TC NVP ddI IDV ABC ATV/r RAL	+		9.8%
17	+	+	M	48	158	872	10	<40	151	58	TDF ABC NVP	AZT ddI d4T 3TC RTV NVP IDV ddC ABC ATV/r TDF	-	fatigue	4.9%
18	+	+	M	40	54	309	152	<40	54	51	TDF FTC EFV	AZT 3TC EFV TDF FTC	-	LVF	0.8%
19	+	+	F	45	71	537	10	<40	70	40	TDF FTC NVP	AZT 3TC EFV TDF FTC NVP	-		0.1%
20	+	+	M	49	193	762	120	<40	193	122	TDF FTC ATV/r	AZT ddC ddI 3TC d4T SQV NVP IDV NFV ABC TDF LPV/r FTC ATV/r	+	LVF, CVD, fatigue	1.3%
21	+	+	M	50	140	669	0	<40	138	75	TDF FTC NVP	AZT d4T IDV NFV SQV 3TC NVP ddI TDF FTC	+	fatigue	2.8%
22	+	-	M	25	32	217	197	1250	-	-	-	-	-		0.0%
23	+	-	M	49	227	223	199	18900	-	-	-	-	-		0.3%
24	+	-	M	46	110	387	328	17000	-	-	-	-	-		0.5%
25	+	-	F	45	99	214	214	1050	-	-	-	-	-		0.2%
26	+	-	F	50	120	1358	541	20	-	-	-	-	-		0.0%
27	+	-	F	32	44	626	522	41600	-	-	-	-	-		0.1%
28	+	-	M	27	45	391	283	34300	-	-	-	-	-		0.1%
29	+	-	M	34	31	422	389	4700	-	-	-	-	-		0.1%
30	+	-	F	32	27	380	380	13900	-	-	-	-	-		0.0%
31	+	-	M	27	37	633	438	12700	-	-	-	-	-		0.0%
32	+	-	M	37	64	1165	1033	4300	-	-	-	-	-		0.0%
33	+	-	M	44	14	332	332	23600	-	-	-	-	-		0.0%
34	-	-	M	35	-	-	-	-	-	-	-	-	-		0.0%
35	-	-	F	31	-	-	-	-	-	-	-	-	-		0.0%
36	-	-	M	26	-	-	-	-	-	-	-	-	-		0.0%
37	-	-	M	47	-	-	-	-	-	-	-	-	-		0.3%
38	-	-	M	36	-	-	-	-	-	-	-	-	-		0.2%
39	-	-	F	42	-	-	-	-	-	-	-	-	-		0.2%
40	-	-	M	24	-	-	-	-	-	-	-	-	-		0.0%
41	-	-	M	21	-	-	-	-	-	-	-	-	-		0.0%
42	-	-	F	20	-	-	-	-	-	-	-	-	-		0.0%
43	-	-	M	52	-	-	-	-	-	-	-	-	-		0.2%

Supplementary Table 2. MtDNA deletion break-points in single muscle fibers

Sequence break-points for large scale mitochondrial DNA (mtDNA) deletions identified in individual COX (cytochrome c oxidase) deficient skeletal muscle fibers from nucleoside analog (NRTI) treated, HIV-infected subjects. Deletion shown as nucleotide positions (rCRS) and size of sequence overlap (where present). Bracketed nucleotides, sequence overlap (where repeat appears once only in the deleted molecule); bold italic nucleotides, partial mismatch in overlap sequences.

Subject	Deletion	Size	Flanking sequence
18	8145-8-14379	6243bp	AAACCACTTT(CA***C***CGCTA)-(CA***T***CGCTA)ACCCCACTAAAA
18	8246---14603	6358bp	AAAAATCTTTGA-AGGCTTAGAAGAAAA
18	9924-2-16070	6149bp	CGAAGCCGCCG(CC)-(CC)CATCAACAACCG
18	8718---14298	5581bp	TACACAACACTAA-TATTCAGCTTCCTA
18	8483-13-13446	4977bp	AAACTACCACCT(ACCTCCCTCACCA)-(ACCTCCCTCACCA)TTGGCAGCCTAGCA
18	9011-12-14931	5933bp	AGCCCTGGCCGT(ACGCCTAACCGC)-(ACGCCT***C***AACCGC)CTTTTCATCAATC
18	7376---13406	6031bp	AACCCTCCATAAA-AAAAATAGGAGGACT
20	7106-11-12082	4988bp	ATTCACTGATT(TCCCC***T***ATTCT)-(TCCCC***C***ATTCT)CCTCCTATCCC
20	7960-12-14481	6534bp	TTCAACTCCTA(CAT***AC***TTCCCCC)-(CAT***CA***TTCCCCC)TAAATAAATTAAAAAAA
15	7129-14-13991	6877bp	TCAGGCTACAC(CCTAGACC***A***AACCT)-(CCTAGACC***T***AACCT)GACTAGAAAA
15	8035-11-11422	3399bp	TACTCCCGATT(GAAGCCCCCAT)-(GAAGCCCCCAT)CGCTGGGTCAATA
12	8483-13-13446	4977bp	AAACTACCACCT(ACCTCCCTCACCA)-(ACCTCCCTCACCA)TTGGCAGCCTAGCA
12	6942---14816	7875bp	AGGATTCATCTTTC-CATCCAACATCTCC
12	6071---12499	6429bp	CCACATCTACAACGTT-TGTGCCTAGACCAAGAA
12	8936---16070	7135bp	CACCTACACCCC-CCCATCAACAACC

Supplementary Table 3. UDS (Roche 454 FLX GS) outputs.

Variance and read depth detected at individual base positions in mtDNA hypervariable segment 2 (*MT-HV2*), COX subunit 3 (*MT-CO3*) and autosomal (*BRCA2*) amplicons, for skeletal muscle DNA extracts from HIV-infected NRTI-treated subjects (HIV+/NRTI+, n=8), HIV-infected untreated subjects (HIV+/NRTI-, n=4), HIV-uninfected healthy controls (HIV-, n=4), subjects with *POLG* defects (*POLG*, n=4) and cloned DNA.

Raw 454 Flowgram output is available from the authors.

Supplementary Table 4. Primers.

All mitochondrial nucleotide positions refer to revised Cambridge Reference Sequence (rCRS, NC_012920).

Long-range PCR from single skeletal muscle fibers

Primary PCR: forward primer, nt5855-5875 (AGATTTACAGTCCAATGCTTC);
reverse primer, nt129-110 (AGATACTGCGACATAGGGTG).

Secondary PCR: forward primer, nt6358-6377 (TAGCAGGTGTCTCCTCTATC);
reverse primer, nt20-1 (AGGGTGATAGACCTGTGATC).

Real-time PCR from skeletal muscle homogenate and single fibers

B2M (nuclear)*:* forward primer, nt9145-9166 (CACTGAAAAAGATGAGTATGCC);
reverse primer, nt9375-9357 (AACATTCCCTGACAATCCC).

MT-ND1: forward primer, nt3458-3481 (ACGCCATAAAACTCTTCACCAAAG);
reverse primer, nt3569-3546 (GGGTTCATAGTAGAAGAGCGATGG).

MT-ND4: forward primer, nt11144-11165 (ACCTTGGCTATCATCACCCGAT);
reverse primer, nt11250-11230 (AGTGCGATGAGTAGGGGAAGG).

CD: forward primer, nt8393-8414 (CCCACCATAATTACCCCCATAC)
rev. primer, nt13509-13486 (GGAGTAGAAACCTGTGAGGAAAGG)

Whole mtDNA genome sequencing from single skeletal muscle fibers

Primary PCR	nt
AF GCTCACATCACCCCATAAAC	627-646
AR CTCGTCTTGCTGTGTTATGC	2721-2702
BF ACCAACAAGTCATTATTACCC	2395-2415
BR ATACTTGATGGCAGCTTCTG	4646-4627
CF GTCAGCTAAATAAGCTATCGG	4408-4428
CR GGACGGATCAGACGAAGAG	6468-6450
DF AATACCCATCATAATCGGAGG	6113-6133
DR GGTGATGAGGAATAGTGTAAG	8437-8417
EF TCAATGCTCTGAAATCTGTGG	8167-8187
ER TCGAAGCCGCACTCGTAAG	10183-10165
FF CTATTGATGAGGGTCTTACTC	9974-9994
FR GAGCTTTCTCGGTAAATAAGG	12216-12196
GF CTGTGCTAGTAACCACGTTC	11898-11917
GR GGTAGAATCCGAGTATGTTGG	13924-13904
HF TATTCGCAGGATTTCTCATTAC	13721-13742
HR GTGCTAATGGTGGAGTTAAAG	15989-15969
IF CCCATCCTCCATATATCCAAAC	15659-15680
IR TCACTGCTGTTTCCCGTGG	823 -805

Secondary PCR

Forward primers with M13 tag for cycle sequencing (TGTAAAACGACGGCCAGT)

1F TGTAAAACGACGGCCAGTTCACCCTCTAAATCACCACG	721-740
2F TGTAAAACGACGGCCAGTTTAAAACTCAAAGGACCTGGC	1157-1177
3F TGTAAAACGACGGCCAGTAACTTAACTTGACCGCTCTGAG	1650-1671
4F TGTAAAACGACGGCCAGTACTGTTAGTCCAAAGAGGAAC	2091-2111
5F TGTAAAACGACGGCCAGTCAGTGACACATGTTTAACGGC	2549-2569
6F TGTAAAACGACGGCCAGTCAGCCGCTATTAAAGGTTCG	3017-3036
7F TGTAAAACGACGGCCAGTACCATCACCCTCTACATCAC	3505-3524
8F TGTAAAACGACGGCCAGTTCGCCCTATTCTTCATAGCC	3965-3984
9F TGTAAAACGACGGCCAGTACACTCATCACAGCGCTAAG	4518-4537
10F TGTAAAACGACGGCCAGTCTCACTCTCTCAATCTTATCC	4932-4952
11F TGTAAAACGACGGCCAGTACCTCAATCACACTACTCCC	5367-5386
12F TGTAAAACGACGGCCAGTAGATTTACAGTCCAATGCTTC	5855-5875
13F TGTAAAACGACGGCCAGTTAGCAGGTGTCTCCTCTATC	6358-6377
14F TGTAAAACGACGGCCAGTATTTAGCTGACTCGCCACAC	6863-6882
15F TGTAAAACGACGGCCAGTGGCTCATTCATTTCTCTAACAG	7272-7293
16F TGTAAAACGACGGCCAGTTCCTAACACTCACAACAAAAC	7713-7723
17F TGTAAAACGACGGCCAGTACAGTTTCATGCCCATCGTC	8196-8215
18F TGTAAAACGACGGCCAGTACCACCCAACAATGACTAATC	8656-8676
19F TGTAAAACGACGGCCAGTATCCTAGAAATCGCTGTCGC	9127-9146
20F TGTAAAACGACGGCCAGTCATCCGTATTACTCGCATCAG	9607-9627
21F TGTAAAACGACGGCCAGTCAACACCCTCCTAGCCTTAC	10085-10104
22F TGTAAAACGACGGCCAGTATCGCTCACACCTCATATCC	10534-10553
23F TGTAAAACGACGGCCAGTTATCCAGTGAACCACTATCAC	11010-11030
24F TGTAAAACGACGGCCAGTTCCTTGTACTATCCCTATGAG	11541-11561
25F TGTAAAACGACGGCCAGTCTCCCTCTACATATTTACCAC	11977-11997

26F TGTAAAACGACGGCCAGTCTCTTCCCCACAACAATATTC 12478-12498

27F TGTAAAACGACGGCCAGTGCCCTTCTAAACGCTAATCC 12940-12959

28F TGTAAAACGACGGCCAGTCGGGTCCATCATCCACAAC 13365-13383

29F TGTAAAACGACGGCCAGTACCTAAAACTCACAGCCCTC 13790-13809

30F TGTAAAACGACGGCCAGTATTAAAGTTTACCACAACCACC 14317-14341

31F TGTAAAACGACGGCCAGTATTCATCGACCTCCCCACC 14797-14815

32F TGTAAAACGACGGCCAGTCATCTTGCCCTTCATTATTGC 15295-15315

D1F TGTAAAACGACGGCCAGTATCGGAGGACAACCAGTAAG 15758-15777

D2F TGTAAAACGACGGCCAGTCTCAACTATCACACATCAACTG 16223-16244

D3F TGTAAAACGACGGCCAGTCCTTAAATAAGACATCACGATG 16548-16569

D4F TGTAAAACGACGGCCAGTGCCACAGCACTTAAACACATC 323-343

Reverse primers with M13 tag (CAGGAAACAGCTATGACC)

1R CAGGAAACAGCTATGACCGATGGCGGTATATAGGCTGAG 1268-1248

2R CAGGAAACAGCTATGACCCTGGTAGTAAGGTGGAGTGGG 1709-1689

3R CAGGAAACAGCTATGACCATTGGTGGCTGCTTTTAGG 2193-2175

4R CAGGAAACAGCTATGACCTCGTGGAGCCATTCATACAG 2644-2625

5R CAGGAAACAGCTATGACCGATTACTCCGGTCTGAACTC 3087-3068

6R CAGGAAACAGCTATGACCGGAGGGGGGTTCATAGTAG 3374- 3356

7R CAGGAAACAGCTATGACCAGAGTGCGTCATATGTTGTTC 4057-4037

8R CAGGAAACAGCTATGACCGTTTATTTCTAGGCCTACTCAG 4577-4556

9R CAGGAAACAGCTATGACCGATTTTGCGTAGCTGGGTTTG 5003-4983

10R CAGGAAACAGCTATGACCTGTAGGAGTAGCGTGGTAAGG 5481-5462

11R CAGGAAACAGCTATGACCTAGTCAACGGTCGGCGAAC 5924-5906

12R CAGGAAACAGCTATGACCATGGCAGGGGGTTTTATATTG 6430-6410

13R CAGGAAACAGCTATGACCAAGAAAGATGAATCCTAGGGC 6944-6924

14R CAGGAAACAGCTATGACCCATCCATATAGTCACTCCAGG 7396-7376

15R CAGGAAACAGCTATGACCGGCAGGATAGTTCAGACGG 7791-7773

16R CAGGAAACAGCTATGACCTACAGTGGGCTCTAGAGGG 8301-8283

17R CAGGAAACAGCTATGACCGTATAAGAGATCAGGTTCGTC 8740-8720

18R CAGGAAACAGCTATGACCGTTGTCGTGCAGGTAGAGG 9201-9183

19R CAGGAAACAGCTATGACCATTAGACTATGGTGAGCTCAG 9661-9641

20R CAGGAAACAGCTATGACCTAGCCGTTGAGTTGTGGTAG 10147-10128

21R CAGGAAACAGCTATGACCAGGCACAATATTGGCTAAGAG 10649-10629

22R CAGGAAACAGCTATGACCATGATTAGTTCTGTGGCTGTG 11109-11089

23R CAGGAAACAGCTATGACCTAGGTCTGTTTGTCGTAGGC 11605-11586

24R CAGGAAACAGCTATGACCCGTGTGAATGAGGGTTTTATG 12054-12034

25R CAGGAAACAGCTATGACCGTGGCTCAGTGTCAGTTCG 12545-12527

26R CAGGAAACAGCTATGACCCTGATTTGCCTGCTGCTGC 13009-12991

27R CAGGAAACAGCTATGACCGGGAGGTTGAAGTGAGAGG 13453-13435

28R CAGGAAACAGCTATGACCGTTAGGTAGTTGAGGTCTAGG 13859-13839

29R CAGGAAACAGCTATGACCAGGATTGGTGCTGTGGGTG 14374-14356

30R CAGGAAACAGCTATGACCAAGGAGTGAGCCGAAGTTTC 14857-14838

31R CAGGAAACAGCTATGACCGGTTGTTTGATCCCGTTTCG 15368-15349

32R CAGGAAACAGCTATGACCTACAAGGACAGGCCCATTTG 15896-15877

D1R CAGGAAACAGCTATGACCAGGGTGATAGACCTGTGATC 19-1

D2R CAGGAAACAGCTATGACCAGATACTGCGACATAGGGTG 129-110

D3R CAGGAAACAGCTATGACCCTGGTTAGGCTGGTGTTAGG 389-370

D4R CAGGAAACAGCTATGACCTGCTGCGTGCTTGATGCTTG 771-752

Amplicon generation for ultra-deep sequencing-by-synthesis (454 GS FLX)

Primers comprised a sequence-specific segment, a barcode, and a fusion primer segment.

Sequence-specific segments

MT-HV2: forward, nt162-184 (CGCACCTACGTTCAATATTACAG)

reverse, nt455-434 (AAAATAATGTGTTAGTTGGGGG)

MT-CO3: forward, nt9307-9329 (GATTTCACTTCCACTCCATAACG)

reverse, nt9591-9572 (CTTCTAGGGGATTTAGCGGG)

Fusion primer segments

Forward: GCCTCCCTCGCGCCATCAG

Reverse: GCCTTGCCAGCCCGCTCAG

Barcode segments

1	AAGGAAGGT	16	ACTTAAGGT
2	TTAAGGACT	17	TTACTTACT
3	TAAGGCCGT	18	TACTTCCGT
4	TTAAGTAAT	19	TCCGGAAGT
5	TAAGTACGT	20	CCGGACGGT
6	AAGTCCGGT	21	TTCCGGCCT
7	TAATTAAGT	22	CCGTAAGGT
8	AATTACGGT	23	TTCCGTACT
9	TTAATTACT	24	TCCGTCCGT
10	TTACGGAAT	25	TTCCTTAAT
11	TACGGACGT	26	TCCTTACGT
12	ACGGCCGGT	27	CCTTCCGGT
13	TACGTAAGT		
14	ACGTACGGT		
15	TTACGTCCT		

Amplicon generation for ultra-deep sequencing-by-synthesis (454 GS FLX Titanium)

Primers comprised a sequence-specific segment, a barcode, and a fusion primer segment.

Sequence-specific segments

HVS2:	forward, nt.109-130 (GCACCCTATGTCGCAGTATCTG)
	reverse, nt.483-458 (GAGATTAGTAGTATGGGAGTGGGAGG)
CO3:	forward, nt.9304-9329 (TGTGATTTCACTTCCACTCCATAACG)
	reverse, nt.9653-9629 (ATGGTGAGCTCAGGTGATTGATACT)

Barcode segments

1	ACGAGTGCGT
2	ACGCTCGACA
3	AGACGCACTC
4	AGCACTGTAG

Fusion primer segments

Forward:	CGTATCGCCTCCCTCGCGCCATCAG
Reverse:	CTATGCGCCTTGCCAGCCCGCTCAG

Supplementary Note.

Clinical Data

All subjects were aged 50 years or below as no COX defect would be expected in skeletal muscle of healthy individuals in this age group (Brierley *et al.*, 1996). All subjects gave informed consent to participation in the study. HIV-infected subjects were classified based on cumulative (lifetime) anti-retroviral drug exposure as HIV-infected, treatment naïve (HIV+/NRTI-) or as nucleoside analogue exposed (HIV+/NRTI+). We predicted that those NRTIs documented to disrupt mtDNA replication through pol γ inhibition may affect somatic mtDNA mutation (Lim and Copeland, 2001; Martin *et al.*, 2003). Therefore all HIV+/NRTI+ subjects studied had history of exposure to at least one of the following NRTIs: zidovudine (AZT), stavudine (d4T), didanosine (ddI) and / or zalcitabine (ddC). Full subject treatment histories are presented in **Supplementary Table 1**. HIV-infected subjects were unselected with respect to the presence or absence of clinical complications of anti-retroviral therapy. Subjects with history of (non-HIV-related) neuromuscular disease, diabetes mellitus or chronic viral hepatitis were specifically excluded.

Lower limb skeletal muscle biopsies from HIV-infected subjects were obtained under local anesthesia. Open biopsies were obtained from HIV-uninfected control subjects (HIV-) at the time of elective orthopedic surgery. Samples were snap-frozen in the liquid phase of isopentane, cooled by liquid nitrogen, within 20 minutes of sampling.

Four patients with inherited defects of *POLG* were used as positive controls for UDS assay. Two of these patients (both 56 year old females) carried a compound heterozygous mutation, R627Q / W748S; one patient (17 year old male) carried compound heterozygous mutation, R627Q / R1096H; and one patient (45 year old female) carried homozygous A467T. All *POLG* patients showed minimal histochemical COX defects.

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CO3 seq	Subject CO3 pos	POLG1 P1	POLG1 P2	POLG1 P3	POLG1 P4	HIV+/NRTI- 29	HIV+/NRTI- 23	HIV+/NRTI- 32	HIV+/NRTI- 25	HIV+/NRTI+ 17	HIV+/NRTI+ 20	HIV+/NRTI+ 18	HIV+/NRTI+ 16	HIV+/NRTI+ 7	HIV+/NRTI+ 12	HIV+/NRTI+ 15	HIV+/NRTI+ 5	HIV- 35	HIV- 38	HIV- 37	HIV- 39	clone
C	9330	20803	17644	18604	14397	15644	18614	15798	18509	10821	13209	15351	14770	15913	16186	14094	16544	17523	15913	15166	18460	12278
T	9331	20803	17644	18604	14397	15645	18614	15799	18509	10821	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18460	12278
C	9332	20803	17644	18604	14397	15646	18614	15799	18509	10821	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18460	12278
C	9333	20803	17644	18604	14397	15646	18614	15799	18509	10821	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18460	12278
T	9334	20803	17644	18604	14397	15646	18614	15799	18509	10821	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18461	12278
C	9335	20803	17644	18604	14397	15646	18614	15799	18509	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18461	12278
A	9336	20804	17644	18605	14397	15646	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18461	12278
T	9337	20805	17644	18605	14397	15646	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15166	18461	12278
A	9338	20805	17644	18606	14397	15646	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15167	18461	12278
C	9339	20805	17644	18606	14397	15647	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15167	18461	12278
T	9340	20805	17644	18606	14397	15647	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15167	18461	12278
A	9341	20805	17644	18607	14397	15647	18614	15799	18511	10822	13209	15351	14770	15913	16187	14094	16544	17525	15913	15167	18461	12278
G	9342	20805	17644	18607	14398	15647	18614	15799	18511	10823	13209	15351	14770	15913	16188	14094	16544	17526	15913	15167	18461	12278
G	9343	20805	17644	18608	14398	15647	18614	15799	18511	10824	13209	15351	14770	15913	16188	14094	16544	17526	15913	15168	18461	12278
C	9344	20805	17644	18608	14398	15648	18615	15799	18511	10824	13210	15351	14770	15913	16188	14094	16544	17526	15914	15168	18461	12278
C	9345	20805	17644	18608	14398	15648	18615	15799	18511	10824	13210	15351	14770	15913	16188	14094	16544	17526	15914	15168	18461	12278
T	9346	20805	17644	18608	14398	15648	18615	15800	18511	10824	13210	15352	14770	15913	16188	14094	16544	17526	15914	15168	18461	12278
A	9347	20805	17644	18608	14398	15648	18615	15800	18511	10824	13210	15352	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9348	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15352	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
T	9349	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15352	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9350	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9351	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9352	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9353	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9354	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9355	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9356	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9357	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9358	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9359	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9360	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
T	9361	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9362	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
A	9363	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9364	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16188	14094	16544	17526	15915	15168	18461	12278
C	9365	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17526	15915	15168	18461	12278
A	9366	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
T	9367	20805	17644	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
A	9368	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
T	9369	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
A	9370	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
C	9371	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
C	9372	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
A	9373	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
A	9374	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18461	12278
T	9375	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
G	9376	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
A	9377	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
T	9378	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
G	9379	20805	17645	18608	14398	15648	18615	15800	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
G	9380	20805	17645	18608	14398	15648	18615	15801	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
C	9381	20805	17645	18608	14398	15648	18615	15801	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
G	9382	20805	17645	18608	14398	15648	18615	15801	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
C	9383	20805	17645	18608	14398	15648	18615	15801	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
G	9384	20805	17645	18608	14398	15648	18615	15801	18511	10824	13211	15354	14770	15913	16189	14094	16544	17527	15915	15168	18462	12278
A	9385	20805	17645	18608	14398	15648	18615	15801	18512	10824	13211	15354	14770	15913	16189	14094	16545	17527	15915	15168	18462	12278
T	9386	20805	17645	18608	14398	15648	18615	15801	18513	10824	13211	15354	14770	15913	16191	14094	16545	17527	15917	15168	18462	12278
G	9387	20805	17645	18608	14398	15648	18615	15801	18513	10824	13211	15354	14770	15913	16191	14094	16545	17527	15917	15168	18462	12278
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G	9547	20808	17645	18608	14397	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16548	17526	15916	15167	18463	12278
G	9548	20808	17645	18607	14397	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16547	17526	15916	15167	18463	12278
C	9549	20808	17643	18606	14396	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16547	17525	15916	15167	18463	12278
A	9550	20808	17643	18606	14396	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16547	17525	15916	15167	18463	12278
C	9551	20808	17642	18606	14396	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16546	17525	15916	15167	18463	12278
T	9552	20808	17642	18606	14396	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16546	17525	15916	15167	18463	12278
G	9553	20808	17642	18606	14396	15646	18614	15806	18509	10826	13214	15357	14773	15912	16189	14091	16546	17525	15916	15167	18463	12278
A	9561	20806	17640	18603	14396	15640	18613	15804	18508	10826	13212	15355	14773	15911	16184	14091	16544	17523	15915	15166	18461	12278
C	9562	20806	17640	18601	14396	15640	18613	15803	18508	10825	13211	15355	14771	15911	16182	14090	16541	17523	15915	15166	18460	12278
A	9563	20806	17639	18601	14396	15640	18613	15802	18507	10825	13211	15355	14771	15911	16182	14090	16540	17522	15914	15165	18460	12278
G	9564	20806	17639	18600	14396	15640	18613	15802	18507	10825	13211	15355	14769	15911	16180	14090	16539	17522	15914	15165	18460	12278
G	9565	20806	17639	18599	14396	15639	18613	15801	18504	10825	13211	15355	14769	15909	16180	14089	16539	17521	15913	15165	18460	12278
C	9566	20806	17639	18599	14396	15639	18612	15801	18504	10823	13211	15355	14769	15909	16180	14088	16539	17521	15913	15165	18459	12278
A	9567	20806	17639	18598	14395	15638	18612	15801	18504	10823	13211	15354	14769	15909	16179	14088	16539	17521	15913	15165	18458	12278
T	9568	20803	17636	18596	14390	15637	18610	15801	18501	10823	13211	15354	14769	15906	16179	14088	16539	17518	15912	15165	18456	12278
C	9569	20790	17622	18569	14377	15632	18604	15799	18493	10820	13208	15351	14767	15898	16172	14083	16535	17506	15902	15164	18442	12278

CO3 seq	Subject CO3 pos	POLG1 P1	POLG1 P2	POLG1 P3	POLG1 P4	HIV+/NRTI- 29	HIV+/NRTI- 23	HIV+/NRTI- 32	HIV+/NRTI- 25	HIV+/NRTI+ 17	HIV+/NRTI+ 20	HIV+/NRTI+ 18	HIV+/NRTI+ 16	HIV+/NRTI+ 7	HIV+/NRTI+ 12	HIV+/NRTI+ 15	HIV+/NRTI+ 5	HIV- 35	HIV- 38	HIV- 37	HIV- 39	clone
C	9330	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%
T	9331	0.02%	0.03%	0.01%	0.00%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.02%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.02%
C	9332	0.05%	0.03%	0.08%	0.04%	0.10%	0.12%	0.06%	0.01%	0.09%	0.06%	0.03%	0.03%	0.09%	0.10%	0.11%	0.04%	0.11%	0.08%	0.07%	0.03%	0.00%
C	9333	0.01%	0.01%	0.02%	0.00%	0.00%	0.01%	0.01%	0.02%	0.00%	0.04%	0.05%	0.01%	0.02%	0.01%	0.01%	0.02%	0.00%	0.01%	0.01%	0.01%	0.11%
T	9334	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.01%	0.01%	0.04%	0.01%	0.01%	0.00%	0.00%	0.01%	0.02%	0.01%	0.02%	0.03%	0.00%	0.01%	0.03%
C	9335	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%
A	9336	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%	0.01%	0.00%	0.00%
T	9337	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
A	9338	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.02%	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%
C	9339	0.01%	0.05%	0.03%	0.01%	0.01%	0.01%	0.01%	0.03%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%
T	9340	0.01%	0.01%	0.03%	0.01%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.02%	0.03%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
A	9341	0.02%	0.03%	0.05%	0.02%	0.07%	0.11%	0.06%	0.04%	0.14%	0.08%	0.04%	0.07%	0.06%	0.06%	0.09%	0.05%	0.07%	0.06%	0.08%	0.08%	0.01%
G	9342	0.35%	0.26%	0.55%	0.18%	0.40%	0.80%	0.56%	0.15%	0.87%	0.11%	0.29%	0.23%	0.62%	0.41%	0.77%	0.47%	0.86%	0.27%	0.57%	0.12%	0.01%
G	9343	0.03%	0.01%	0.03%	0.03%	0.03%	0.01%	0.01%	0.01%	0.02%	0.02%	0.01%	0.00%	0.01%	0.04%	0.01%	0.01%	0.02%	0.01%	0.05%	0.01%	0.06%
C	9344	0.00%	0.03%	0.04%	0.03%	0.01%	0.01%	0.03%	0.02%	0.02%	0.02%	0.02%	0.05%	0.02%	0.02%	0.01%	0.01%	0.02%	0.01%	0.01%	0.03%	0.02%
C	9345	0.01%	0.03%	0.01%	0.01%	0.04%	0.01%	0.01%	0.01%	0.01%	0.03%	0.01%	0.01%	0.02%	0.01%	0.04%	0.02%	0.01%	0.02%	0.00%	0.00%	0.01%
T	9346	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
A	9347	0.03%	0.03%	0.02%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.05%	0.01%	0.00%
C	9348	0.01%	0.00%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%	0.03%	0.03%
T	9349	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%
A	9350	0.00%	0.01%	0.01%	0.01%	0.03%	0.01%	0.02%	0.01%	0.01%	0.02%	0.00%	0.00%	0.01%	0.02%	0.04%	0.01%	0.01%	0.03%	0.02%	0.04%	0.00%
A	9351	0.02%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%	0.02%	0.02%	0.00%	0.01%	0.01%	0.03%	0.00%	0.01%	0.02%
C	9352	0.01%	0.02%	0.03%	0.01%	0.02%	0.04%	0.03%	0.00%	0.01%	0.00%	0.02%	0.00%	0.01%	0.02%	0.03%	0.01%	0.03%	0.00%	0.02%	0.00%	0.02%
C	9353	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.05%
A	9354	0.01%	0.01%	0.01%	0.00%	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.02%	0.00%	0.01%	0.00%	0.03%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%
A	9355	0.00%	0.01%	0.01%	0.01%	0.00%	0.02%	0.01%	0.04%	0.00%	0.02%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%	0.03%	0.02%	0.00%	0.03%	0.00%
C	9356	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.02%
A	9357	0.01%	0.01%	0.03%	0.02%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.02%	0.01%	0.01%	0.00%	0.01%
C	9358	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
A	9359	0.00%	0.01%	0.03%	0.01%	0.04%	0.01%	0.03%	0.00%	0.00%	0.01%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%
C	9360	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.03%
T	9361	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
A	9362	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.00%	0.01%	0.02%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.02%	0.02%	0.01%	0.00%	0.01%	0.00%
A	9363	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.02%	0.01%	0.02%	0.01%	0.01%	0.00%	0.02%	0.01%	0.06%	0.01%	0.02%	0.01%	0.01%	0.02%	0.01%
C	9364	0.28%	0.22%	0.62%	0.16%	0.35%	0.82%	0.53%	0.16%	0.72%	0.18%	0.35%	0.28%	1.24%	0.43%	0.94%	0.44%	0.89%	0.35%	0.59%	0.19%	0.00%
C	9365	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%
A	9366	0.00%	0.01%	0.00%	0.05%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%
T	9367	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%
A	9368	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%
T	9369	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
A	9370	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.02%	0.03%	0.01%	0.01%	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%
C	9371	0.14%	0.11%	0.37%	0.13%	0.22%	0.45%	0.30%	0.08%	0.50%	0.05%	0.16%	0.11%	0.31%	0.22%	0.47%	0.16%	0.52%	0.18%	0.34%	0.10%	0.00%
C	9372	0.09%	0.10%	0.29%	0.07%	0.17%	0.38%	0.26%	0.05%	0.38%	0.03%	0.15%	0.10%	0.23%	0.18%	0.43%	0.13%	0.43%	0.13%	0.30%	0.07%	0.02%
A	9373	0.18%	0.15%	0.29%	0.14%	0.17%	0.90%	0.31%	0.12%	0.50%	0.15%	0.17%	0.20%	0.51%	0.32%	0.82%	0.26%	0.74%	0.27%	0.35%	0.11%	0.00%
A	9374	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.08%
T	9375	0.00%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
G	9376	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%
A	9377	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%
T	9378	0.01%	0.01%	0.02%	0.03%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%
G	9379	0.41%	0.36%	0.87%	0.31%	0.57%	3.17%	0.97%	0.24%	1.31%	0.18%	0.55%	0.51%	1.18%	0.81%	2.75%	0.64%	2.40%	0.56%	1.23%	0.20%	0.00%
G	9380	0.01%	0.02%	0.04%	0.01%	0.04%	0.03%	0.06%	0.02%	0.08%	0.05%	0.02%	0.01%	0.04%	0.04%	0.02%	0.02%	0.02%	0.02%	0.01%	0.03%	0.01%
C	9381	0.02%	0.01%	0.02%	0.01%	0.01%	0.00%	0.00%	0.05%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.02%	0.01%	0.01%	0.01%	0.01%	0.01%
G	9382	0.02%	0.03%	0.09%	0.03%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.02%	0.02%	0.01%	0.02%
C	9383	0.05%	0.02%	0.03%	0.04%	0.01%	0.01%	0.01%	0.01%	0.02%	0.04%	0.01%	0.00%	0.01%	0.04%	0.02%	0.04%	0.02%	0.01%	0.04%	0.01%	0.03%
G	9384	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
A	9385	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	9386	0.01%	0.03%	0.02%	0.01%	0.00%	0.00%	0.04%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%
G	9387	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%
T	9388	0.02%	0.02%	0.01%	0.00%	0.01%	0.03%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%	0.01%	0.01%	0.00%
A	9389	0.02%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	9390	0.01%	0.02%	0.03%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.02%	0.02%	0.01%	0.01%	0.00%	0.02%	0.01%
C	9391	0.00%	0.01%	0.00%	0.03%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.03%	0.01%	0.00%	0.00%	0.01%	0.02%
A	9392	0.01%	0.01%	0.01%	0.01%	0.01%	0.03%	0.01%	0.02%	0.02%	0.01%	0.03%	0.02%	0.01%	0.00%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.00%
C	9393	0.01%	0.02%	0.04%	0.00%	0.00%	0.00%	0.01%	0.00%	0.05%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.00%	0.00%	0.03%
G	9394	0.04%	0.06%	0.07%	0.08%	0.04%	0.04%	0.04%	0.04%	0.03%	0.04%	0.05%	0.03%	0.06%	0.06%	0.06%	0.06%	0.02%	0.08%	0.07%	0.03%	0.01%
A	9395	0.02%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.01%	0.00%	0.03%	0.03%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%	0.01%	0.00%	0.00%
G	9396	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%
A	9397	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
A	9398	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.03%	0.00%	0.04%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%
A	9399	0.00%	0.01%	0.02%	0.00%	0.00%	0.01%	0.04%	0.00%	0.03%	0.01%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.05%	0.00%	0.01%	0.02%
G	9400	0.00%	0.01%	0.04%	0.00%	0.01%	0.03%	0.00%	0.02%	0.00%	0.00%	0.01%	0.01%	0.04%	0.01%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%	0.02%
C	9401	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%
A	9402	0.02%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.02%	0.00%	0.03%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
C	9403	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%
A	9404	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%
T	9405	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%
A	9406	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.03%	0.02%	0.00%	0.02%	0.03%	0.01%	0.01%	0.00%
C	9407	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.00%	0.03%	0.02%	0.01%	0.00%	0.02%	0.01%	0.01%	0.01%	0.00%
C	9408	0.03%	0.02%	0.01%	0.01%	0.01%	0.02%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.04%	0.01%
A	9409	0.03%	0.03%	0.01%	0.00%	0.01%	0.03%	0.03%	0.02%	0.04%	0.02%	0.01%	0.05%	0.01%	0.02%	0.01%	0.02%	0.02%	0.00%	0.02%	0.03%	0.02%
A	9410	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.02%	0.02%	0.01%	0.04%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.06%
G	9411	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%
G	9412	0.02%	0.02%	0.02%	0.10%	0.00%	0.01%	0.03%	0.01%	0.02%	0.02%	0.01%	0.03%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%	0.03%	0.00%	0.01%
C	9413	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.00%	0.01%	0.01%	0.00%	0.01%	0.02%	0.04%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%
C	9414	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.04%
A	9415	0.01%	0.01%	0.01%	0.03%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.01%	0.03%	0.00%	0.02%	0.00%
C	9416	0.01%	0.02%	0.02%	0.00%	0.03%	0.02%	0.07%	0.01%	0.00%	0.01%	0.00%	0.01%	0.03%	0.01%	0.00%	0.02%	0.02%	0.01%	0.03%	0.00%	0.01%
C	9417	0.00%	0.00%	0.02%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.02%	0.01%
A	9418	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.02%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.02%	0.02%	0.02%	0.00%	0.01%	0.01%
C	9419	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%
A	9420	0.01%	0.02%	0.01%	0.01%	0.00%	0.02%	0.01%	0.01%	0.02%	0.02%	0.02%	0.01%	0.00%	0.03%	0.03%	0.01%	0.02%	0.02%	0.01%	0.01%	0.02%
C	9421	0.00%	0.00%	0.03%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%
A	9422	0.01%	0.02%	0.01%	0.02%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%
C	9423	0.02%	0.03%	0.08%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.02%	0.01%	0.03%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.03%	0.01%	0.02%
C	9424	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.03%
A	9425	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.06%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%
C	9426	0.01%	0.00%	0.05%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%	0.02%	0.02%	0.00%	0.01%	0.01%	0.01%	0.03%	0.02%	0.01%	0.02%	0.01%	0.00%
C	9427	0.05%	0.03%	0.00%	0.00%	0.00%	0.02%	0.03%	0.01%	0.01%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.03%	0.01%	0.00%	0.01%	0.01%
T	9428	0.01%	0.02%	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%	0.01%	0.02%	0.01%	0.01%	0.04%	0.03%	0.00%	0.01%	0.01%	0.01%	0.02%
G	9429	0.01%	0.07%	0.02%	0.04%	0.01%	0.08%	0.03%	0.04%	0.03%	0.04%	0.03%	0.07%	0.03%	0.03%	0.04%	0.05%	0.02%	0.02%	0.05%	0.04%	0.01%
T	9430	0.02%	0.03%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.01%	0.04%
C	9431	0.00%	0.02%	0.05%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%	0.04%	0.00%	0.03%	0.03%	0.02%	0.02%	0.01%	0.00%	0.01%	0.03%	0.03%
G	9439	0.01%	0.03%	0.02%	0.00%	0.00%	0.03%	0.01%	0.01%	0.03%	0.05%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.02%
C	9440	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.02%	0.01%	0.02%	0.02%
C	9441	0.00%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.01%	0.02%	0.01%	0.01%	0.00%	0.00%	0.04%
T	9442	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%	0.02%	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.03%	0.01%	0.02%	0.02%
T	9443	0.05%	0.08%	0.08%	0.03%	0.08%	0.07%	0.08%	0.05%	0.02%	0.03%	0.04%	0.05%	0.08%	0.04%	0.09%	0.05%	0.05%	0.04%	0.07%	0.04%	0.01%
C	9444	0.02%	0.01%	0.04%	0.05%	0.00%	0.00%	0.01%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.04%	0.01%	0.00%	0.01%	0.01%	0.02%	0.02%	0.00%
G	9445	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
A	9446	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%
T	9447	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.00%
A	9448	0.00%	0.02%	0.04%	0.01%	0.02%	0.04%	0.02%	0.04%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.05%	0.01%
C	9449	0.01%	0.01%	0.04%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.02%	0.01%	0.03%
G	9450	0.01%	0.02%	0.02%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.02%	0.01%	0.01%	0.01%	0.00%	0.02%	0.01%	0.00%	0.00%
G	9451	0.00%	0.01%	0.07%	0.01%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.04%	0.01%	0.02%	0.01%	0.00%	0.01%
G	9452	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.11%
A	9453	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
T	9454	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	9455	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%
A	9456	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
T	9457	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.03%	0.00%	0.02%	0.01%
C	9458	0.01%	0.01%	0.03%	0.01%	0.02%	0.00%	0.00%	0.02%	0.02%	0.01%	0.01%	0.02%	0.00%	0.02%	0.00%	0.01%	0.03%	0.03%	0.01%	0.00%	0.01%
C	9459	0.02%	0.01%	0.03%	0.01%	0.01%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.02%	0.00%
T	9460	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
A	9461	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	9462	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	9463	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	9464	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%
A	9465	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	9466	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%
T	9467	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%
A	9468	0.00%	0.01%	0.01%	0.01%	0.03%	0.00%	0.02%	0.01%	0.00%	0.01%	0.02%	0.01%	0.01%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%
C	9469	0.02%	0.04%	0.03%	0.02%	0.01%	0.02%	0.01%	0.03%	0.00%	0.01%	0.03%	0.01%	0.02%	0.06%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%
C	9470	0.01%	0.03%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%
T	9471	0.01%	0.01%	0.00%	0.02%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.02%
C	9472	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%
A	9473	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
G	9474	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	9476	0.01%	0.02%	0.01%	0.03%	0.01%	0.01%	0.01%	0.01%	0.03%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%
T	9486	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%
T	9487	0.01%	0.04%	0.02%	0.03%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.02%	0.04%	0.01%	0.02%	0.00%	0.03%	0.01%	0.03%	0.01%
C	9488	0.01%	0.02%	0.03%	0.02%	0.00%	0.02%	0.04%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%	0.03%	0.01%	0.01%	0.02%
G	9489	0.00%	0.01%	0.05%	0.00%	0.01%	0.01%	0.00%	0.00%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.02%	0.00%
C	9490	0.00%	0.00%	0.01%	0.01%	0.03%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.03%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%
A	9491	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.01%	0.02%	0.00%	0.00%	0.01%	0.02%	0.00%	0.01%	0.01%
G	9492	0.01%	0.01%	0.02%	0.00%	0.01%	0.01%	0.00%	0.03%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.02%	0.01%	0.00%	0.01%	0.00%	0.00%
G	9493	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
T	9501	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%
G	9502	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.03%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%
A	9503	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.01%	0.02%	0.00%	0.01%	0.01%
G	9504	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.02%	0.01%	0.01%	0.01%
C	9505	0.01%	0.00%	0.02%	0.01%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%
C	9512	0.01%	0.03%	0.02%	0.02%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.03%	0.02%	0.02%	0.01%	0.03%	0.04%	0.00%	0.01%	0.02%
C	9513	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.04%
A	9514	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%
C	9515	0.01%	0.03%	0.02%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.03%	0.01%	0.01%	0.03%	0.01%	0.00%	0.01%
T	9516	0.00%	0.00%	0.03%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.02%
C	9517	0.01%	0.02%	0.05%	0.04%	0.01%	0.02%	0.01%	0.02%	0.01%	0.03%	0.02%	0.01%	0.03%	0.01%	0.00%	0.01%	0.01%	0.03%	0.03%	0.02%	0.01%
C	9518	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.02%	0.01%
A	9519	0.00%	0.03%	0.02%	0.01%	0.00%	0.04%	0.01%	0.02%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%
G	9520	0.01%	0.01%	0.03%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.01%	0.04%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%
C	9521	0.01%	0.01%	0.03%	0.02%	0.01%	0.01%	0.06%	0.02%	0.05%	0.01%	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.05%	0.01%	0.01%	0.00%	0.02%
C	9522	0.00%	0.01%	0.01%	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%
T	9523	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%
A	9524	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.02%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%
T	9540	0.02%	0.03%	0.02%	0.01%	0.03%	0.02%	0.08%	0.49%	0.02%	0.05%	0.65%	0.00%	0.21%	0.03%	0.01%	0.01%	0.01%	0.72%	0.01%	0.09%	0.01%
T	9541	0.00%	0.02%	0.01%	0.02%	0.00%	0.00%	0.01%	0.02%	0.03%	0.01%	0.02%	0.01%	0.01%	0.02%	0.04%	0.02%	0.00%	0.01%	0.00%	0.02%	0.00%
A	9542	0.00%	0.02%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%
G	9543	0.03%	0.03%	0.03%	0.05%	0.04%	0.03%	0.06%	0.03%	0.01%	0.05%	0.01%	0.04%	0.06%	0.03%	0.03%	0.04%	0.06%	0.01%	0.03%	0.04%	0.01%
G	9544	0.04%	0.03%	0.02%	0.06%	0.03%	0.02%	0.03%	0.03%	0.04%	0.02%	0.01%	0.03%	0.04%	0.02%	0.04%	0.04%	0.01%	0.02%	0.01%	0.03%	0.12%
A	9545	0.01%	0.01%	0.03%	0.02%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.02%	0.01%	0.03%	0.01%	0.01%	0.01%	0.02%	0.02%
G	9546	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%
G	9547	0.04%	0.02%	0.06%	0.02%	0.01%	0.02%	0.04%	0.02%	0.02%	0.01%	0.02%	0.01%	0.06%	0.02%	0.03%	0.02%	0.04%	0.01%	0.01%	0.03%	0.00%
G	9548	0.00%	0.01%	0.03%	0.01%	0.01%	0.01%	0.04%	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.15%
C	9549	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.01%
A	9550	0.01%	0.01%	0.03%	0.03%	0.01%	0.01%	0.06%	0.02%	0.02%	0.02%	0.01%	0.02%	0.02%	0.01%	0.02%	0.03%	0.01%	0.03%	0.01%	0.01%	0.00%
C	9551	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.04%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%
T	9552	0.02%	0.04%	0.03%	0.01%	0.01%	0.01%	0.03%	0.05%	0.00%	0.02%	0.02%	0.01%	0.01%	0.02%	0.01%	0.02%	0.01%	0.00%	0.01%	0.02%	0.00%
G	9553	0.05%	0.09%	0.06%	0.07%	0.03%	0.08%	0.06%	5.51%	0.06%	0.08%	0.08%	0.09%	0.16%	0.11%	0.09%	0.06%	0.03%	0.14%	0.03%	0.05%	0.02%
A	9561	0.01%	0.03%	0.01%	0.03%	0.01%	0.02%	0.03%	0.04%	0.03%	0.01%	0.07%	0.01%	0.02%	0.02%	0.04%	0.02%	0.01%	0.08%	0.01%	0.01%	0.07%
C	9562	0.01%	0.00%	0.02%	0.01%	0.02%	0.00%	0.01%	0.02%	0.00%	0.00%	0.01%	0.00%	0.02%	0.03%	0.01%	0.03%	0.02%	0.01%	0.01%	0.01%	0.00%
A	9563	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.02%	0.02%
G	9564	0.01%	0.02%	0.02%	0.03%	0.00%	0.02%	0.01%	0.01%	0.02%	0.02%	0.01%	0.01%	0.04%	0.01%	0.08%	0.01%	0.01%	0.02%	0.02%	0.01%	0.00%
G	9565	0.00%	0.02%	0.04%	0.03%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.01%	0.01%	0.02%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.00%
C	9566	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%
A	9567	0.00%	0.00%	0.01%	0.02%	0.01%	0.00%	0.00%	0.03%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%
T	9568	0.00%	0.01%	0.01%	0.04%	0.00%	0.01%	0.02%	0.02%	0.00%	0.03%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.02%	0.00%
C	9569	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.01%

HVS2 seq	Subject HVS2 pos	POLG1 P1	POLG1 P2	POLG1 P3	POLG1 P4	HIV+/NRTI- 29	HIV+/NRTI- 23	HIV+/NRTI- 32	HIV+/NRTI- 25	HIV+/NRTI+ 17	HIV+/NRTI+ 20	HIV+/NRTI+ 18	HIV+/NRTI+ 16	HIV+/NRTI+ 7	HIV+/NRTI+ 12	HIV+/NRTI+ 15	HIV+/NRTI+ 5	HIV- 35	HIV- 38	HIV- 37	HIV- 39	clone
G	185	8774	8936	9450	9354	8489	6414	9148	8816	9242	9385	9257	9679	9732	9750	10369	8229	9144	8488	8100	10077	8733
C	186	8778	8936	9450	9355	8489	6419	9149	8819	9246	9387	9259	9679	9732	9751	10370	8229	9144	8488	8100	10078	8733
G	187	8778	8936	9450	9356	8489	6425	9149	8819	9246	9387	9259	9679	9732	9751	10370	8229	9144	8488	8100	10078	8733
A	188	8812	8970	9450	9358	8489	6542	9153	8848	9273	9453	9273	9683	9733	9756	10387	8229	9155	8492	8101	10123	8733
A	189	8814	8971	9450	9358	8489	6542	9153	8849	9274	9454	9273	9683	9733	9756	10387	8229	9156	8492	8101	10124	8733
C	190	8814	8971	9450	9358	8489	6546	9153	8849	9274	9455	9273	9683	9733	9756	10387	8229	9156	8492	8101	10124	8733
A	191	8814	8971	9450	9359	8489	6584	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
T	192	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
A	193	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
C	194	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
T	195	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
T	196	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
A	197	8815	8971	9450	9359	8489	6600	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
C	198	8815	8971	9450	9359	8489	6608	9153	8849	9274	9455	9273	9684	9733	9756	10387	8229	9156	8492	8101	10124	8733
T	199	8815	8971	9450	9359	8489	6632	9153	8849	9274	9455	9273	9684	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	200	8815	8972	9450	9359	8489	6640	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	201	8815	8972	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	202	8815	8972	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	203	8815	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	204	8815	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	205	8815	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	206	8815	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	207	8817	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	208	8817	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	209	8817	8973	9450	9361	8489	6641	9153	8849	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	210	8818	8973	9450	9361	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	211	8818	8973	9450	9361	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	212	8819	8973	9450	9362	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	213	8819	8973	9450	9362	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	214	8819	8973	9450	9362	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	215	8819	8973	9450	9362	8489	6641	9153	8850	9274	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	216	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	217	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	218	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	219	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	220	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	221	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
C	222	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	223	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	224	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	225	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
T	226	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	227	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	228	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
G	229	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
A	230	8819	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8492	8101	10124	8733
C	231	8820	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8493	8101	10124	8733
A	232	8821	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8493	8101	10124	8733
T	233	8822	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9685	9733	9756	10387	8230	9156	8493	8101	10124	8733
A	234	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8493	8101	10124	8733
A	235	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8493	8101	10124	8733
T	236	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8493	8101	10124	8733
A	237	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	238	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	239	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	240	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	241	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
C	242	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	243	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	244	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	245	8825	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	246	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
G	247	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	248	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
A	249	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	250	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
G	251	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	252	8826	8973	9450	9362	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
C	253	8828	8973	9450	9363	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
T	254	8828	8973	9450	9363	8489	6641	9153	8850	9275	9455	9273	9686	9733	9756	10387	8230	9156	8494	8101	10124	8733
G	255	8828	8973	9450	9363	8489	6641	9153	8851	9275	9456	9273	9686	9733	9757	10388	8230	9156	8494	8101	10124	8733
C	256	8828	8973	9452	9363	8489	6642	9153	8851	9275	9456	9273	9687	9735	9759	10388	8230	9156	8494	8101	10124	8733
A	257	8829	8973	9452	9363	8490	6642	9153	8851	9275	9457	9273	9687	9735	9759	10388	8230	9156	8496	8101	10124	8733
C	258	8829	8973	9452	9364	8490	6642	9153	8851	9275	9457	9273	9687	9737	9759	10388	8230	9156	8496	8101	10124	8733
A	259	8829	8973	9452	9364	8490	6642	9153	8851	9275	9457	9273	9687	9737	9759	10388	8230	9156	8496	8101	10124	8733
G	260	8829	8974	9452	9364	8490	6642	9153	8851	9275	9457	9273	9687	9737	9759	10388	8230	9156	8496	8101	10124	8733
C	261	8829	8974	9452	9364	8490	6642	9154	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	262	8829	8974	9452	9364	8490	6642	9154	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
A	263	8829	8974	9452	9365	8491	6643	9154	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	264	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
T	265	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
T	266	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
T	267	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	268	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	269	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
A	270	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	271	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
A	272	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
C	273	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8231	9157	8496	8101	10124	8733
A	274	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8232	9157	8496	8101	10124	8733
G	275	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8232	9157	8496	8101	10124	8733
A	276	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8232	9157	8496	8101	10124	8733
C	277	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8232	9157	8496	8101	10124	8733
A	278	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10389	8232	9157	8496	8101	10124	8733
T	279	8829	8974	9452	9365	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
C	280	8829	8974	9453	9366	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
A	281	8829	8974	9453	9366	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
T	282	8829	8974	9453	9366	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
A	283	8829	8975	9453	9367	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
A	284	8829	8975	9453	9367	8491	6643	9155	8851	9275	9457	9274	9687	9737	9760	10390	8232	9157	8496	8101	10124	8733
T	293	8830	8980	9456	9374	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	295	8830	8980	9458	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	296	8830	8980	9459	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	297	8830	8980	9459	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	298	8830	8980	9459	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	300	8830	8980	9459	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	301	8830	8980	9459	9375	8491	6644	9155	8852	9277	9458	9274	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	317	8830	8979	9458	9374	8491	6644	9155	8852	9277	9458	9273	9689	9739	9760	10391	8233	9157	8498	8102	10124	8733
T	318	8830	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8233	9157	8498	8102	10124	8733
T	319	8830	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8233	9157	8498	8102	10124	8733
C	320	8830	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8233	9157	8498	8102	10124	8733
T	321	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8233	9157	8498	8102	10124	8733
G	322	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
G	323	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	324	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	325	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	326	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9689	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	327	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	328	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
G	329	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	330	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	331	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	332	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	333	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	334	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	335	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	336	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	337	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	338	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	339	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	340	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	341	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	342	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	343	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	344	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	345	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	346	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
G	347	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	348	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	349	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	350	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	351	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	363	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	364	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	365	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
G	366	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	367	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	368	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	369	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	370	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	371	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
T	372	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	373	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	374	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	375	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9688	9739	9760	10391	8232	9157	8498	8102	10124	8733
A	376	8829	8979	9458	9374	8491	6644	9155	8852	9277	9457	9273	9687	9739	9760	10391	8232	9157	8498	8102	10124	8733
C	377	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
C	378	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
A	379	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
G	380	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
C	381	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
C	382	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
T	383	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
A	384	8829	8979	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
A	385	8829	8978	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
C	386	8829	8978	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
C	387	8829	8978	9458	9374	8491	6644	9155	8852	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8102	10124	8733
A	388	8829	8978	9458	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8101	10124	8733
G	389	8829	8978	9458	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8101	10124	8733
A	390	8829	8978	9458	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9157	8497	8101	10124	8733
T	391	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
T	392	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
T	393	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
C	394	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
A	395	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
A	396	8829	8978	9457	9374	8490	6643	9155	8851	9277	9456	9273	9687	9739	9760	10391	8232	9156	8496	8101	10124	8733
T	403	8825	8976	9457	9374	8490	6643	9155	8849	9276	9456	9273	9687	9739	9759	10391	8232	9155	8496	8101	10124	8733
G	410	8824	8976	9457	9374	8490	6643	9155	8849	9274	9456	9273	9687	9739	9759	10390	8232	9155	8496	8101	10124	8733
C	411	8824	8976	9457	9374	8490	6643	9155	8849	9274	9456	9273	9687	9739	9759	10390	8231	9155	8496	8101	10124	8733
G	412	8824	8976	9457	9374	8490	6643	9154	8849	9274	9456	9273	9687	9739	9759	10389	8231	9155	8496	8101	10124	8733
G	413	8824	8975	9457	9374	8490	6643	9154	8849	9272	9456	9273	9687	9739	9759	10389	8231	9155	8496	8101	10124	8733
T	414	8824	8975	9456	9374	8490	6643	9154	8849	9272	9456	9273	9687	9739	9759	10389	8231	9155	8496	8101	10124	8733
A	415	8823	8975	9456	9374	8490	6643	9154	8849	9271	9456	9273	9687	9738	9759	10389	8231	9155	8496	8101	10124	8733
T	416	8823	8975	9455	9373	8489	6643	9154	8848	9271	9456	9272	9687	9738	9759	10389	8231	9155	8496	8101	10123	8733
G	417	8823	8975	9455	9373	8489	6643	9154	8848	9271	9456	9272	9687	9738	9759	10389	8231	9155	8496	8101	10123	8733
C	418	8823	8975	9455	9373	8489	6643	9154	8848	9271	9456	9272	9687	9738	9759	10389	8231	9155	8496	8101	10123	8733
A	419	8823	8975	9455	9373	8489	6643	9154	8848	9271	9456	9272	9687	9738	9759	10389	8231	9155	8496	8101	10123	8733
A	426	8823	8974	9453	9372	8489	6643	9154	8848	9271	9456	9271	9685	9738	9757	10388	8231	9154	8496	8101	10123	8733
C	427	8822	8972	9453	9371	8488	6640	9152	8848	9271	9455	9271	9685	9736	9756	10387	8231	9153	8496	8100	10122	8733
A	428	8822	8971	9453	9371	8488	6639	9152	8848	9271	9455	9271	9685	9735	9756	10387	8231	9153	8496	8100	10122	8733
G	429	8822	8970	9453	9371	8488	6635	9152	8848	9271	9455	9271	9685	9735	9756	10387	8231	9153	8496	8100	10122	8733
T	430	8821	8969	9453	9371	8488	6606	9152	8848	9270	9455	9271	9685	9735	9756	10387	8230	9152	8496	8100	10122	8733
C	431	8818	8966	9453	9371	8488	6501	9152	8848	9270	9455	9270	9685	9735	9755	10387	8229	9152	8496	8100	10122	8733

HVS2 seq	Subject HVS2 pos	POLG1 P1	POLG1 P2	POLG1 P3	POLG1 P4	HIV+/NRTI- 29	HIV+/NRTI- 23	HIV+/NRTI- 32	HIV+/NRTI- 25	HIV+/NRTI+ 17	HIV+/NRTI+ 20	HIV+/NRTI+ 18	HIV+/NRTI+ 16	HIV+/NRTI+ 7	HIV+/NRTI+ 12	HIV+/NRTI+ 15	HIV+/NRTI+ 5	HIV- 35	HIV- 38	HIV- 37	HIV- 39	clone
G	185	0.74%	0.67%	0.13%	0.10%	0.05%	2.67%	0.37%	0.34%	0.38%	0.72%	0.35%	0.13%	0.13%	0.08%	0.31%	0.05%	0.10%	0.16%	0.23%	0.79%	0.05%
C	186	0.98%	1.32%	0.03%	18.66%	0.04%	0.89%	0.11%	0.50%	0.65%	0.94%	0.51%	0.18%	0.09%	0.08%	0.26%	0.04%	0.13%	0.06%	0.04%	1.08%	0.06%
G	187	0.15%	0.26%	0.05%	0.03%	0.01%	0.62%	0.02%	0.08%	0.08%	0.26%	0.12%	0.05%	0.01%	0.04%	0.06%	0.00%	0.01%	0.01%	0.00%	0.25%	0.02%
A	188	0.27%	0.43%	0.01%	0.00%	0.04%	0.64%	0.03%	0.11%	0.14%	0.36%	0.15%	0.04%	0.01%	0.01%	0.11%	0.00%	0.03%	0.01%	0.00%	0.51%	0.00%
A	189	3.00%	4.96%	0.01%	0.09%	0.39%	7.55%	0.45%	1.41%	2.63%	3.41%	1.67%	0.37%	0.26%	0.27%	1.20%	0.01%	0.54%	0.54%	0.20%	3.65%	0.01%
C	190	0.02%	0.01%	0.00%	0.03%	0.02%	1.56%	0.00%	0.02%	0.00%	0.02%	0.02%	0.03%	0.01%	0.01%	0.02%	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%
A	191	0.00%	0.01%	0.00%	0.07%	0.00%	2.72%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	192	0.00%	0.00%	0.01%	0.73%	0.00%	0.09%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	193	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%
C	194	0.05%	0.03%	0.03%	0.06%	0.07%	0.00%	0.02%	0.00%	0.01%	0.04%	0.00%	0.03%	0.03%	0.04%	0.00%	0.01%	0.02%	0.00%	0.04%	0.02%	0.02%
T	195	0.06%	0.03%	0.04%	0.06%	0.06%	8.58%	0.02%	1.25%	0.02%	0.14%	0.53%	0.05%	0.75%	0.06%	0.42%	0.01%	0.03%	0.68%	0.05%	0.28%	0.00%
T	196	0.10%	0.08%	0.01%	0.03%	0.04%	5.36%	0.07%	0.67%	0.08%	0.19%	0.26%	0.12%	0.20%	0.07%	0.23%	0.09%	0.05%	0.21%	0.04%	0.12%	0.00%
A	197	0.01%	0.01%	0.01%	0.00%	0.00%	4.06%	0.00%	0.00%	0.01%	0.03%	0.00%	0.04%	0.01%	0.01%	0.00%	0.04%	0.00%	0.00%	0.02%	0.03%	0.00%
C	198	0.00%	0.00%	0.00%	0.00%	0.01%	0.55%	0.00%	0.05%	0.02%	0.01%	0.06%	0.03%	0.02%	0.02%	0.10%	0.01%	0.03%	0.75%	0.01%	0.18%	0.01%
T	199	0.02%	0.02%	0.00%	0.03%	0.00%	0.11%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%
A	200	0.01%	0.01%	0.00%	0.01%	0.00%	49.98%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.09%	0.01%	0.00%	0.00%
A	201	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	202	0.12%	0.08%	0.15%	0.04%	0.08%	0.15%	0.09%	0.07%	0.04%	0.10%	0.12%	0.10%	0.11%	0.09%	0.10%	0.06%	0.04%	0.01%	0.07%	0.11%	0.06%
G	203	0.01%	0.01%	0.00%	0.03%	0.01%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%	0.01%	0.00%	0.01%	0.01%	0.00%	0.01%	0.02%	0.01%	0.01%
T	204	0.02%	0.03%	0.00%	0.00%	0.04%	0.15%	0.02%	0.02%	0.01%	0.04%	0.03%	0.02%	0.00%	0.02%	0.01%	0.16%	0.00%	0.47%	0.01%	0.00%	0.00%
G	205	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%
T	206	0.00%	0.00%	0.00%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.02%	0.01%	0.01%	0.02%	0.00%	0.00%
G	207	0.02%	0.00%	0.00%	0.12%	0.00%	0.00%	0.02%	0.01%	0.02%	0.01%	0.03%	0.00%	0.02%	0.01%	0.03%	0.01%	0.03%	0.86%	0.01%	0.20%	0.02%
T	208	0.00%	0.00%	0.03%	0.00%	0.01%	0.00%	0.02%	0.00%	0.00%	0.02%	0.02%	0.01%	0.00%	0.01%	0.02%	0.01%	0.00%	0.11%	0.01%	0.00%	0.00%
T	209	0.00%	0.01%	0.05%	0.00%	0.01%	0.03%	0.03%	0.00%	0.00%	0.02%	0.06%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.12%	0.04%	0.01%	0.01%
A	210	0.00%	0.00%	0.04%	0.00%	0.01%	0.02%	0.02%	0.00%	0.00%	0.02%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.12%	0.02%	0.00%	0.01%
A	211	0.10%	0.07%	0.14%	0.10%	0.09%	0.09%	0.12%	0.09%	0.05%	0.14%	0.10%	0.11%	0.03%	0.03%	0.09%	0.10%	0.03%	0.02%	0.07%	0.05%	0.00%
T	212	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	213	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
A	214	11.58%	9.21%	1.82%	1.99%	0.00%	0.54%	0.01%	0.16%	0.28%	0.34%	0.02%	0.03%	0.04%	0.00%	0.00%	0.01%	0.01%	0.20%	0.19%	0.08%	0.00%
A	215	6.66%	4.47%	1.80%	1.51%	0.01%	0.54%	0.03%	0.49%	0.25%	0.32%	0.03%	0.03%	0.10%	0.04%	0.01%	0.02%	0.12%	0.15%	0.37%	0.12%	0.05%
T	216	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	217	0.06%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.02%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	218	0.02%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.01%	0.02%	0.02%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%
A	219	0.05%	0.03%	0.01%	0.03%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.03%	0.03%	0.03%	0.02%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%
T	220	0.02%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
G	221	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%
C	222	0.02%	0.00%	0.06%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%
T	223	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	224	0.03%	0.02%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.00%
G	225	0.05%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.03%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.03%	0.00%	0.00%	0.02%	0.00%	0.00%	0.02%
T	226	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%
A	227	0.03%	0.01%	0.00%	0.01%	0.02%	0.00%	0.01%	0.02%	0.00%	0.01%	0.01%	0.02%	0.02%	0.02%	0.06%	0.00%	0.01%	0.01%	0.00%	0.00%	0.02%
G	228	0.02%	0.04%	0.06%	0.03%	0.01%	0.14%	0.23%	0.18%	0.00%	0.02%	0.02%	0.00%	0.01%	0.04%	0.04%	0.00%	0.05%	0.02%	0.15%	0.01%	0.00%
G	229	0.03%	0.03%	0.02%	0.14%	0.00%	0.15%	0.10%	0.05%	0.00%	0.01%	0.00%	0.02%	0.05%	0.02%	0.00%	0.00%	0.05%	0.15%	0.04%	0.00%	0.05%
A	230	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
C	231	0.00%	0.01%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.01%
A	232	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	233	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
A	234	0.05%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
A	235	0.24%	0.30%	0.03%	0.02%	0.01%	0.05%	0.01%	0.01%	0.04%	0.02%	0.01%	0.03%	0.03%	0.03%	0.07%	0.00%	0.00%	0.02%	0.02%	0.03%	0.00%
T	236	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
A	237	0.11%	0.23%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.05%	0.00%
A	238	0.20%	0.11%	0.02%	0.15%	0.00%	0.00%	0.00%	0.00%	0.04%	0.03%	0.01%	0.00%	0.03%	0.01%	0.01%	0.00%	0.01%	0.01%	0.02%	0.00%	0.06%
T	239	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	240	0.29%	0.21%	0.00%	0.16%	0.00%	0.02%	0.00%	0.08%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%
A	241	0.25%	0.18%	0.02%	0.06%	0.00%	0.06%	0.01%	0.00%	0.09%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.01%	0.00%
C	242	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
A	243	0.14%	0.07%	0.00%	0.10%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.03%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
A	244	0.12%	0.09%	0.00%	0.12%	0.00%	0.00%	0.00%	0.08%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
T	245	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	246	0.12%	0.06%	0.01%	0.41%	0.00%	0.00%	0.00%	0.02%	0.03%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.02%	0.05%	0.00%	0.00%
G	247	0.02%	0.01%	0.01%	0.03%	0.00%	0.02%	0.00%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	248	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%
A	249	0.01%	0.02%	0.00%	0.01%	0.00%	0.02%	0.00%	0.00%	0.01%	0.02%	0.00%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%
T	250	0.03%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
G	251	0.85%	0.71%	0.00%	0.37%	0.00%	0.02%	0.00%	0.05%	0.04%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.04%	0.12%	0.24%	0.00%
T	252	0.02%	0.02%	0.01%	0.05%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%
C	253	0.43%	0.50%	0.05%	0.12%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%
T	254	0.19%	0.19%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%
G	255	0.02%	0.02%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%
C	256	0.01%	0.02%	0.01%	0.00%	0.01%	0.02%	0.02%	0.00%	0.01%	0.01%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
A	257	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.03%	0.01%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%
C	258	0.02%	0.01%	0.02%	0.01%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.02%	0.04%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%
A	259	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
G	260	0.01%	0.00%	0.02%	0.02%	0.01%	0.00%	0.00%	0.03%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%
C	261	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.01%
C	262	0.00%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.02%	0.01%	0.01%
A	263	0.03%	0.03%	0.05%	0.07%	0.01%	0.03%	0.01%	0.01%	0.04%	0.01%	0.01%	0.07%	0.06%	0.00%	0.07%	0.04%	0.01%	0.04%	0.04%	0.04%	0.14%
C	264	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
T	265	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%
T	266	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	267	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.06%
C	268	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C	269	0.01%	0.02%	0.05%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.01%	0.02%	0.01%	0.04%	0.01%	0.02%	0.02%	0.02%	0.01%
A	270	0.00%	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.05%	0.00%	0.00%	0.00%	0.00%	0.01%
C	271	0.01%	0.02%	0.01%	0.03%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.04%	0.01%
A	272	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%
C	273	0.70%	0.40%	0.01%	0.07%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.03%	0.00%	0.00%	0.06%	0.05%	0.04%	0.02%
A	274	0.19%	0.19%	0.01%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
G	275	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%
A	276	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%
C	277	0.58%	0.57%	0.00%	0.33%	0.00%	0.00%	0.02%	0.06%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.00%	0.00%	0.02%	0.01%	0.00%	0.02%	0.00%
A	278	0.01%	0.04%	0.02%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	279	0.08%	0.08%	0.01%	0.07%	0.00%	0.00%	0.00%	0.18%	0.00%	0.01%	0.05%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
C	280	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
A	281	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
T	282	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.08%	0.00%	0.61%	0.01%	0.01%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%
A	283	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%
A	284	0.24%	0.30%	0.53%	0.38%	0.60%	0.69%	0.23%	0.16%	0.50%	0.41%	0.26%	0.44%	0.27%	0.32%	0.22%	0.55%	0.41%	0.14%	0.65%	0.37%	0.02%
T	293	0.01%	0.02%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%
C	295	0.01%	0.01%	0.01%	0.02%	0.01%	0.03%	0.10%	0.01%	0.00%	0.00%	0.01%	0.02%	0.00%	0.00%	0.02%	0.01%	0.02%	0.00%	0.07%	0.00%	0.00%
C	296	0.02%	0.02%	0.03%	0.12%	0.00%	0.21%	0.15%	0.01%	0.04%	0.01%	0.01%	0.03%	0.01%	0.01%	0.02%	0.07%	0.04%	0.14%	0.10%	0.00%	0.00%
A	297	0.02%	0.03%	0.02%	0.00%	0.01%	0.05%	0.01%	0.02%	0.05%	0.00%	0.01%	0.03%	0.01%	0.01%	0.05%	0.07%	0.02%	0.01%	0.02%	0.01%	0.00%
C	298	0.00%	0.01%	0.03%	0.00%	0.00%	0.02%	0.00%	0.00%	0.02%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
A	300	0.00%	0.03%	0.04%	0.01%	0.02%	0.02%	0.02%	0.00%	0.02%	0.01%	0.00%	0.01%	0.01%	0.02%	0.01%	0.04%	0.00%	0.01%	0.01%	0.02%	0.05%
A	301	0.00%	0.00%	0.03%	0.01%	0.00%	0.03%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
C	317	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
T	318	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.03%	0.00%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.02%	0.00%
T	319	0.03%	0.02%	0.03%	0.01%	0.01%	0.03%	0.01%	0.02%	0.02%	0.03%	0.02%	0.01%	0.05%	0.00%	0.01%	0.02%	0.00%	0.01%	0.02%	0.02%	0.34%
C	320	0.01%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.02%	0.01%	0.00%
T	321	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.02%	0.00%	0.01%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.01%
G	322	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
G	323	0.02%	0.02%	0.02%	0.00%	0.01%	0.02%	0.00%	0.00%	0.02%	0.00%	0.01%	0.03%	0.03%	0.00%	0.01%	0.04%	0.00%	0.00%	0.01%	0.01%	0.22%
C	324	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%
C	325	0.00%	0.01%	0.06%	0.05%	0.01%	0.02%	0.01%	0.00%	0.02%	0.01%	0.02%	0.03%	0.00%	0.03%	0.03%	0.00%	0.07%	0.01%	0.00%	0.02%	0.03%
A	326	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%
C	327	0.01%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.04%	0.01%	0.00%	0.00%	0.00%	0.01%
A	328	0.02%	0.00%	0.00%	0.07%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.03%	0.00%
G	329	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
C	330	0.02%	0.00%	0.02%	0.00%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%
A	331	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
C	332	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.02%	0.00%
T	333	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
T	334	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.02%	0.02%
A	335	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	336	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
A	337	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
C	338	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.02%	0.01%	0.02%	0.01%	0.02%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%
A	339	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
C	340	0.03%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.02%	0.04%	0.00%	0.01%	0.00%	0.00%	0.03%
A	341	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.01%	0.00%	0.00%
T	342	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C	343	0.00%	0.00%	0.01%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
T	344	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C	345	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%
T	346	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%
G	347	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%
C	348	0.01%	0.00%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
C	349	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.05%	0.01%	0.00%	0.01%	0.05%	0.00%	0.02%	0.00%	0.02%	0.00%	0.01%	0.00%	0.01%	0.06%
A	350	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%
A	351	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	363	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
A	364	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
A	365	0.11%	0.11%	0.11%	0.16%	0.13%	0.09%	0.14%	0.06%	0.12%	0.11%	0.19%	0.12%	0.08%	0.10%	0.12%	0.19%	0.16%	0.18%	0.10%	0.13%	0.25%
G	366	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
A	367	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A	368	0.01%	0.01%	0.01%	0.02%	0.00%	0.02%	0.00%	0.01%	0.02%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.04%	0.01%	0.01%	0.01%	0.05%	0.02%
C	369	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
C	370	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
C	371	0.02%	0.01%	0.01%	0.02%	0.04%	0.00%	0.01%	0.00%	0.03%	0.04%	0.00%	0.03%	0.04%	0.00%	0.01%	0.00%	0.03%	0.01%	0.01%	0.00%	0.00%
T	372	0.01%	0.00%	0.02%	0.02%	0.02%	0.00%	0.04%	0.02%	0.03%	0.01%	0.01%	0.03%	0.01%	0.00%	0.02%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%
A	373	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
A	374	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.04%	0.01%	0.00%	0.00%	0.02%
C	375	0.00%	0.00%	0.01%	0.01%	0.02%	0.02%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.02%	0.04%	0.05%	0.01%	0.01%	0.02%	0.00%
A	376	0.00%	0.00%	0.00%	0.01%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%
C	377	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
C	378	0.01%	0.00%	0.02%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.04%	0.00%	0.00%	0.00%	0.03%	0.00%
A	379	0.00%	0.00%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.03%	0.04%	0.01%	0.01%	0.00%	0.00%	0.01%
G	380	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
C	381	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%
C	382	0.00%	0.07%	0.04%	0.03%	0.07%	0.02%	0.02%	0.00%	0.04%	0.00%	0.01%	0.06%	0.02%	0.02%	0.01%	0.02%	0.05%	0.06%	0.01%	0.02%	0.02%
T	383	0.02%	0.03%	0.02%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%
A	384	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
A	385	0.16%	0.06%	0.00%	0.05%	0.01%	0.02%	0.01%	0.02%	0.08%	0.01%	0.00%	0.02%	0.00%	0.01%	0.01%	0.00%	0.02%	0.01%	0.04%	0.01%	0.00%
C	386	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
C	387	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%	0.02%	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%
A	388	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
G	389	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.02%
A	390	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	391	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
T	392	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	393	0.11%	0.08%	0.10%	0.05%	0.12%	0.12%	0.10%	0.14%	0.14%	0.14%	0.14%	0.12%	0.05%	0.14%	0.08%	0.09%	0.12%	0.07%	0.11%	0.08%	0.05%
C	394	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.02%	0.02%	0.00%	0.01%	0.03%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%
A	395	0.05%	0.01%	0.02%	0.02%	0.00%	0.00%	0.01%	0.02%	0.03%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%
A	396	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
T	403	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%
G	410	0.02%	0.03%	0.05%	0.03%	0.04%	0.06%	0.02%	0.02%	0.05%	0.03%	0.02%	0.05%	0.02%	0.03%	0.03%	0.01%	0.07%	0.05%	0.02%	0.06%	0.10%
C	411	0.53%	0.53%	0.06%	0.15%	0.06%	0.08%	0.04%	0.05%	0.36%	0.03%	0.08%	0.01%	0.01%	0.03%	0.13%	0.02%	0.07%	0.06%	0.09%	0.06%	0.02%
G	412	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
G	413	0.00%	0.06%	0.01%	0.01%	0.02%	0.02%	0.03%	0.01%	0.03%	0.00%	0.02%	0.03%	0.03%	0.01%	0.00%	0.00%	0.01%	0.00%	0.01%	0.03%	0.05%
T	414	0.48%	0.65%	0.01%	0.09%	0.01%	0.51%	0.01%	0.16%	3.69%	0.23%	0.06%	0.18%	0.05%	0.05%	0.05%	0.05%	0.00%	0.00%	0.06%	0.03%	0.00%
A	415	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
T	416	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
G	417	0.01%	0.00%	0.04%	0.02%	0.00%	0.03%	0.01%	0.00%	0.01%	0.01%	0.01%	0.01%	0.02%	0.00%	0.03%	0.01%	0.02%	0.00%	0.01%	0.00%	0.00%
C	418	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.04%	0.01%	0.00%	0.00%	0.01%	0.00%
A	419	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
A	426	0.01%	0.00%	0.01%	0.00%	0.01%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.01%
C	427	0.00%	0.00%	0.02%	0.03%	0.04%	0.03%	0.01%	0.00%	0.02%	0.00%	0.01%	0.03%	0.03%	0.13%	0.00%	0.01%	0.02%	0.00%	0.02%	0.01%	0.05%
A	428	0.00%	0.01%	0.03%	0.01%	0.01%	0.00%	0.01%	0.00%	0.02%	0.01%	0.02%	0.01%	0.01%	0.02%	0.00%	0.04%	0.04%	0.01%	0.00%	0.01%	0.01%
G	429	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
T	430	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%
C	431	0.01%	0.00%	0.10%	0.00%	0.00%	0.00%	0.01%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%

		BRCA2 (genomic 1)		BRCA2 (genomic 2)		BRCA2 (clonal)	
osition Chr1	seq	coverage		coverage		coverage	
32907125	T	53488	0.0000%	6906	0.0000%	12264	0.0245%
32907126	C	53489	0.0019%	6906	0.0145%	12264	0.0082%
32907127	T	53491	0.0150%	6906	0.0000%	12264	0.0163%
32907128	A	53491	0.0000%	6906	0.0000%	12264	0.0000%
32907129	T	53492	0.0019%	6906	0.0290%	12264	0.0082%
32907130	A	53495	0.0019%	6906	0.0000%	12264	0.0000%
32907131	T	53499	0.0019%	6906	0.0000%	12264	0.0082%
32907132	T	53500	0.0075%	6906	0.1448%	12264	0.1223%
32907133	C	53501	0.0075%	6906	0.0000%	12264	0.0163%
32907134	A	53503	0.0224%	6906	0.0145%	12264	0.0326%
32907135	G	53561	0.0000%	6906	0.0145%	12264	0.0082%
32907136	A	53575	0.0019%	6906	0.0145%	12264	0.0000%
32907137	A	53584	0.0093%	6906	0.0290%	12264	0.0000%
32907138	T	53584	0.0037%	6906	0.0000%	12264	0.0000%
32907139	A	53585	0.0000%	6906	0.0000%	12264	0.0000%
32907140	A	53586	0.0056%	6906	0.0000%	12264	0.0000%
32907141	G	53586	0.0019%	6906	0.0145%	12264	0.0000%
32907142	A	53587	0.0019%	6906	0.0145%	12264	0.0000%
32907143	G	53588	0.0000%	6906	0.0145%	12264	0.0163%
32907144	A	53588	0.0037%	6906	0.0000%	12264	0.0082%
32907145	A	53589	0.0000%	6906	0.0000%	12264	0.0000%
32907146	T	53589	0.0000%	6906	0.0000%	12264	0.0000%
32907147	C	53590	0.0000%	6906	0.0145%	12264	0.0000%
32907148	A	53591	0.0000%	6906	0.0000%	12264	0.0000%
32907149	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907150	C	53592	0.0019%	6906	0.0000%	12264	0.0245%
32907151	T	53592	0.0000%	6906	0.0434%	12264	0.0163%
32907152	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907153	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907154	A	53592	0.0019%	6906	0.0579%	12264	0.0245%
32907155	G	53592	0.0000%	6906	0.0290%	12264	0.0245%
32907156	A	53592	0.0000%	6906	0.0000%	12264	0.0082%
32907157	G	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907158	A	53592	0.0000%	6906	0.0145%	12264	0.0000%
32907159	C	53592	0.0019%	6906	0.0290%	12264	0.0408%
32907160	T	53592	0.0000%	6906	0.0145%	12264	0.0000%
32907161	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907162	T	53592	0.0056%	6906	0.1014%	12264	0.0897%
32907163	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907164	A	53592	0.0000%	6906	0.0000%	12264	0.0163%
32907165	A	53592	0.0075%	6906	0.0000%	12264	0.0000%
32907166	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907167	G	53592	0.0037%	6906	0.0145%	12264	0.0082%
32907168	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907169	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907170	A	53592	0.0243%	6906	0.0000%	12264	0.0163%
32907178	A	53592	0.0037%	6906	0.0434%	12264	0.0245%
32907179	G	53592	0.0000%	6906	0.0000%	12264	0.0082%
32907180	G	53592	0.0019%	6906	0.0290%	12264	0.0000%
32907181	T	53592	0.0075%	6906	0.0145%	12264	0.0815%
32907182	C	53592	0.0000%	6906	0.0000%	12264	0.0245%
32907183	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907184	T	53592	0.0093%	6906	0.0145%	12264	0.0000%
32907185	A	53592	0.0131%	6906	0.0000%	12264	0.0082%
32907186	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907187	G	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907188	A	53592	0.0485%	6906	0.0145%	12264	0.0082%
32907189	C	53592	0.0000%	6906	0.0579%	12264	0.1060%
32907190	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907191	G	53592	0.0019%	6906	0.0000%	12264	0.0000%
32907192	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907193	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907194	C	53592	0.0019%	6906	0.0145%	12264	0.0163%
32907195	C	53592	0.0056%	6906	0.0000%	12264	0.0082%
32907196	A	53592	0.0019%	6906	0.0000%	12264	0.0000%
32907197	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907198	A	53592	0.0299%	6906	0.0145%	12264	0.0082%
32907199	C	53592	0.0037%	6906	0.0145%	12264	0.0082%
32907200	T	53592	0.0019%	6906	0.0000%	12264	0.0000%
32907201	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907210	A	53592	0.0000%	6906	0.0145%	12264	0.0000%
32907211	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907212	A	53592	0.0205%	6906	0.0000%	12264	0.0408%
32907213	C	53592	0.0000%	6906	0.0000%	12264	0.0163%
32907214	T	53592	0.0000%	6906	0.0000%	12264	0.0082%
32907215	G	53592	0.0000%	6906	0.0000%	12264	0.0245%
32907216	A	53592	0.0075%	6906	0.0000%	12264	0.0082%
32907217	A	53592	0.0056%	6906	0.0290%	12264	0.0163%
32907218	G	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907219	C	53592	0.0019%	6906	0.0000%	12264	0.0082%
32907220	C	53592	0.0149%	6906	0.0145%	12264	0.0163%
32907221	T	53592	0.0019%	6906	0.0145%	12264	0.0082%
32907222	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907223	T	53592	0.0112%	6906	0.0579%	12264	0.0000%
32907224	G	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907225	A	53592	0.0000%	6906	0.0000%	12264	0.0082%
32907226	A	53592	0.0056%	6906	0.0000%	12264	0.0000%
32907227	A	53592	0.0131%	6906	0.0145%	12264	0.0245%
32907228	G	53592	0.0075%	6906	0.0145%	12264	0.0082%
32907229	T	53592	0.0000%	6906	0.0434%	12264	0.0163%
32907230	G	53592	0.0000%	6906	0.0000%	12264	0.0082%
32907231	G	53592	0.0037%	6906	0.0145%	12264	0.0082%
32907232	A	53592	0.0000%	6906	0.0145%	12264	0.0000%
32907233	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907234	T	53592	0.0019%	6906	0.0145%	12264	0.0000%
32907235	G	53592	0.0019%	6906	0.0000%	12264	0.0000%
32907236	G	53592	0.0056%	6906	0.0145%	12264	0.0000%
32907237	A	53592	0.0019%	6906	0.0145%	12264	0.0000%
32907238	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907239	A	53592	0.0019%	6906	0.0290%	12264	0.0489%
32907240	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907241	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907242	C	53592	0.0056%	6906	0.0290%	12264	0.0163%
32907243	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907244	T	53592	0.0056%	6906	0.0000%	12264	0.0000%
32907245	A	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907246	C	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907247	T	53592	0.0000%	6906	0.0000%	12264	0.0000%
32907248	G	53592	0.0000%	6906	0.0145%	12264	0.0245%
32907249	T	53591	0.0000%	6906	0.0000%	12264	0.0000%
32907250	T	53591	0.0000%	6906	0.0000%	12264	0.0000%
32907251	T	53590	0.0019%	6906	0.1014%	12264	0.0734%
32907252	G	53590	0.0056%	6906	0.0290%	12264	0.0245%
32907253	C	53590	0.0019%	6906	0.0145%	12264	0.0000%
32907254	T	53590	0.0037%	6906	0.0000%	12264	0.0082%
32907255	C	53590	0.0000%	6906	0.0000%	12264	0.0000%
32907256	A	53588	0.0000%	6906	0.0145%	12264	0.0082%
32907257	C	53584	0.0112%	6906	0.0000%	12264	0.0000%
32907258	A	53572	0.0037%	6906	0.0000%	12264	0.0163%
32907259	G	53545	0.0000%	6906	0.0000%	12264	0.0000%
32907260	A	53542	0.0019%	6906	0.0290%	12264	0.0000%
32907261	A	53542	0.0131%	6906	0.0145%	12264	0.0245%
32907262	G	53541	0.0000%	6906	0.0145%	12264	0.0000%
32907263	G	53541	0.0019%	6906	0.0145%	12264	0.0163%
32907264	A	53538	0.0112%	6906	0.0434%	12264	0.0163%
32907265	G	53537	0.0019%	6906	0.0145%	12264	0.0163%
32907266	G	53537	0.0262%	6906	0.1158%	12264	0.0734%
32907267	A	53537	0.0037%	6906	0.0000%	12264	0.0082%
32907268	C	53537	0.0448%	6906	0.0145%	12264	0.0163%
32907269	T	53536	0.0318%	6906	0.0145%	12264	0.0000%
32907270	C	53535	0.0411%	6906	0.0000%	12264	0.0082%
32907271	C	53535	0.2821%	6906	0.1014%	12263	0.0815%
32907272	T	53535	0.1476%	6906	0.0000%	12263	0.0163%
32907273	T	53535	0.1662%	6906	0.1158%	12263	0.0652%
32907274	A	53535	0.0411%	6906	0.0000%	12263	0.0245%
32907275	T	53534	0.0747%	6906	0.0000%	12263	0.0163%

Universal heteroplasmy of human mitochondrial DNA

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Abstract

Mammalian cells contain thousands of copies of mitochondrial DNA (mtDNA). At birth, these are thought to be identical in most humans. Here, we use long read length ultra-deep resequencing-by-synthesis to interrogate regions of the mtDNA genome from related and unrelated individuals at unprecedented resolution. We show that very low-level heteroplasmic variance is present in all tested healthy individuals, and is likely to be due to both inherited and somatic single base substitutions. Using this approach, we demonstrate an increase in mtDNA mutations in the skeletal muscle of patients with a proofreading-deficient mtDNA polymerase γ due to *POLG* mutations. In contrast, we show that *OPA1* mutations, which indirectly affect mtDNA maintenance, do not increase point mutation load. The demonstration of universal mtDNA heteroplasmy has fundamental implications for our understanding of mtDNA inheritance and evolution. Ostensibly *de novo* somatic mtDNA mutations, seen in mtDNA maintenance disorders and neurodegenerative disease and aging, will partly be due to the clonal expansion of low-level inherited variants.

Introduction

Nucleated mammalian cells contain thousands of copies of the mitochondrial genome. Until recently, it has been generally accepted that, for the vast majority of humans, all mitochondrial DNA (mtDNA) molecules are identical at birth (homoplasmy) (Parsons *et al.*, 1997; Taylor and Turnbull, 2005). However, recent work has shown that ~25% of healthy individuals inherit a mixture of wild-type and variant mtDNA (heteroplasmy), which almost exclusively involves the non-coding mtDNA D-loop (Li *et al.*, 2010). Even less frequently, <1 in 200 inherit a potentially pathogenic variant of mtDNA in the coding region (Elliott *et al.*, 2008). Being exclusively maternally inherited, mtDNA undergoes negligible recombination manifested at the population level. As a result, homoplasmic variation of mtDNA has played a key role in determining population migrations on a global scale (Torroni *et al.*, 2006) and in the confident identification of biological samples in forensic medicine (Just *et al.*, 2009). Heteroplasmic variants can confound the situation in both circumstances.

MtDNA is highly mutable, with an estimated mutation rate of at least ~5–15 times that of the nuclear genome. This is partly a result of proximity to the electron transport chain that is the major intracellular source of oxidative free radicals, and partly a result of the relatively limited mtDNA protection and repair mechanisms (Wallace, 2010a). The high mutation rate contributes to the high levels of mtDNA diversity and also the generation of somatic mtDNA mutations with aging (Wallace, 2010b). Once present, the intracellular level of both inherited and somatic mtDNA mutations can change during life through the unequal partitioning of mitochondrial genotypes, which can occur during cytokinesis in dividing cells (vegetative segregation) or within a non-dividing cell through the relaxed replication of mtDNA. An increase in heteroplasmy is often referred to as ‘clonal expansion’ that may be enhanced by a replicative advantage for specific mutation types (Birky, 1994). If deleterious mutations exceed a critical threshold level within a cell, they can cause a biochemical defect in the mitochondrial respiratory chain (Schon *et al.*, 1997). This often involves cytochrome c oxidase (COX), leads to increased free radical production, a defect of ATP synthesis, cell dysfunction and ultimately cell death (Taylor and Turnbull, 2005). High percentage levels of inherited mtDNA mutations cause multisystem mitochondrial diseases in ~1 in 10 000 of the population (Schaefer *et al.*, 2008), and high percentage levels of somatic mutations have been described in several age-associated degenerative diseases and

possibly contribute to the aging process (Park and Larsson, 2011). Understanding the origin of mtDNA variants, therefore, has far-reaching implications, with impact on medicine, human anthropology and forensic science.

Although generally assumed that age-related mtDNA mutations originate during life, it is equally plausible that some ostensibly somatic variants are actually inherited, but fall below the detection threshold of previously applied technologies. With the recent development of massively parallel sequencing, it is now possible to definitively address this issue through the detection of very low-level heteroplasmic variants. Recent studies using whole mtDNA genome sequencing on the Illumina GA / Solexa platforms have suggested that a single heteroplasmic variant can be detected in ~25% of individuals (He *et al.*, 2010; Li *et al.*, 2010; Tang and Huang, 2010; Goto *et al.*, 2011). However, the relatively conservative detection thresholds (a minor allele frequency of >1.5–10%) used in these studies mean that lower frequency variants were not reported, despite the fact that, from first principles, most somatic mtDNA variants will be expected to fall below this level.

We, therefore, sought to improve the depth of resolution for very low-frequency heteroplasmy by developing an amplicon-based resequencing method on the Roche 454 GS FLX platform. Despite the very great depth of coverage per base position achieved in previous studies using other platforms (He *et al.*, 2010; Li *et al.*, 2010; Tang and Huang, 2010; Goto *et al.*, 2011), the achievable lower limit of resolution for heteroplasmic mtDNA variants is pragmatically limited by the 'noise' generated principally during the sequencing reaction. Amplicon resequencing on the 454 GS FLX platform has, thus, become the method of choice for ultra-deep resequencing (UDS) of mutants and quasi-species within other small genomes, for example viruses (Daly *et al.*, 2011) and bacteria (Soares *et al.*, 2012), where resolution in the 0.1–1% variant frequency range has been described.

Results

Developing a highly sensitive and specific method of detecting very low-level mtDNA sequence variants

At the population level, *MT-HV2* within the mtDNA non-coding control region is observed to be more polymorphic than the coding region (Macaulay *et al.*, 1999). We, therefore, designed two mtDNA amplicons to compare the base substitution rate in two regions in different subjects: one in *MT-HV2* (nt 162–455, 294 bp) and one in the coding region *MT-CO3* (nt 9307–9591, 285 bp) (see Supplementary Material, Table S1). As nuclear pseudogenes [nuclear mitochondrial sequences (NumtS)] have the potential to confound deep resequencing of mtDNA, we took a three-stage process to exclude the possibility of this affecting our data. Firstly, we compared our amplicon loci with published NumtS to determine the extent to which they nested within these regions and the corresponding degree of sequence identity (Simone *et al.*, 2011). Only one of the NumtS had a high sequence identity with modern mtDNA (>90%) and was predicted to lie within our *MT-CO3* amplicon (Supplementary Material, Table S2). However, this NumtS was still predicted to differ by six nucleotide substitutions from modern mtDNA, which significantly exceeded the number of variants ever seen on single reads within our dataset. Secondly, we used Primer-BLAST analysis of each amplicon primer pair (hg19 build), which showed no predicted non-mtDNA PCR product generation (Ye *et al.*, 2012). Finally, we attempted to PCR amplify a product using genomic DNA extracted from rho0 cells that contained no mtDNA, following ethidium bromide-induced mtDNA depletion to directly confirm our predictions and to look for unexpected NumtS hits. No product was obtained with either amplicon primer set, despite a strong band with a positive control (nuclear gene primers). We, therefore, concluded that nuclear pseudogene amplification was highly unlikely with the specific primers that we designed to amplify *MT-HV2* and *MT-CO3*. In the 454 assay, the mean depth of coverage using these primers was 8391 reads (range 4158 – 20 803).

Although commercially available lambda phage controls can be used to estimate the level of background sequencing artifacts, differences in guanine-cytosine content of the cloned templates can influence the outcome. We, therefore, designed several in-house controls to determine the level of background noise using the 454 platform on mtDNA templates: (i) a nuclear DNA amplicon in *BRCA2* (NC_000013.10, 32907099–32907295, 197 bp) was designed to compare the intrinsic properties of mtDNA and

nuclear genomic DNA, (ii) a cloned nuclear DNA template corresponding exactly to the *BRCA2* amplicons was generated from genomic DNA and (iii) we cloned mtDNA templates from exactly the same mtDNA sequences (*MT-HV2, MT-CO3*). On this basis, any difference in sequence variants between the cloned and genomic DNA templates from the same individual is highly likely to reflect 'biological' sequence variants generated *in vivo* and not technical artifact arising from the sequencing process.

Being based on pyrosequencing, the base-calling errors on the 454 platform are very largely dependent on poly-mononucleotide tracts (Quince *et al.*, 2009). As our goal was to maximize specificity in calling very low-level variants, we, therefore, excluded these tracts from all of the analysis. We also introduced a further quality-control step, requiring observed variants to be present in both forward and reverse reads at comparable frequencies. A 3-fold difference was permitted to allow for the effects of a binomial sampling distribution at very low variant levels.

Using this highly stringent approach, there were no variants present at >0.2% heteroplasmy in any of the cloned or genomic DNA templates (Fig. 1). This demonstrated the low background noise level using the 454 UDS approach and indicated that variants detected at >0.2% heteroplasmy are highly likely to be generated *in vivo* and to be of biological origin.

Further validation of this approach came from repeated experiments performed using the same 454 platform, but different chemistry (Titanium) and different amplicon generation primers. Duplicate experiments were performed on four samples of genomic DNA. There was 100% consistency of variant detection between runs, but 6% of the variants present in the first run fell below the 0.2% threshold in the second run. The heteroplasmy level range for these variants in the second run was 0.12 – 0.19%, demonstrating that these fell just outside our defined threshold for detection, but were indeed present.

Frequency of very low-level mtDNA sequence variants in healthy control subjects

Next, we studied genomic DNA extracted from whole blood and the skeletal muscle from unrelated and related healthy individuals at different ages. Demographic and genotypic details of the subjects are shown in Supplementary Material, Table S3. Detected mtDNA sequence variants are shown in Supplementary Material, Table S4.

All of the healthy control subjects showed variants at >0.2% heteroplasmy in both the blood and skeletal muscle DNA samples at one or more base positions. These data indicate that very low-level single-base heteroplasmy appears to be a universal finding among different control subjects (Fig. 2A and B). The total number of variant base positions was the same in the blood and skeletal muscle samples, but variants at higher percentage heteroplasmy levels (>2%) were present only in the skeletal muscle of some individuals (three of seven healthy subjects) and never seen in the blood. Higher level variants (>2%) were only found in the *MT-HV2* amplicon.

Overall, the number of variants per base position was significantly more common in the skeletal muscle than in the blood ($P = 0.001$), and this difference was greater for *MT-HV2* than *MT-CO3* ($P < 0.001$). In addition, the number of variants per base position was significantly greater in *MT-HV2* than *MT-CO3* ($P < 0.001$). In summary, the greatest frequency of very low-frequency variants was seen in the *MT-HV2* region, and both the greatest number of different variants and the highest heteroplasmy levels were seen in the skeletal muscle.

The frequency of very low-level variants in disorders of mtDNA maintenance

We studied patients with inherited defects of *POLG* encoding the sole mtDNA polymerase γ, predicted to have error-prone mtDNA replication (Van Goethem *et al.*, 2001; Naviaux and Nguyen, 2004; Hakonen *et al.*, 2005). For comparison, we also studied subjects with defects of *OPA1*. Skeletal muscle biopsies from these individuals show an excess of cells containing clonally amplified mtDNA somatic mutations; however, unlike for *POLG* defects, the mechanism is believed to be indirect, mediated through defects in mitochondrial fission and fusion, rather than by any direct effect on

the polymerase (Alexander *et al.*, 2000; Amati-Bonneau *et al.*, 2008; Hudson *et al.*, 2008). Clinical details and pedigrees are shown in Supplementary Material, Table S3 and Supplementary Material, Figure S1.

First, we studied eight patients with a variety of *POLG* mutations (A467T/A467T, A467T/G848S, A467T/W748S, R627Q/W748S and R627Q/R1096H). In skeletal muscle biopsies, the overall burden of mtDNA variants was greater in patients with *POLG* mutations when compared with healthy control subjects, but only for *MT-HV2*, where the majority of variants were seen ($P = 0.004$) (Fig. 2A). The excess of mtDNA variants was present across all levels of heteroplasmy, and increased significantly with age ($r = 0.86$, $P = 0.006$), suggestive of a time-dependent accumulation of low-level somatic mutations. There was no significant correlation of mutation burden with age in healthy controls ($r = 0.62$, $P = 0.14$). Given that the mutational burden was significantly greater at lower heteroplasmy levels in *POLG* when compared with control subjects (for <1% heteroplasmy level variants, $P = 0.019$), these findings imply that that the higher mutational burden is primarily due to *de novo* somatic mutation in *POLG* patients.

We, then, studied multiple tissues from patients with mutations in *OPA1* (M1fsX208, S545R, V294fsX667, R905X and intron 8 and intron 15 splicing defects). In contrast to the *POLG* patients, there was no significant increase in mutational burden in *OPA1* patients when compared with healthy controls in either the blood or skeletal muscle for the *MT-HV2* or *MT-CO3* amplicons (Fig. 2A and B). These findings argue against an increased rate of *de novo* mutagenesis in patients with *OPA1* mutations, consistent with there being no direct effect on the polymerase γ and in keeping with previous suggestions that accelerated clonal expansion of existing somatic mutations is responsible for the cellular cytochrome c oxidase defects (Yu-Wai-Man *et al.*, 2010). We studied the mutational burden in the skeletal muscle of one subject on two occasions 10 years apart. Six heteroplasmic variants were detected in the first muscle sample of this individual. All six variants were also detectable at comparable levels in the follow-up muscle sample taken 10 years later, although two variants in the second sample fell below the 0.2% threshold (0.17%, 0.06%). Overall, five variants showed a decrease in heteroplasmy level and one showed an increase over the 10 year period; however, the absolute changes were small (Supplementary Material, Table S5).

Determining whether very low-level variants are maternally inherited

Having identified low-level heteroplasmic mtDNA variants in the different pedigrees, we, then, compared related and unrelated individuals to determine the extent to which the mtDNA variants arose through maternal transmission or somatic mutation. We focused on families with known nuclear genetic defects associated with mtDNA maintenance because of the larger number of variants available for scrutiny (i.e. increased signal strength).

We compared 13 pairs of samples from 12 maternal first-degree relatives, including one subject with repeated blood and muscle samples taken 10 years apart. To determine the background level of similarity between unrelated individuals, we compared the same tissue between 79 different random pairs of unrelated subjects. Out of 485 variants, 59 variants were common in unrelated subjects (12%). In contrast, when the analysis was restricted to first-degree maternal relatives, 25/63 variants were shared (40%, $P < 0.001$, Table 1). We also compared the frequency of sharing between 13 pairs of related individuals and 13 random pairs of unrelated controls. This also repeatedly revealed a highly significant difference ($P < 0.001$), adding further weight to our conclusions. On average, 39% of variants in a given individual were shared with their maternal relative. Interestingly, shared variants in related subjects were principally seen in the skeletal muscle mtDNA, where 17/24 variants were shared (71%), compared with 50/395 variants shared in unrelated subjects (13%, $P < 0.001$). In contrast, variant sharing in related subjects was rather uncommon in the blood mtDNA, where 8/39 variants were shared (21%) when compared with 9/90 variants in unrelated subjects (10%, $P = 0.10$).

These findings have two implications. First, some of the more common *MT-HV2* variants are present in the background population at very low heteroplasmy levels. Second, 4-fold more variants are family specific and are transmitted between first-degree maternal relatives. Although it is not possible, without longitudinal study, to state exactly what proportion of variants are due to somatic mutation, the data clearly show that a large proportion appear to be inherited at very low heteroplasmy levels.

Discussion

Using amplicon-based UDS at unprecedented depth, we have shown that detectable very low-frequency mtDNA variants (0.2 – 2% heteroplasmy) are present in all tested healthy subjects.

It is highly unlikely that nuclear pseudogene (NumtS) contamination influenced our results for the following reasons: Primer-BLAST analysis (hg19 build) failed to identify any predicted non-mtDNA PCR products using our specific *MT-HV2* and *MT-CO3* primers; we were unable to generate a product from rho0 genomic DNA using these primers. Moreover, further evidence came from the *post hoc* analysis of individual sequence reads and scrutiny of all predicted nuclear pseudogenes (Supplementary Material, Table S2) (Simone *et al.*, 2011). Based on this analysis, a minimum of six changes from the mtDNA consensus sequence would be expected in a read from a pseudogene with our *MT-CO3* primers and ~48 changes for the *MT-HV2* primers. Read-by-read analysis never identified this number of variants, so pseudogene contamination is extremely unlikely in our dataset. We conclude, therefore, that heteroplasmy is a universal finding in humans. Although our approach does not allow us to state with absolute certainty whether any one variant is inherited or a somatic mutation, a comparison of maternal relatives shows substantial sharing between first-degree relatives, and our findings, therefore, show that inherited variants make a significant contribution to the overall mutation load in any one individual.

None of the heteroplasmic variants detected were haplogroup defining or haplogroup specific. Twelve of 40 base positions containing heteroplasmic variants are reported to be polymorphic (>1% of reported sequences with variants) at the population level (http://www.mtdb.igp.uu.se/). None of the variants detected have been ascribed definite pathogenic potential, although a single mutation at position m.9544 has previously been associated with optic neuropathy. We also examined whether variants detected had previously been reported as somatic mutations. In our data, 21 of 31 control region (*MT-HV2*) heteroplasmic variants we detected, and 2 of 9 coding region (*MT-CO3*) variants have previously been reported as somatic variants (http://www.mitomap.org/MITOMAP) (Supplementary Material, Table S4).

Using this approach, we also show that a disrupted mtDNA polymerase γ due to a *POLG* defect leads to increased levels of mutations, and that these increase during life, mirroring observations in *POLG*-deficient mice (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). In contrast, we did not observe a correlation between age and mutation burden in control subjects, whether in the blood or muscle. This suggests that *de novo* mutagenesis throughout life is unlikely to contribute significantly to the cellular COX defects observed in healthy aged individuals. Rather, age-related COX defects are more likely to be the result of clonal expansions of mutations occurring during early life (Elson *et al.*, 2001). This notion has recently been demonstrated in aged colonic crypt cells, where multi-method measurement of mtDNA mutation load failed to demonstrate an age-dependent increase (Greaves *et al.*, 2010). However, our observation of very low-frequency heteroplasmy transmission indicates that that many of these mutations will be inherited down the maternal line. As a result, all clonally expanded species need not be the result of somatic mutation events in early life, and some could have arisen from low-level inherited variants.

The changes in low-level heteroplasmy value between each mother and offspring (Supplementary Material, Figure S1 and Supplementary Material, Table S4) are relatively small when compared with the shifts observed in patient pedigrees carrying higher levels of pathogenic mutations (Chinnery *et al.*, 2000). However, this is consistent with the neutral drift theory (Wonnapinij *et al.*, 2010) which predicts that the variance in the offspring heteroplasmy due to the mtDNA bottleneck decreases as the mother's mutation level decreases. These are the first data to show the inheritance of such low-level mtDNA mutations. The high rate of inheritance of the low-level mutation in muscle (71%) may be surprising, considering our expectations of the effect of the mtDNA inheritance bottleneck; however, two points should be considered here. First, that muscle data is from only two sibling pairs [subjects A-B and P-Q (Supplementary Material, Figure S1 and Supplementary Material, Table S4)]. Second, as noted above, the neutral drift theory predicts that very low-level mutations will have low variance in the offspring, making them more likely to be preserved through the mtDNA inheritance bottleneck.

The different patterns of mutation observed in the blood and skeletal muscle are, at least in part, likely to be a consequence of different rates of cell and mtDNA turnover between dividing (blood) and post mitotic tissues. Rapid turnover of blood cells can lead to the loss of mtDNA mutations during life, either through selection against a particular mutation (Pyle *et al.*, 2007) or simply by genetic drift (Chinnery and Samuels, 1999). On the other hand, the loss of mutations is much less likely in a post mitotic tissue such as the skeletal muscle, where the replication of mtDNA can lead to an increase in mutation load during life within individual cells and the tissue as a whole, even from very low levels of heteroplasmy (Elson *et al.*, 2001). Thus, it is plausible that an inherited mutation is lost from the blood, but detected in the muscle, explaining why some inherited mutations are more likely to be detected in the muscle than in the blood.

It is intriguing that the frequency of low-level variants in *MT-HV2* is significantly greater than in *MT-CO3* in healthy control subjects (OR 3.3). Why should this be the case, given that our UDS of cloned mtDNA showed no intrinsic sequence-specific difference between the two templates? One possible explanation is that much of the non-coding D-loop heteroplasmy is actually inherited at a very low level. Being a non-coding region, these substitutions may be tolerated during transmission, unlike coding region variants that undergo strong negative selection during transmission (Stewart *et al.*, 2008).

Perhaps most importantly, we show here that next generation sequencing has the potential to reliably detect very low levels of heteroplasmy, when a very stringent analytical approach is employed. It is important to note that we did not perform a head-to-head comparison of different next generation sequencing platforms, so it would be premature to conclude that the 454 approach is superior to other platforms. However, using this method, we have gained novel insight into mtDNA within individuals and within pedigrees. Prospective studies in larger family-based cohorts will substantiate these findings. However, given that mtDNA heteroplasmy levels can change dramatically during life, and during maternal transmission, the finding of universal mtDNA heteroplasmy has significant implications for our understanding of mtDNA at the population, family and individual level. If deleterious mutations are inherited, these have the potential to accumulate within single cells during life and thus contribute to neurodegenerative disease. Or, if they segregate rapidly through the mtDNA bottleneck,

they could lead to a maternally inherited mitochondrial disorder. This places greater emphasis on the importance of developing techniques to prevent the transmission of mtDNA heteroplasmy and preventing the clonal expansion of pre-existing mtDNA mutations.

Materials and Methods

Patient and samples

All subjects gave informed consent for participation in research. Characteristics of patient and control samples are shown in Supplementary Material, Table S3 and Supplementary Material, Figure S1.

Molecular analyses

Amplicon resequencing was performed on the Roche 454 GS FLX platform. Two mtDNA amplicons were used: *MT-HV2* (NC_012920, nt.162–455) and *MT-CO3* (nt.9307–9591). Confirmatory experiments on a subset of samples used the following amplicon positions: *MT-HV2* (nt.109–483) and *MT-CO3* (nt.9304–9653). Full primer sequences are shown in Supplementary Material, Table S1. Owing to the potential of nuclear pseudogenes to confound deep resequencing studies of mtDNA, we used BLAST to ensure that our primers avoided these areas and confirmed this by lack of amplification from mtDNA deplete rho0 DNA. Amplicon-specific mtDNA clones comprised the following inserts in a pGEM-T-easy vector (Promega): *MT-HV2* clone (nt.16548–771) and *MT-CO3* clone (nt.9127–9661). Autosomal negative control comprised amplicon *BRCA2* (NC_000013.10, 32907099–32907295) and *BRCA2* clone (32906828–32907480). Amplicon generation PCR reactions were performed in a 50 μl volume comprising: 1x buffer for KOD Hot Start DNA Polymerase (Novagen), 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.3 μM primers, 1 U KOD Hot Start DNA Polymerase (Novagen) and 100 ng DNA. Cycling conditions were 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 60°C for 10 s and 70°C for 4 s. EmPCR and bidirectional sequencing were performed according to the manufacturer's instructions (Roche 454).

Bioinformatic analyses

Base calling and alignment were performed using the algorithm of PyroBayes and Mosaik. Subsequent analysis of variants was performed using the custom R library flowgram. To maximize specificity for very low-level variants, base positions within or immediately adjacent to poly-mononucleotide tracts were excluded from analysis. Variant calls were then validated by comparison of read directions.

Primer-BLAST (Ye *et al.*, 2012) analysis to confirm complete specificity of amplicon generation primer pairs was performed using standard stringent parameters: human genome assembly hg19, blast E value 30 000, and unintended targets with <6 mismatches were considered.

Statistical analyses

Comparison of proportions of base positions between groups was done using the chi-squared test.

Figure 1. **Resolution of ultra-deep sequencing-by-synthesis assay.**

Demonstration of very low levels of noise in negative controls after quality-control filtering for poly-mononucleotide tracts and bidirectional validation of variants. An amplicon was produced from cloned DNA for each mtDNA amplicon (*MT-HV2*, *MT-CO3*) along with an autosomal amplicon (*BRCA2*) and a clone. All negative controls showed minimal numbers of base positions with any variants, and none at >0.2% heteroplasmy level, with no inherent differences between the different DNA templates.

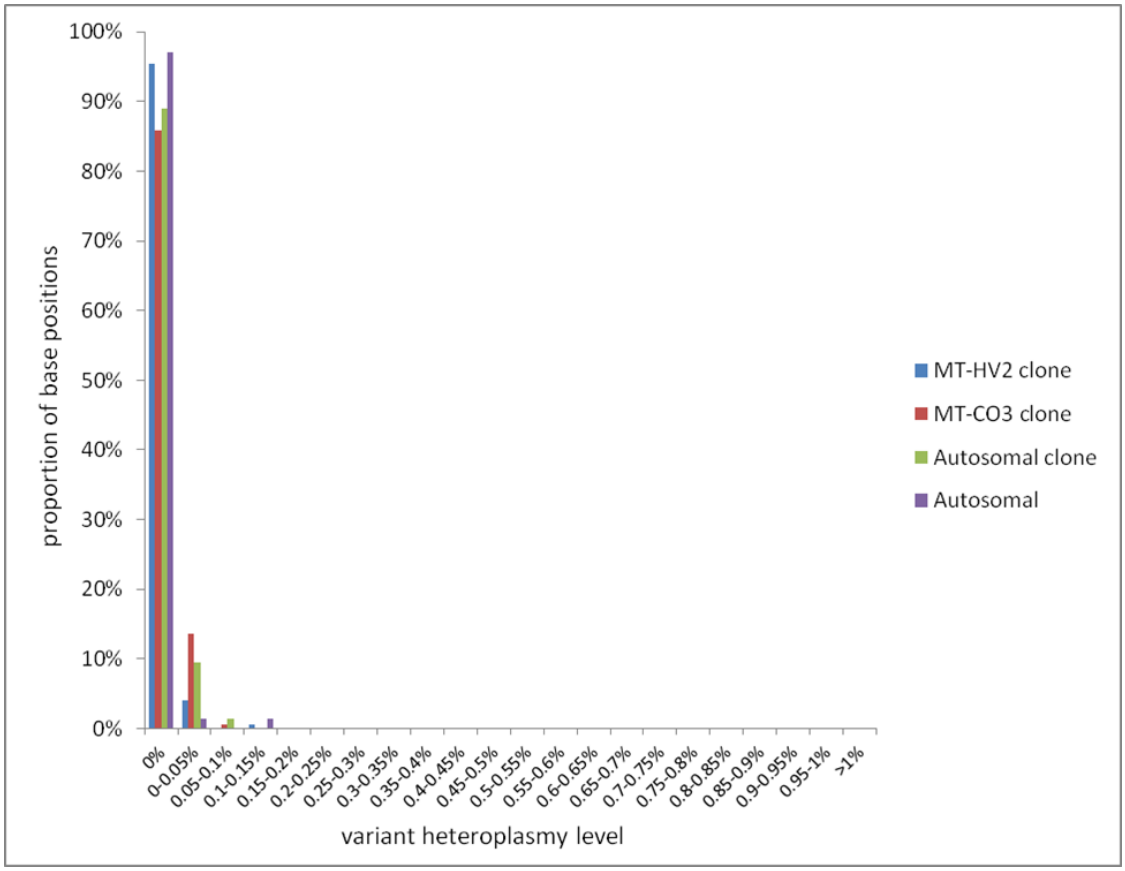


Figure 2. Very low-level heteroplasmic mtDNA variance was detected by ultra-deep amplicon resequencing.

(A) Comparison of variants detected in the skeletal muscle (skm) DNA within the*MT-HV2* amplicon in different patient groups shows no difference between healthy control (*n* = 7) and *OPA1* (*n* = 8) subjects, but a significant excess of variance at all heteroplasmy levels in *POLG* subjects (*n*= 8). (B) Comparison of variants detected in the blood DNA in healthy control (*n* = 7), *POLG* (*n* = 4) and *OPA1* (*n* = 7) subjects shows that variants are present, but less common than in the skeletal muscle DNA with the absence of higher level variants (>2% heteroplasmy) and no difference between patient groups.

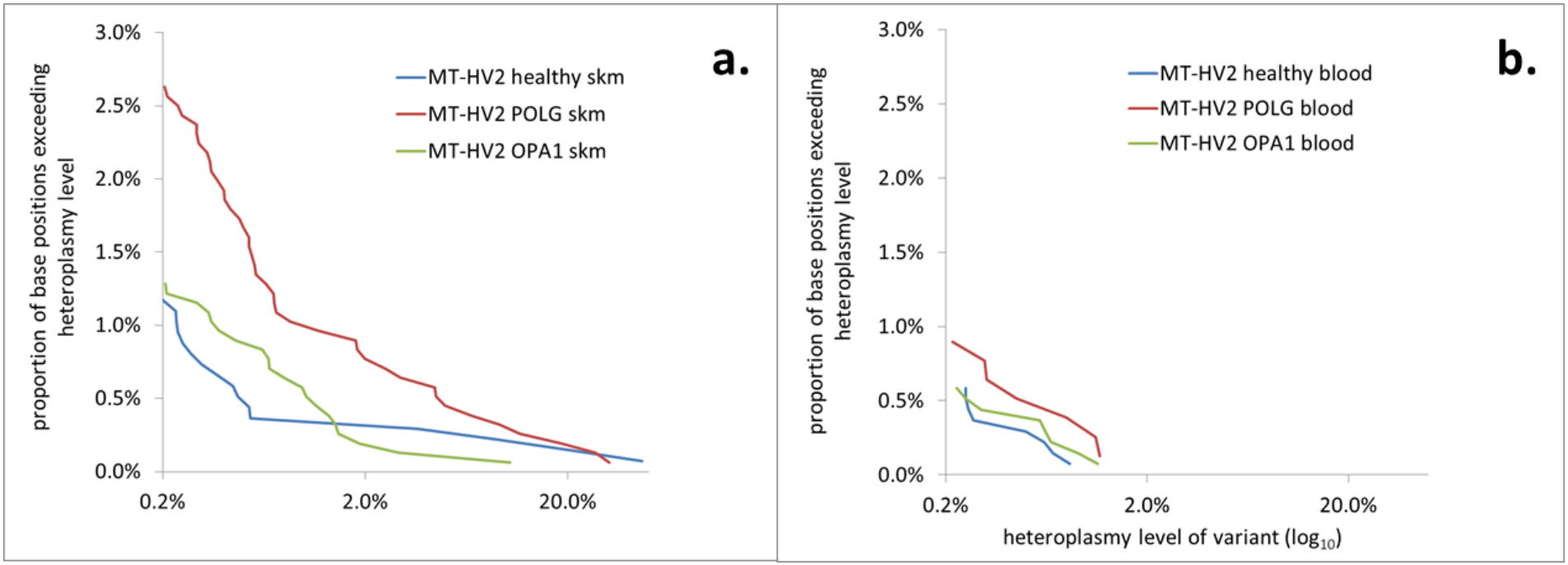


Table 1. Extent of sharing of variants in 13 pairs of maternally related and 79 pairs of unrelated samples

	Maternally related samples			Unrelated samples			p-value
	Shared variants (n)	Unshared variants (n)	Shared variants (%)	Shared variants (n)	Unshared variants (n)	Shared variants (%)	
Skeletal muscle	17	7	70.8%	50	345	12.7%	<0.0001
Blood	8	31	20.5%	9	81	10.0%	0.10
All samples	25	38	39.7%	59	426	12.2%	<0.0001

Supplementary Material

Supplementary Figure 1: Pedigrees.

Supplementary Table 1: Amplicon generation for ultra-deep sequencing-by-synthesis.

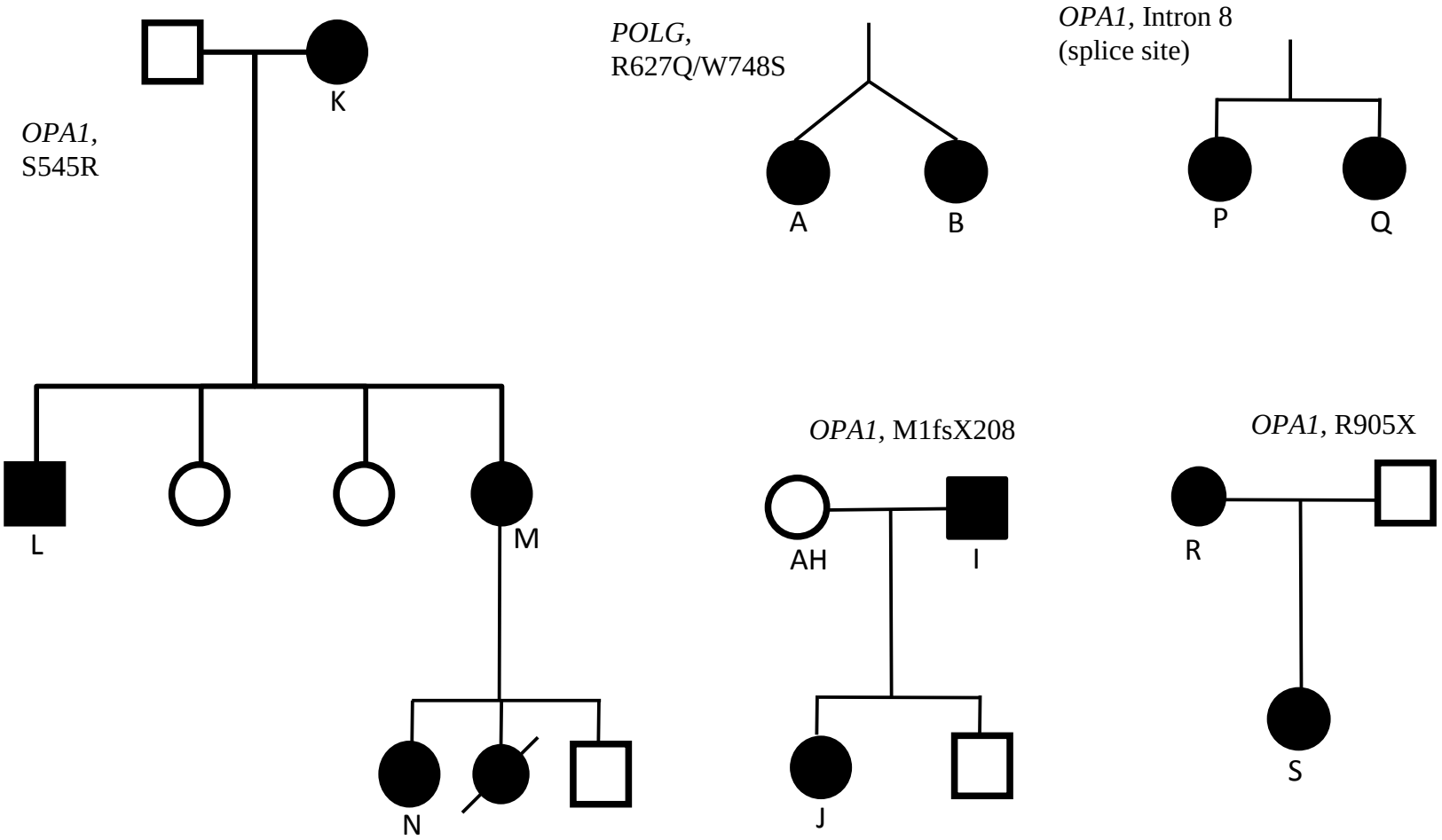
Supplementary Table 2: Published nuclear pseudogenes corresponding to mtDNA amplicons.

Supplementary Table 3: Characteristics of patient and control samples.

Supplementary Table 4: Variants detected in patient and control samples.

Supplementary Table 5: Variants detected in skeletal muscle mtDNA of two samples taken from same individual at 10 year interval.

Supplementary Figure 1. Pedigrees.



Supplementary Table 1.

Amplicon generation for ultra-deep sequencing-by-synthesis (454 GS FLX Standard Chemistry)

Primers comprised a sequence-specific segment, a barcode, and a fusion primer segment.

Fusion primer segments

Forward:	GCCTCCCTCGCGCCATCAG
Reverse:	GCCTTGCCAGCCCGCTCAG

Sequence-specific segments

HVS2:	forward, nt162-184 (CGCACCTACGTTCAATATTACAG)
	reverse, nt455-434 (AAAATAATGTGTTAGTTGGGGG)
CO3:	forward, nt9307-9329 (GATTTCACTTCCACTCCATAACG)
	reverse, nt9591-9572 (CTTCTAGGGGATTTAGCGGG)

Barcode segments

1	AAGGAAGGT	15	TTACGTCCT
2	TTAAGGACT	16	ACTTAAGGT
3	TAAGGCCGT	17	TTACTTACT
4	TTAAGTAAT	18	TACTTCCGT
5	TAAGTACGT	19	TCCGGAAGT
6	AAGTCCGGT	20	CCGGACGGT
7	TAATTAAGT	21	TTCCGGCCT
8	AATTACGGT	22	CCGTAAGGT
9	TTAATTACT	23	TTCCGTACT
10	TTACGGAAT	24	TCCGTCCGT
11	TACGGACGT	25	TTCCTTAAT
12	ACGGCCGGT	26	TCCTTACGT
13	TACGTAAGT	27	CCTTCCGGT
14	ACGTACGGT		

Amplicon generation for ultra-deep sequencing-by-synthesis (454 GS FLX Titanium XLR70 chemistry)

Primers comprised a sequence-specific segment, a barcode, and a fusion primer segment.

Sequence-specific segments

HVS2:	forward, nt.109-130 (GCACCCTATGTCGCAGTATCTG)
	reverse, nt.483-458 (GAGATTAGTAGTATGGGAGTGGGAGG)
CO3:	forward, nt.9304-9329 (TGTGATTTCACTTCCACTCCATAACG)
	reverse, nt.9653-9629 (ATGGTGAGCTCAGGTGATTGATACT)

Barcode segments

1	ACGAGTGCGT
2	ACGCTCGACA
3	AGACGCACTC
4	AGCACTGTAG
5	ATCAGACACG

Fusion primer segments

Forward:	CGTATCGCCTCCCTCGCGCCATCAG
Reverse:	CTATGCGCCTTGCCAGCCCGCTCAG

Supplementary Table 2. List of published nuclear mitochondrial pseudogene (Nuclear mitochondrial sequences, NumtS) loci within which our 454 FLX amplicons would potentially nest. NumtS were defined according to Simone et al. (19). All but one NumtS locus shows low sequence identity (compared between NumtS and rCRS (NC_012920)). Only one high sequence identity NumtS (HSA_NumtS_001, indicated *) was identified, and would be predicted to show 6 nucleotide substitutions from our mitochondrial DNA amplicon sequence.

454 FLX Amplicon	NumtS ID	Chromosome	Sequency identity (%)
MT-HV2	HSA_NumtS_227	5	82
MT-HV2	HSA_NumtS_508	17	80
MT-CO3	HSA_NumtS_001	1	98*
MT-CO3	HSA_NumtS_075	2	73
MT-CO3	HSA_NumtS_080	2	76
MT-CO3	HSA_NumtS_084	2	76
MT-CO3	HSA_NumtS_090	2	71
MT-CO3	HSA_NumtS_100	2	65
MT-CO3	HSA_NumtS_101	2	74
MT-CO3	HSA_NumtS_146	3	74
MT-CO3	HSA_NumtS_160	3	75
MT-CO3	HSA_NumtS_199	4	76
MT-CO3	HSA_NumtS_222	5	88
MT-CO3	HSA_NumtS_237	6	78
MT-CO3	HSA_NumtS_258	6	72
MT-CO3	HSA_NumtS_269	7	74
MT-CO3	HSA_NumtS_270	7	74
MT-CO3	HSA_NumtS_272	7	76
MT-CO3	HSA_NumtS_312	8	73
MT-CO3	HSA_NumtS_329	9	73
MT-CO3	HSA_NumtS_344	9	77
MT-CO3	HSA_NumtS_346	9	76
MT-CO3	HSA_NumtS_407	11	75
MT-CO3	HSA_NumtS_495	16	74
MT-CO3	HSA_NumtS_508	17	83
MT-CO3	HSA_NumtS_561	22	69

Supplementary Table 3. Characteristics of patient and control samples (skm, skeletal muscle; bld, blood). * patient O had two blood (1997 and 2009) and two skeletal muscle (1999 and 2009) DNA samples analyzed.

SUBJECT	DISEASE	MUTATION	TISSUE	AGE
A	*POLG*	R627Q / W748S	SKM / BLD	57
B	*POLG*	R627Q / W748S	SKM / BLD	57
C	*POLG*	R627Q / R1096H	SKM / BLD	17
D	*POLG*	A467T (homozygous)	SKM / BLD	45
E	*POLG*	A467T (homozygous)	SKM	35
F	*POLG*	A467T / G848S	SKM	4
G	*POLG*	A467T / W748S	SKM	28
H	*POLG*	A467T (homozygous)	SKM	30
I	*OPA1*	M1fsX208	SKM / BLD	59
J	*OPA1*	M1fsX208	BLD	33
K	*OPA1*	S545R	BLD	63
L	*OPA1*	S545R	SKM / BLD	30
M	*OPA1*	S545R	BLD	38
N	*OPA1*	S545R	BLD	3
O	*OPA1*	V294fsX667	SKM* /	50 / 60*
P	*OPA1*	Intron 8 (splice site)	SKM	37
Q	*OPA1*	Intron 8 (splice site)	SKM	56
R	*OPA1*	R905X	SKM	60
S	*OPA1*	R905X	SKM	41
T	*OPA1*	Intron 15 (splice site)	SKM	54
U	CONTROL	N/A	SKM	31
V	CONTROL	N/A	SKM	36
W	CONTROL	N/A	SKM	47
X	CONTROL	N/A	SKM	42
Y	CONTROL	N/A	SKM	1
Z	CONTROL	N/A	SKM	67
AA	CONTROL	N/A	SKM	94
AB	CONTROL	N/A	BLD	25 - 30
AC	CONTROL	N/A	BLD	25 - 30
AD	CONTROL	N/A	BLD	25 - 30
AE	CONTROL	N/A	BLD	85 - 90
AF	CONTROL	N/A	BLD	85 - 90
AG	CONTROL	N/A	BLD	85 - 90
AH	CONTROL	N/A	BLD	59

Supplementary Table 4: Variants detected in patient and control samples.

Supplementary Table 5: Variants detected in skeletal muscle mtDNA of two samples taken from same individual (patient O) at 10 year interval. Italicized heteroplasmy levels indicate that variant was detected but level is below the established experimental threshold (>0.2%).

Timepoint	**Baseline**	**plus 10 years**
Variant	**Heteroplasmy level**	
189	0.38%	0.28%
214	0.62%	0.51%
235	0.67%	1.47%
411	0.29%	*0.17%*
414	0.80%	0.30%
9565	0.49%	*0.06%*

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SUBJECT SAMPLE GROUP	A SKM *POLG*	A BLD *POLG*	B SKM *POLG*	B BLD *POLG*	C SKM *POLG*	C BLD *POLG*	D SKM *POLG*	D BLD *POLG*	E SKM *POLG*	F SKM *POLG*	G SKM *POLG*	H SKM *POLG*	I SKM *OPA1*	I BLD *OPA1*	J BLD *OPA1*	K BLD *OPA1*	L SKM *OPA1*	L BLD *OPA1*	M BLD *OPA1*	N BLD *OPA1*	O SKM(1) *OPA1*	O SKM(2) *OPA1*	O BLD(1) *OPA1*	O BLD(2) *OPA1*	P SKM *OPA1*	Q SKM *OPA1*	R SKM *OPA1*	S SKM *OPA1*	T SKM *OPA1*	U SKM *CONT*	V SKM *CONT*	W SKM *CONT*	X SKM *CONT*	Y SKM *CONT*	Z SKM *CONT*	AA SKM *CONT*	AB BLD *CONT*	AC BLD *CONT*	AD BLD *CONT*	AE BLD *CONT*	AF BLD *CONT*	AG BLD *CONT*	AH BLD *CONT*	population level polymorphic?	%	reported pathogenic?	reported somatic mutation?	codon position
nt (*MT-HV2*)																																																	
185												27.31%		0.30%																		0.23%				8.89%									Y	3.1%		Y	
186							18.66%																																						N			Y	
189	3.00%		4.96%						2.52%				2.92%			1.14%					0.38%	0.28%			0.21%	1.48%	1.32%	0.67%	10.38%	0.54%	0.54%		3.65%		46.64%									Y	4.5%		Y	
192							0.73%																																						N			Y	
194																																		20.08%											Y	3.6%		Y	
195										32.07%						0.66%			0.25%													0.28%		0.25%									Y	15.6%		Y	
196								0.22%																																					N			N	
199																											1.15%																	Y	6.5%		Y	
204		1.11%		1.16%		0.45%		0.79%								0.63%	0.21%								0.98%		1.02%				0.47%											0.68%	0.26%	Y	6.6%		Y	
207																0.59%																											Y	6.6%		Y	
214	11.58%		9.21%		1.82%		1.99%		4.41%		0.35%		0.46%								0.62%	0.51%									0.20%													N			Y	
215	6.66%		4.47%		1.80%	0.32%		0.31%																																				N			Y	
217																													1.43%															N			N	
235	0.24%		0.30%													0.91%	1.87%			0.23%	0.67%	1.47%																						Y	3.2%		Y	
238	0.20%																																											N			N	
240	0.29%		0.21%																																									N			Y	
241	0.25%																																											N			N	
246							0.41%																																					N			N	
251	0.85%		0.71%				0.37%		0.55%																							0.24%											N			Y	
253	0.43%		0.50%																																									N			Y	
255									1.17%																																			N			N	
263																																											N			Y	
273	0.70%		0.40%																																									N			Y	
277	0.58%		0.57%				0.33%																																					N			Y	
279																	0.34%																											N			N	
295												0.34%																																Y	4.2%		Y	
297																																												N			Y	
299										0.29%																																		N			N	
365																																												N			N	
385																																												N			Y	
411	0.53%		0.53%																		0.29%																							N			N	
414	0.48%		0.65%										0.35%								0.80%	0.30%													0.23%	0.31%								N			Y	
nt (*MT-CO3*)																																			0.44%	0.37%												
9342																			0.62%																									N	N	1
9377																																												Y	1.6%	N	N	3
9384																	0.36%																											N	N	N	1
9386																																												N	N	N	3
9540														0.20%			0.61%		0.51%	0.69%												0.21%				0.29%							Y	34.9%	N	Y	1
9544																	0.28%																											N	possible	Y	2
9545										13.66%							0.65%	0.20%	0.53%	0.73%																0.27%								Y	2.0%	N	N	3
9548																	0.55%		0.52%	0.75%																0.25%			0.21%					N	N	N	3
9565																					0.49%																							N		N	N	2

N

Elevated serum fibroblast growth factor 21 levels correlate with immune recovery but not mitochondrial dysfunction in HIV infection

Brendan AI Payne, D Ashley Price, and Patrick F Chinnery

AIDS Research and Therapy (2013) 10:27

Abstract

Background

Anti-retroviral treated HIV-infected patients are at risk of mitochondrial toxicity, but non-invasive markers are lacking. Serum FGF-21 (fibroblast growth factor 21) levels correlate strongly with muscle biopsy findings in inherited mitochondrial disorders. We therefore aimed to determine whether serum FGF-21 levels correlate with muscle mitochondrial dysfunction in HIV-infected patients.

Findings

We performed a cross-sectional study of anti-retroviral treated HIV-infected subjects (aged 29 – 71 years, n = 32). Serum FGF-21 levels were determined by quantitative ELISA. Cellular mitochondrial dysfunction was assessed by COX (cytochrome *c* oxidase) histochemistry of lower limb skeletal muscle biopsy. Serum FGF-21 levels were elevated in 66% of subjects. Levels correlated significantly with current CD4 lymphocyte count ($p = 0.042$) and with total CD4 count gain since initiation of anti-retroviral therapy ($p = 0.016$), but not with the nature or duration of past or current anti-retroviral treatment. There was no correlation between serum FGF-21 levels and severity of the muscle mitochondrial (COX) defect.

Conclusions

Serum FGF-21 levels are a poor predictor of muscle mitochondrial dysfunction in contemporary anti-retroviral treated patients. Serum FGF-21 levels are nevertheless commonly elevated, in association with the degree of immune recovery, suggesting a non-mitochondrial metabolic disturbance with potential implications for future comorbidity.

Findings

Mitochondrial dysfunction is a well-described complication of anti-retroviral therapy (Dalakas *et al.*, 1990; Arnaudo *et al.*, 1991; Shikuma *et al.*, 2001; Zaera *et al.*, 2001; Miro *et al.*, 2003; van der Valk *et al.*, 2004; Walker *et al.*, 2004). It is most strongly associated with several of the older nucleoside analogue reverse transcriptase inhibitors (NRTIs): zidovudine (AZT), stavudine (d4T), didanosine (ddI), and zalcitabine (ddC). Although these drugs are no longer in common usage in industrialised countries, there are nevertheless large numbers of patients who have had extensive prior exposure to these drugs, and some remain in common usage in developing countries. We have recently demonstrated that patients with previous exposure to these NRTIs may have persistent cellular mitochondrial COX (cytochrome *c* oxidase) defects in skeletal muscle, consequent on an NRTI-induced accumulation of somatic (acquired) mitochondrial DNA (mtDNA) mutations (Payne *et al.*, 2011).

Non-invasive measures of mitochondrial damage would be very valuable in the HIV clinic, both for the diagnosis of anti-retroviral associated mitochondrial dysfunction and the serial monitoring of such patients. The determination of mtDNA content in peripheral blood mononuclear cells (PBMCs) has previously been proposed as such a measure (Cote *et al.*, 2002; Montaner *et al.*, 2003; Chiappini *et al.*, 2004; van der Valk *et al.*, 2004). This consideration arises from the fact that the mitochondrially-toxic NRTIs (as listed above) cause a reduction in cellular mtDNA content (depletion) during therapy (Arnaudo *et al.*, 1991; Dalakas *et al.*, 2001; Lim and Copeland, 2001; Shikuma *et al.*, 2001; Cherry *et al.*, 2002; Cote *et al.*, 2002; Hammond *et al.*, 2004; Walker *et al.*, 2004; Hoschele, 2006). However, modern N(t)RTIs such as tenofovir (TDF) and abacavir (ABC) do not cause mtDNA depletion (Birkus *et al.*, 2002), and as a result, mtDNA levels return to normal with a switch away from a mitochondrially-toxic NRTI. Thus, measuring mtDNA levels is not a useful measure of on-going mitochondrial dysfunction due to an NRTI exposure in the distant past.

In contrast, FGF-21 (fibroblast growth factor 21) has recently been proposed as a valuable serum measure in inherited mitochondrial disease (Suomalainen *et al.*, 2011). In these patients, serum FGF-21 levels showed a very strong correlation with mitochondrial dysfunction on skeletal muscle biopsy, as determined by the percentage

of cells expressing a COX defect (Suomalainen *et al.*, 2011). FGF-21 is thought to regulate mitochondrial activity and enhance oxidative capacity, mediated via PGC-1α (peroxisome proliferator-activated receptor gamma co-activator 1-alpha) expression (Moyers *et al.*, 2007). To date, one study has assessed serum FGF-21 in HIV infection, and demonstrated elevated levels (Affandi *et al.*, 2008). Given the recently described association between serum FGF-21 elevation and muscle COX defects in inherited mitochondrial disorders (Suomalainen *et al.*, 2011), and the recent observation of significant COX defects in long-term anti-retroviral treated HIV-infected patients (Payne *et al.*, 2011), we speculated that muscle mitochondrial dysfunction might also drive the FGF-21 elevation in anti-retroviral treated HIV infection.

Patient characteristics

All subjects gave informed written consent for participation, and the study was approved by local research ethics committee. We performed a cross-sectional study of adult HIV-1 infected patients, receiving ambulatory care at one of two specialist clinics in Newcastle-upon-Tyne, UK. Patients with current active hepatitis B or C co-infection, known inherited or non-HIV-associated neuromuscular disease, and diabetes mellitus were excluded. No subjects were clinically obese (BMI >30). 32 HIV-infected subjects participated, of whom 81% were male. 84% were of white Caucasian ethnicity and the remainder black African. Mean age was 48.7 years, with age range of 29 - 71 years. Mean duration of diagnosed HIV infection was 10.8 years. Mean current CD4 lymphocyte count was 663 cells/μl, and 61% of subjects had nadir CD4 count of <200 cells/μl. All subjects were currently receiving combination anti-retroviral therapy, with a mean duration of treatment of 9.2 years. 97% of patients had fully suppressed HIV plasma viral load (<50 HIV-1 RNA copies/ml). 81% of subjects were receiving a non-nucleoside reverse transcriptase inhibitor (NNRTI) and 22% a ritonavir-boosted protease inhibitor (PI). Regarding past (lifetime) NRTI treatment experience, 72% of patients had a history of AZT exposure, and 25% had a history of prior d-drug (dideoxynucleoside analogue) exposure. Characteristics of individual subjects are shown in **Table 1**.

FGF-21 determination

Serum FGF-21 levels were determined by quantitative ELISA (BioVendor, Brno, Czech Republic), performed in triplicate, and normalised by $\log_{10}$ transformation. A serum FGF-21 level of <200 pg/ml was considered as normal in keeping with recent data (Suomalainen *et al.*, 2011). Statistical analyses were performed in SPSS 19, using student's t-test to compare binary variables and Pearson's correlation coefficient (r) to examine the relationship between $\log_{10}$ serum FGF-21 levels and continuous variables. Twenty-one of 32 subjects (66%) had serum FGF-21 levels greater than the normal range, with four being very elevated (>800 pg/ml). On univariate analysis, serum FGF-21 levels were positively correlated with current CD4 lymphocyte count ($r = 0.36$, $p = 0.042$), but more strongly correlated with total CD4 cell count gain since initiation of anti-retroviral therapy (current minus nadir) ($r = 0.45$, $p = 0.016$) (**Figure 1**). In addition, plasma glucose levels correlated with serum FGF-21 levels, although this did not quite reach statistical significance ($r = 0.34$, $p = 0.06$, **Figure 2**), whereas as serum lipids and liver function did not. No other demographic or treatment variables were significantly associated with serum FGF-21 levels, including the nature of current or prior anti-retroviral therapy (**Table 2**). FGF-21 levels did not differ significantly between patients with or without clinical lipodystrophy syndrome. Only CD4 lymphocyte count gain was independently associated with serum FGF-21 levels on multivariate linear regression analysis ($p = 0.016$).

Skeletal muscle mitochondrial histochemistry

COX histochemistry was performed on cryo-sections obtained from lower limb skeletal muscle biopsies on 31 of the 32 subjects (biopsy data for one subject was not analysable). Results of 22 of these biopsies have been reported in our previous work (Payne *et al.*, 2011), whereas the remaining 9 have not. COX contains respiratory chain subunits encoded by the mitochondrial genome, and fibres stain brown (positive) in the presence of intact respiratory chain activity (**Figure 3**). Proportional COX defect was determined by counting ≥500 fibres per biopsy, and normalised by $\log_{10}$ transformation. There was no correlation between serum FGF-21 levels and percentage COX defects on biopsy ($r = -0.02$, $p = 0.9$, **Figure 4**).

Discussion

We have shown that serum FGF-21 levels are frequently elevated in contemporary anti-retroviral treated HIV-infected patients, but do not correlate with the severity of muscle mitochondrial (COX) defect. In contrast, a previous study has shown a very strong correlation between these parameters in patients with inherited mitochondrial disorders (Suomalainen *et al.*, 2011). Ours is the first study to attempt to link serum FGF-21 levels with biopsy-proven mitochondrial defects in HIV-infected patients. What is the reason for this apparent discrepancy in findings? Firstly, the prior study demonstrating serum FGF-21 elevation in mitochondrial disease included a large number of patients with childhood-onset disease. Such patients typically have very severe muscle COX defects (affecting up to ~60% of fibres). In contrast, patients with late-onset inherited mitochondrial disorders typically have more modest COX defects, comparable with those seen in our HIV-infected patients (up to ~10% of fibres). The fact that some patients in our study with a biopsy COX defect of >5% of fibres had relatively normal FGF-21 levels suggests that this serum measure is not particularly sensitive for mild to moderate muscle mitochondrial defects. Secondly, the markedly abnormal serum FGF-21 levels seen in some patients with no significant COX defect suggest a non-mitochondrial origin, as has been observed in other metabolic disorders (Chen *et al.*, 2008; Zhang *et al.*, 2008; Dushay *et al.*, 2010). In the only previous study of FGF-21 levels in HIV infection, the authors found associations of FGF-21 levels with obesity, glycaemia, dyslipidaemia and liver dysfunction, in line with literature from HIV-uninfected patients (Domingo *et al.*, 2010). In our study, we specifically excluded diabetic and obese subjects (as we wished to maximise the likelihood of detecting any association with NRTI-induced mitochondrial dysfunction). Interestingly however, the strongest predictor of serum FGF-21 levels seen in our study was a novel association with total CD4 lymphocyte count gain. This is an intriguing finding. It is plausible that patients who have low nadir CD4 lymphocyte counts may experience more profound metabolic changes as they undergo immune reconstitution on anti-retroviral therapy, switching from a catabolic state to an excessively anabolic state associated with a 'return to health'. This association with CD4 count gain should be further explored by longitudinal study.

In conclusion, serum FGF-21 levels do not appear to be a sensitive or specific marker of muscle mitochondrial dysfunction in contemporary anti-retroviral treated patients.

Nevertheless they are commonly elevated in association with immune recovery. As serum FGF-21 levels in the HIV-uninfected population are elevated in conditions associated with increased cardiovascular risk, it is very plausible that serum FGF-21 elevation in anti-retroviral treated HIV infection may also be a marker of an adverse metabolic risk in this patient group. Given the known increase in cardiovascular disease in anti-retroviral treated patients (Friis-Moller *et al.*, 2003), the prognostic significance of our findings merits further research.

Figure 1. Correlation of serum FGF-21 levels with immune reconstitution.

Correlation of $\log_{10}$ serum FGF-21 (fibroblast growth factor 21) levels in HIV-infected subjects and CD4 lymphocyte count gain on treatment (current minus nadir) (r = 0.45, p = 0.016). (* Serum FGF-21 >1920 pg/ml, the upper limit of quantitation of the assay.)

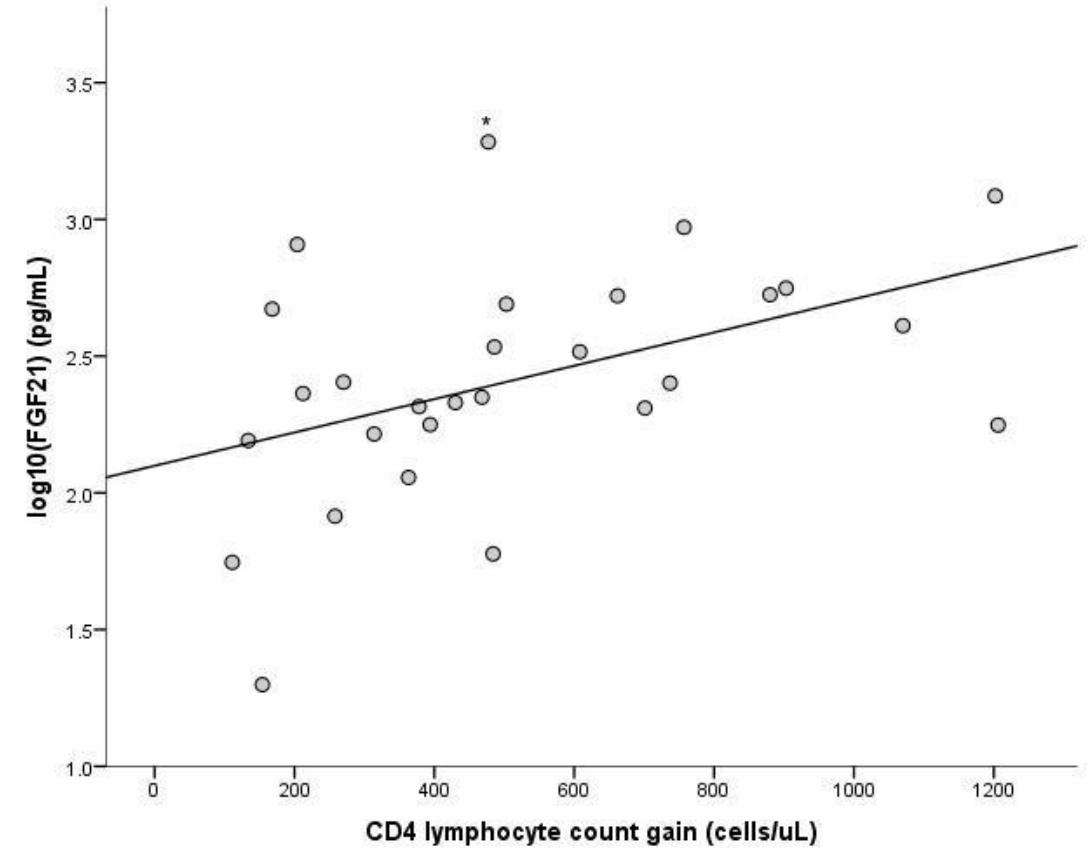

Figure 2. Correlation of serum FGF-21 levels with plasma glucose.

Correlation of log_{10} serum FGF-21 (fibroblast growth factor 21) levels in HIV-infected subjects and random plasma glucose concentration (r = 0.34, p = 0.06). (* Serum FGF-21 >1920 pg/ml, the upper limit of quantitation of the assay.)

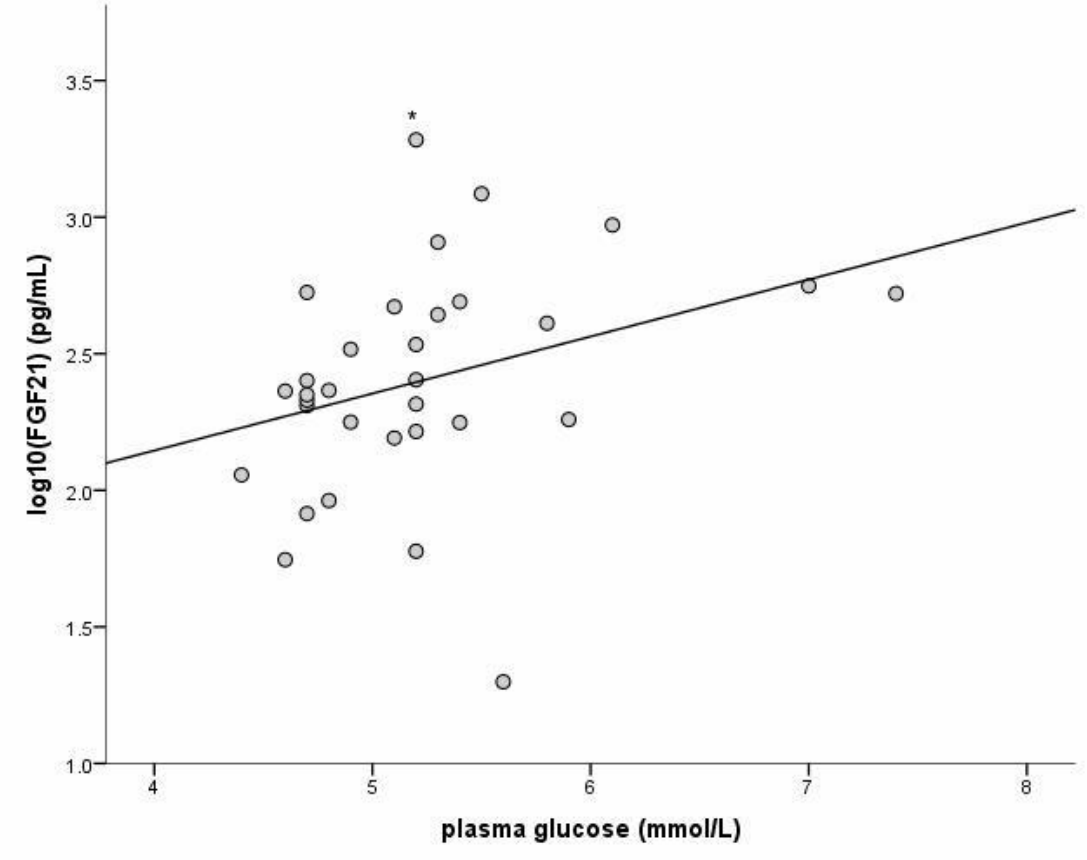

Figure 3. COX histochemistry.

Example of mitochondrial COX/SDH (cytochrome c oxidase / succinate dehydrogenase) histochemistry on lower limb skeletal muscle biopsy of an anti-retroviral treated HIV-infected patient. Normal (COX positive) fibres stain brown, whereas COX deficient fibres counterstain blue due to preserved SDH activity.

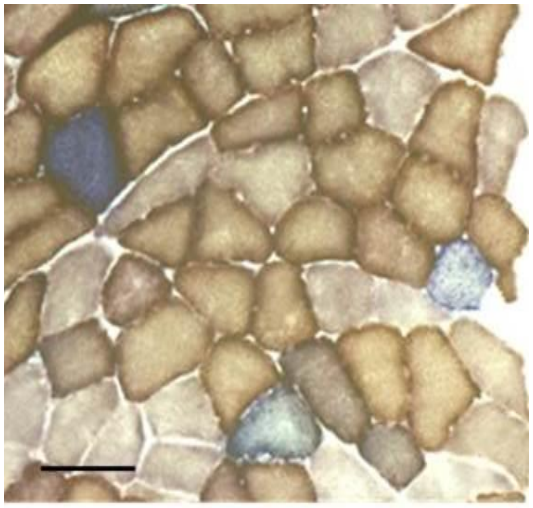

Figure 4. Correlation of serum FGF-21 levels and mitochondrial defects.

Correlation of $\log_{10}$ serum FGF-21 (fibroblast growth factor 21) levels in HIV-infected subjects and percentage COX (cytochrome *c* oxidase) defect on lower limb skeletal muscle biopsy (r = -0.02, p = 0.9). (* Serum FGF-21 >1920 pg/ml, the upper limit of quantitation of the assay.)

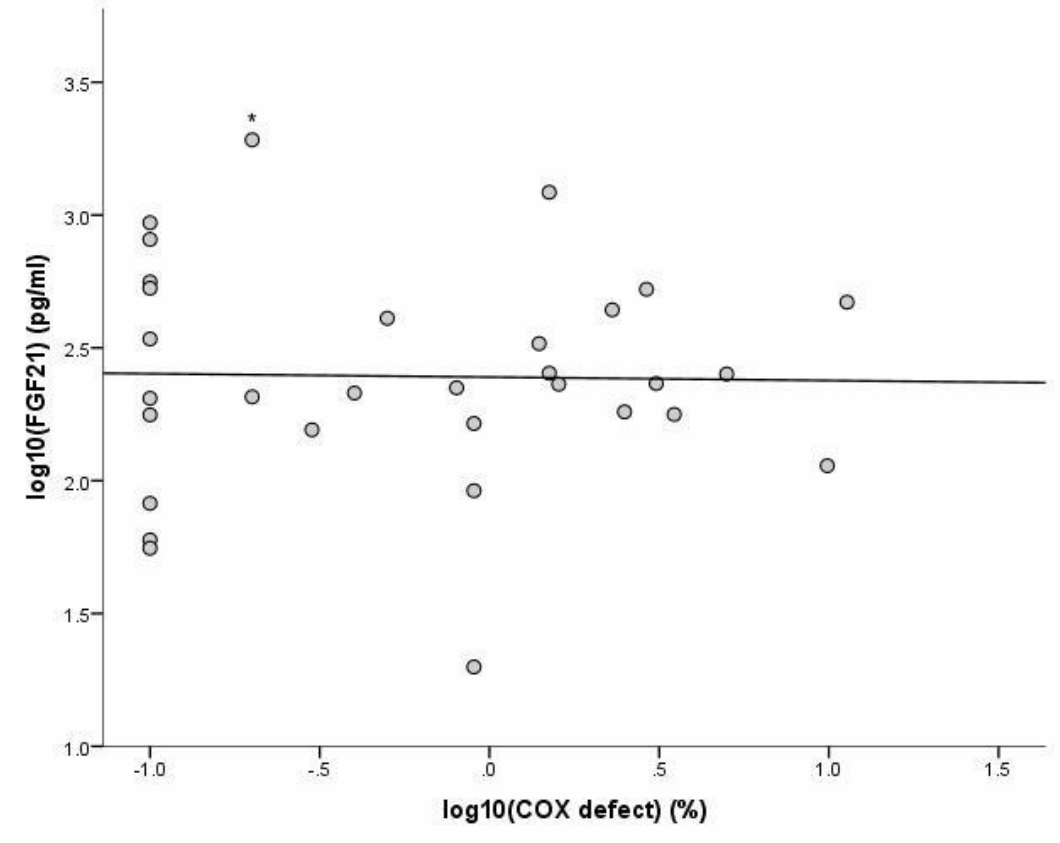

Table 1. Patient characteristics.

Summary characteristics of individual HIV-infected subjects. (WB, white British; BA, black African; ART, anti-retroviral therapy; LDS, lipodystrophy syndrome; AZT, zidovudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir; FTC, emtricitabine; EFV, efavirenz; NVP, nevirapine; ATV, atazanavir; DRV, darunavir; LPV, lopinavir; SQV, saquinavir; NFV, nelfinavir; IDV, indinavir; RTV, ritonavir at therapeutic dose; /r, ritonavir at pharmacokinetic boosting dose; RAL, raltegravir; COX, cytochrome c oxidase; FGF-21, fibroblast growth factor 21; NA, not available.)

Age (y)	Gender	Ethnicity	Duration of diagnosed HIV (mo)	ART duration (mo)	nadir CD4 count (cells/uL)	current CD4 count (cells/uL)	LDS	ART (current)	ART (lifetime)	COX defect (%)	serum FGF-21 (pg/mL)
71	M	WB	130	130	UK	530	Y	TDF FTC EFV	ddi AZT 3TC EFV TDF FTC	3.0%	232
48	F	BA	100	99	10	487	N	TDF FTC NVP	AZT 3TC EFV TDF FTC NVP	0.1%	>1920
34	F	WB	88	86	218	1121	Y	ABC 3TC NVP	AZT 3TC NVP ABC	0.0%	560
55	F	BA	64	27	112	426	N	TDF FTC AZT DRV/r	TDF FTC LPV/r AZT DRV/r	0.8%	164
43	M	BA	87	87	152	306	N	TDF FTC EFV	AZT 3TC EFV TDF FTC	0.8%	20
42	M	WB	185	147	150	636	N	AZT 3TC NVP	AZT 3TC NVP	0.0%	342
63	M	WB	97	97	169	870	N	ABC 3TC EFV	AZT 3TC EFV ABC	0.0%	204
29	M	WB	84	32	197	401	N	TDF FTC EFV	TDF FTC EFV	0.0%	809
63	M	WB	238	221	NA	438	N	ABC 3TC NVP	AZT ddI d4T 3TC ddC IDV NVP ABC	2.2%	440
62	M	WB	63	62	56	190	N	TDF FTC NVP	TDF FTC NVP	0.2%	156
52	M	WB	225	225	120	728	Y	TDF FTC ATV/r	AZT ddC ddI 3TC d4T SQV NVP IDV NFV ABC TDF LPV/r FTC ATV/r	1.3%	328
36	M	WB	139	138	197	627	N	AZT 3TC EFV	AZT 3TC EFV TDF FTC	0.3%	214
51	M	WB	190	183	10	747	N	ABC TDF NVP	AZT ddI d4T 3TC RTV NVP IDV ddC ABC ATV/r TDF	4.9%	252
33	F	WB	96	95	83	1289	N	TDF FTC EFV	AZT 3TC EFV TDF FTC	0.0%	177
48	M	WB	102	100	259	1329	Y	AZT 3TC EFV	AZT 3TC EFV	0.4%	409
51	M	WB	145	144	151	421	N	AZT 3TC NVP	AZT 3TC NVP	1.4%	254
66	M	WB	71	26	287	455	N	TDF FTC EFV	TDF FTC EFV	11.2%	470
46	M	WB	158	157	250	1452	N	TDF FTC EFV	AZT 3TC IDV EFV ABC TDF FTC	1.4%	1218
61	M	WB	116	113	NA	498	Y	TDF FTC NVP	AZT 3TC EFV NVP TDF FTC	2.4%	182
30	M	WB	88	23	283	661	N	TDF FTC DRV/r	TDF FTC EFV DRV/r	0.1%	207
62	M	WB	284	202	NA	422	N	ABC NVP LPV/r	SQV AZT ddC 3TC d4T ddI IDV ABC NVP NFV LPV/r	0.8%	92
45	M	WB	159	158	176	660	N	TDF FTC NVP	AZT 3TC IDV NVP TDF FTC	0.0%	60
54	M	WB	79	38	244	638	N	TDF FTC DRV/r	TDF FTC EFV DRV/r	3.4%	178
52	M	WB	166	164	0	662	Y	TDF FTC NVP	AZT d4T IDV NFV SQV 3TC NVP ddI TDF FTC	2.8%	525
51	M	WB	243	171	327	539	Y	TDF FTC EFV	AZT ddI RTV NFV TDF FTC EFV	1.5%	231
35	F	BA	62	25	380	638	N	TDF FTC EFV	TDF FTC EFV	0.0%	82
53	M	WB	NA	48	301	804	N	TDF FTC EFV	TDF FTC EFV	NA	490
36	M	WB	130	130	18	898	N	TDF FTC ATV/r	AZT 3TC EFV TDF FTC ATV/r	0.0%	530
48	M	WB	53	14	332	443	N	TDF FTC EFV	TDF FTC EFV	0.0%	56
52	F	BA	83	81	17	485	Y	TDF FTC EFV	AZT 3TC EFV TDF FTC	0.7%	224
38	M	WB	129	128	4	761	Y	TDF FTC EFV	AZT 3TC EFV TDF FTC	0.0%	935
47	M	WB	183	164	305	668	Y	ABC RAL ATV/r	d4T 3TC NVP ddI IDV ABC ATV/r RAL	9.8%	114

Table 2. Associations of serum FGF-21 levels.

(a) binary variables; (b) continuous variables. (PI, protease inhibitor; AZT, zidovudine; d-drug, dideoxynucleoside analogue; ART, anti-retroviral therapy; ALT, alanine transaminase; HDL, high density lipoprotein).

(a)

Variable	(n)	Log_{10} Serum FGF-21, mean (SD)	p value
Gender	Male (26)	2.39 (0.40)	
	Female (6)	2.46 (0.48)	0.71
Ethnicity	Caucasian (27)	2.44 (0.33)	
	Black African (5)	2.21 (0.72)	0.27
Current ART	PI (7)	2.29 (0.26)	
	No PI (25)	2.43 (0.44)	0.43
Lifetime ART	d-drugs (8)	2.38 (0.26)	
	No d-drugs (24)	2.41 (0.45)	0.86
	AZT (23)	2.44 (0.42)	
	No AZT (9)	2.30 (0.39)	0.41
Lipodystrophy	Yes (10)	2.50 (0.27)	
	No (22)	2.36 (0.46)	0.39
Lipid-lowering therapy	Yes (8)	2.32 (0.52)	
	No (24)	2.43 (0.38)	0.53

(b)

Variable		Correlation coefficient (r)	p value
Age		-0.07	0.69
Duration of diagnosed HIV infection		-0.08	0.67
Duration of lifetime ART	Total	0.12	0.50
	d-drug	0.01	0.97
	AZT	0.13	0.47
CD4 lymphocyte count	Nadir	-0.29	0.14
	Current	**0.36**	**0.042**
CD4 count gain	(Current minus nadir)	**0.45**	**0.016**
Serum ALT		0.27	0.14
Plasma glucose		**0.34**	**0.06**
Serum lipids	Total cholesterol	-0.03	0.87
	HDL cholesterol	0.02	0.90
	Non-HDL cholesterol	-0.06	0.75
Mitochondrial histochemistry	COX defect (log_{10})	-0.02	0.91

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In vivo mitochondrial function in HIV-infected persons treated with contemporary anti-retroviral therapy: a magnetic resonance spectroscopy study.

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Abstract

Modern anti-retroviral therapy is highly effective at suppressing viral replication and restoring immune function in HIV-infected persons. However, such individuals show reduced physiological performance and increased frailty compared with age-matched uninfected persons. Contemporary anti-retroviral therapy is thought to be largely free from neuromuscular complications, whereas several anti-retroviral drugs previously in common usage have been associated with mitochondrial toxicity. It has recently been established that patients with prior exposure to such drugs exhibit irreversible cellular and molecular mitochondrial defects. However the functional significance of such damage remains unknown.

Here we use phosphorus magnetic resonance spectroscopy (^{31}P-MRS) to measure *in vivo* muscle mitochondrial oxidative function, in patients treated with contemporary anti-retroviral therapy, and compare with biopsy findings (cytochrome c oxidase (COX) histochemistry).

We show that dynamic oxidative function (post-exertional ATP (adenosine triphosphate) resynthesis) was largely maintained in the face of mild to moderate COX defects (affecting up to ~10% of fibers): $\tau_{½}$ ADP (half-life of adenosine diphosphate clearance), HIV-infected 22.1 ±9.9 s, HIV-uninfected 18.8 ±4.4 s, p = 0.09. In contrast, HIV-infected patients had a significant derangement of resting state ATP metabolism compared with controls: ADP / ATP ratio, HIV-infected 1.24 ±0.08 $x10^{-3}$, HIV-uninfected 1.16 ±0.05 $x10^{-3}$, p = 0.001.

These observations are broadly reassuring in that they suggest that *in vivo* mitochondrial function in patients on contemporary anti-retroviral therapy is largely maintained at the whole organ level, despite histochemical (COX) defects within individual cells. Basal energy requirements may nevertheless be increased.

Introduction

Combination anti-retroviral therapy (cART) has transformed the prognosis for HIV-infected persons since the late 1990s. However, patients are at risk of mitochondrial toxicity, thought to be mediated very largely through exposure to certain nucleoside analog reverse transcriptase inhibitor (NRTI) anti-retrovirals. NRTIs were the first class of licensed anti-retroviral drug, and several of the older members of this class, zidovudine, stavudine, zalcitabine and didanosine, are known to inhibit the sole mitochondrial DNA (mtDNA) polymerase, pol γ, resulting in chain termination during mtDNA replication. During therapy, the molecular consequence of this inhibition is reduction in cellular mtDNA content (mtDNA depletion). A wealth of previous studies has demonstrated this phenomenon both *in vitro,* and in a variety of tissues *in vivo* (Arnaudo *et al.*, 1991; Cherry *et al.*, 2002; Cote *et al.*, 2002; Hoschele, 2006). These older NRTIs are no longer in common usage in industrialized countries owing to concerns over their toxicity profiles, although zidovudine and stavudine have been very extensively used in anti-retroviral therapy 'roll-out' programs in developing countries in recent years. Currently used NRTIs, such as tenofovir (a nucleo*tide* RTI) and abacavir, have been shown to be essentially free from pol γ inhibition *in vitro* and to cause no significant mtDNA depletion *in vivo* (Lim and Copeland, 2001; Birkus *et al.*, 2002). If a patient's therapy is switched away from a pol γ inhibiting NRTI, the impairment of mtDNA replication is removed and mtDNA content returns to normal (McComsey *et al.*, 2005). Therefore, although most patients are no longer exposed to pol γ inhibiting NRTIs, a significant cohort of long-term patients will have extensive *prior* exposure to such drugs. Although such patients do not have persistent mtDNA depletion, it has recently been established that they may have persistent histochemical mitochondrial defects evidenced by an increased proportion of COX (cytochrome *c* oxidase) deficient skeletal muscle fibers. These COX deficient fibers contain high levels of individual somatic (acquired) mtDNA mutations (principally large-scale deletion mutations) (Payne *et al.*, 2011). The relevance of this persistent cellular and molecular damage on mitochondrial function remains unknown. It is therefore unclear to what extent mitochondria may be functionally impaired in HIV-infected patients treated with contemporary cART.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) allows the dynamic measurement of *in vivo* skeletal muscle oxidative function through assessment of ATP

(adenosine triphosphate) metabolites as well as acid handling. ^{31}P-MRS has previously been employed in the longitudinal study of subjects with inherited mitochondrial disorders, both primary mtDNA defects, and secondary mtDNA defects consequent on nuclear gene disorders of mtDNA maintenance (Penn *et al.*, 1992; Trenell *et al.*, 2006; Yu-Wai-Man *et al.*, 2011). Limited data also suggests that ^{31}P-MRS abnormalities in skeletal muscle may be demonstrated in the setting of acute exposure to pol γ inhibiting NRTIs: early in the HIV epidemic, in infected patients exposed to high-dose zidovudine therapy; and in uninfected volunteers treated with stavudine. Such measurements have not been performed in contemporary cART treated patients (Sinnwell *et al.*, 1995; Fleischman *et al.*, 2007).

We have therefore used ^{31}P-MRS to determine whether patients on contemporary anti-retroviral therapy have abnormal *in vivo* mitochondrial oxidative function, and whether this correlates with biopsy COX defects.

Methods

Participants.

Participants were adult HIV-1 infected patients, receiving ambulatory care at one of four specialist clinics (2 hospital-based, 2 community-based setting). Patients with current active hepatitis B or C co-infection were excluded. Participants were unselected with respect to the presence or absence of complications of HIV or anti-retroviral therapy. Patients with known inherited or non-HIV-associated neuromuscular disease were excluded. Demographic data, surrogate markers (CD4 T lymphocyte count, and HIV-1 RNA plasma viral load) and detailed lifetime anti-retroviral treatment history were obtained by case note review.

HIV-uninfected control subjects for ^{31}P-MRS studies were age and sex matched to our cases, and we excluded persons with diabetes mellitus or abnormal glucose handling, thyroid disease, previous muscle injury, or diagnosed neuromuscular disease.

Research was approved by the Newcastle and North Tyneside Local Research Ethics Committee (ref. 06/Q0905/137). All subjects gave informed written consent for participation.

Phosphorus magnetic resonance spectroscopy.

MR studies were performed on calf muscle using a 3T Intera Achieva magnet (Philips). ^{31}P-MRS measurements were obtained using a calf coil with a voxel within soleus muscle during: a 1 minute baseline resting period; a 3 minute period of calf flexion exercise at 25% of maximal voluntary contractile force; and a 6 minute recovery period (Trenell *et al.*, 2006; Hollingsworth *et al.*, 2008). This exercise paradigm was specifically designed to keep metabolism within the aerobic phase. Analysis was performed in jMRUI v3.0 (Java Magnetic Resonance User Interface) using AMARES with appropriate prior knowledge parameters for skeletal muscle (Naressi *et al.*, 2001) and metabolite levels were calculated as previously described (Hollingsworth *et al.*, 2008). Phosphorylation potential was calculated from the concentration of ATP ([ATP], buffered at 8.2 mM), and the empirically calculated concentrations of adenosine diphosphate and inorganic phosphate ([ADP], [P_i]), as [ATP]/([ADP][Pi]) (Harris *et al.*, 1974).

Skeletal muscle biopsies and mitochondrial (COX) histochemistry.

Percutaneous lower limb skeletal muscle biopsies were performed under local anesthesia and snap-frozen in the liquid phase of isopentane, cooled in liquid nitrogen within 20 minutes of collection. Sequential COX-SDH (cytochrome *c* oxidase / succinate dehydrogenase) histochemistry was performed on 20μm transverse cryo-sections. COX contains respiratory chain subunits encoded by the mitochondrial genome, and fibers stain brown (positive) in the presence of intact respiratory chain activity. SDH contains subunits encoded entirely by the nuclear genome and thus provides an effective counterstain (blue) as activity will be preserved in the presence of a cellular mtDNA defect. Proportional COX defect was determined by counting ≥500 fibers per biopsy.

Statistical comparisons.

Student's paired t-test was used to compare MRS parameters between cases and controls. Correlation coefficients were calculated between COX and MRS data. All analyses were performed in SPSS 19.

Results

Patient characteristics.

23 HIV-infected subjects participated; 78% were male. Mean age was 57.6 years, with age range of 45-74 years. Mean duration of diagnosed HIV infection was 11.8 years. Mean current CD4 T lymphocyte count was 551 cells/μl; mean nadir CD4 count was 183 cells/μl. All subjects were currently receiving cART, of whom 96% had a fully suppressed HIV plasma viral load (<40 HIV-1 RNA copies/ml). In addition to their NRTIs, 70% of treated subjects were receiving a non-nucleoside reverse transcriptase inhibitor (NNRTI) and 35% a protease inhibitor (PI). With respect to the pol γ inhibiting NRTIs, 61% of patients had a prior history of zidovudine exposure, and 48% had dideoxynucleoside analog (stavudine, zalcitabine or didanosine) exposure (treatment details of individual patients are shown in **Table 1**).

Measures of muscle ATP and acid metabolism by ^{31}P-MRS.

In the resting state, baseline ATP metabolites and pH values were significantly higher in cART-treated HIV-infected subjects compared to age and gender-matched controls (mean ±SD): ADP/ATP ratio, HIV-infected 1.24 ±0.08 $x10^{-3}$, HIV-uninfected 1.16 ±0.05 $x10^{-3}$, p = 0.001; phosphocreatine/ATP (PCr/ATP) ratio, HIV-infected 5.04 ±1.89, HIV-uninfected 3.75 ±0.26, p = 0.004; pH, HIV-infected 7.07 ±0.03, HIV-uninfected 7.04 ±0.02, p = 0.002. Correspondingly, calculated basal phosphorylation potential was significantly lower in HIV-infected subjects compared with controls: HIV-infected 227 ±86 mM^{-1}, HIV-uninfected 292 ±53 mM^{-1}, p = 0.003 (**Figure 1a-c**). (Further details of calculated ^{31}P-MRS parameters are shown in the **Supplementary Table.**)

In terms of dynamic oxidative function, mean post-exercise ATP metabolite recovery rates did not differ significantly between HIV-infected subjects and controls. For

example, $\tau_{½}$ ADP: HIV-infected 22.1 ±9.9 s, HIV-uninfected 18.8 ±4.4 s, p = 0.09 (**Figure 1d**). None of the clinical variables (age, duration of diagnosed HIV infection, CD4 T lymphocyte count, or anti-retroviral treatment history) correlated significantly with any of the baseline or post-exercise ^{31}P-MRS parameters in HIV-infected subjects.

Mitochondrial (COX) histochemistry and correlation with ATP metabolism.

A wide range of COX defects were observed across the subject group (0 to >10% of muscle fibers affected per biopsy). Interestingly, we observed significant COX defects both in patients with prior exposure to pol γ inhibiting NRTIs, and in some patients without such exposure (COX defects for individual patients are shown in **Table 1**). Resting state ADP/ATP ratio showed a moderate correlation with biopsy proportional COX defect (Kendall's $\tau = 0.34$, $p = 0.034$) (**Figure 2a**). There was no correlation between dynamic ATP metabolism, for example as estimated by $\tau_{½}$ ADP, and biopsy COX defect (**Figure 2b**).

Discussion

In our study, most anti-retroviral treated HIV-infected subjects demonstrated dynamic *in vivo* tissue mitochondrial function comparable with uninfected control subjects, whereas it is generally impaired in inherited mitochondrial disorders. Our subject group included patients with very long durations of HIV infection and extensive anti-retroviral drug treatment histories, including past exposure to pol γ inhibiting NRTIs. Interestingly, in the present study we observed COX defects both in subjects with prior exposure to pol γ inhibiting NRTIs, and in some subjects without such exposure. This observation contrasts with our previous work in younger HIV-infected patients (all aged ≤50 years), where COX defects appeared to be attributable almost entirely to exposure to pol γ inhibiting NRTIs (Payne *et al.*, 2011). In the present study, the heterogeneous COX defects are most likely to reflect the significantly older subject age range (45-74 years). In this age group it is expected to see some COX defects due to normal aging (Brierley *et al.*, 1996), although it is also possible that there are other unmeasured HIV or treatment-associated factors driving COX defects in some of these patients. As with NRTI-associated COX defects, these COX deficient fibers also contain high levels of individual somatic mtDNA mutations (Bua *et al.*, 2006). What is therefore the likely explanation of our finding of largely normal *in vivo* muscle mitochondrial function?

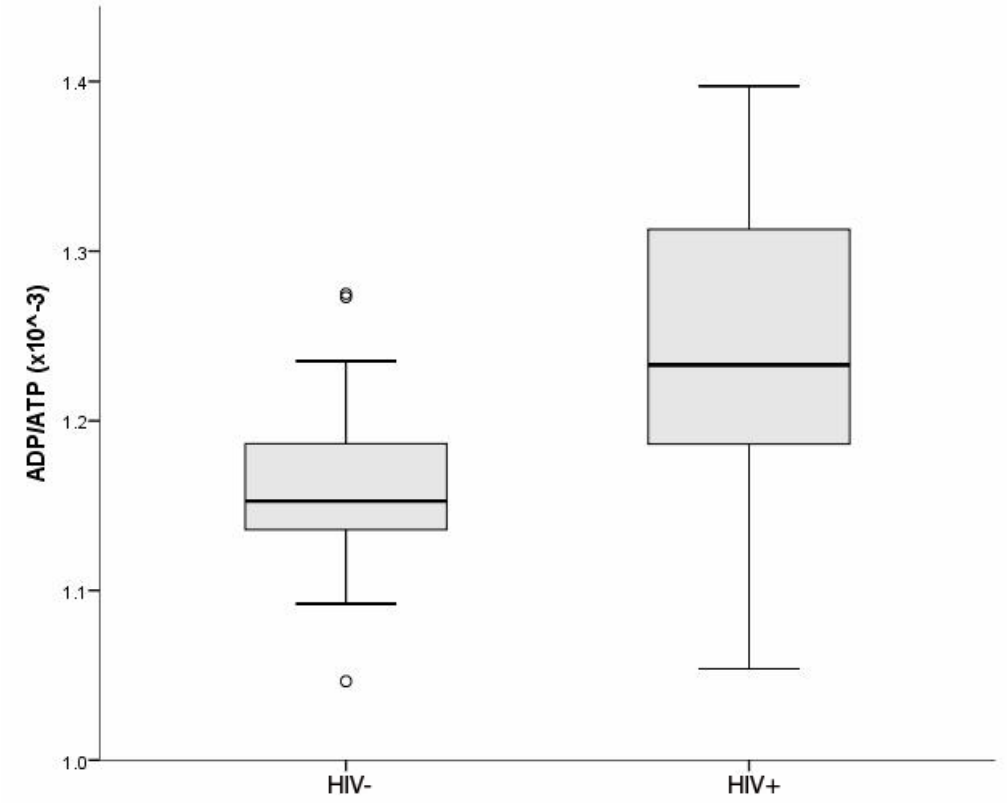
COX-deficient fibers contain high levels of mutant mtDNA, whereas COX positive fibers contain almost exclusively wild-type mtDNA. Therefore function at the whole tissue level is presumably compensated by the larger number of fibers with normal COX function. In contrast, in the historical context when patients were actively treated with a pol γ inhibiting NRTI, the molecular defect was one of mtDNA depletion, and the impairment of oxidative function would be expected to affect all fibers (Sinnwell *et al.*, 1995). We therefore conclude that although somatic mtDNA mutations and associated cellular COX defects are frequently present in contemporary cART treated patients, there is some ability to compensate for *in vivo* exertional oxidative function in the muscle as a whole.

The decreased basal phosphorylation potential, as we have observed in our patients, implies an increased rate of ATP synthesis at rest (Jeneson *et al.*, 1996). Cytosolic ATP concentration is tightly buffered, and results from the balance of the ATP hydrolysis required for the maintenance of cellular integrity and the synthesis of ATP from oxidative phosphorylation (Harris *et al.*, 1974). The rate of ATP synthesis is strongly dependent on the phosphorylation potential in resting muscle, with a decreased phosphorylation potential, as we have observed in our patients, implying an increased rate of ATP synthesis (Jeneson *et al.*, 1996). This notion suggests that there is a requirement for an increased basal rate of intracellular ATP hydrolysis in HIV-infected subjects, and an increased basal rate of ATP synthesis is therefore required to maintain ATP homeostasis. Although this might imply increased basal energy expenditure in these patients compared with healthy subjects, given that dynamic *in vivo* mitochondrial function is unimpaired, the physiological significance of this observation remains uncertain. Further work should therefore examine correlates of this finding, such as fatigue (Payne *et al.*, 2013).

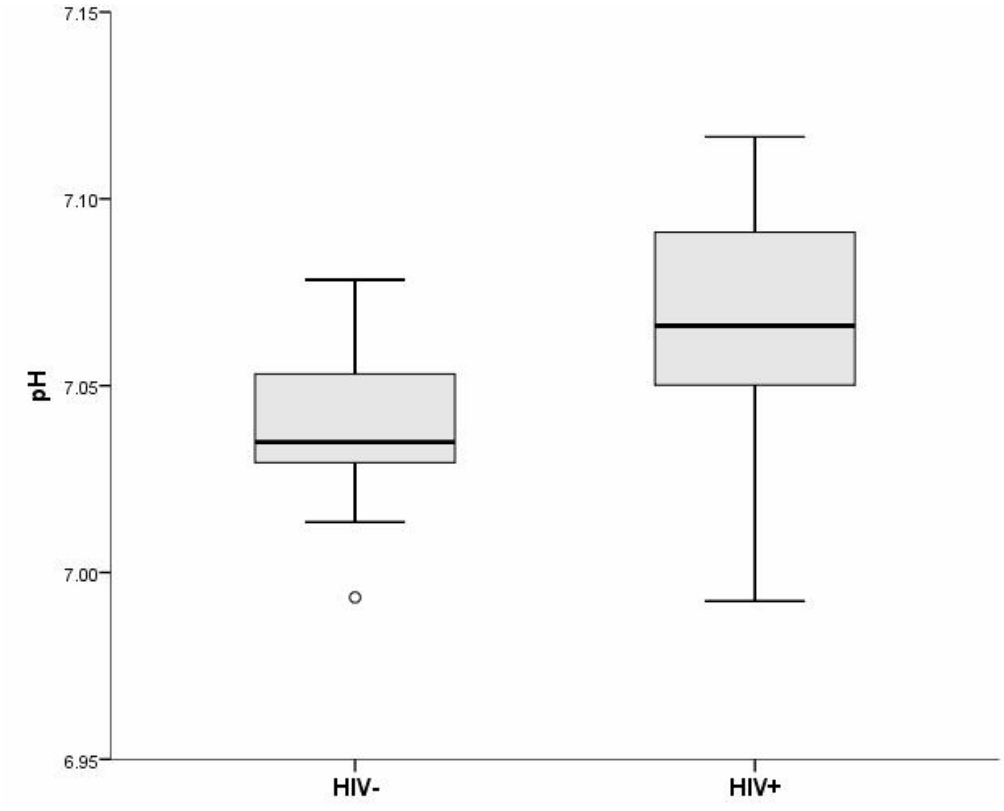
In conclusion, in a cohort of predominantly older HIV-infected patients with longstanding cART, we observed frequent histochemical COX defects both in patients with and without prior exposure to pol γ inhibiting NRTIs. It is, however, broadly reassuring that *in vivo* whole tissue mitochondrial function in most contemporary anti-retroviral treated patients appears to be largely maintained, despite the presence of this frequent mitochondrial damage within individual cells.

Figure 1. Phosphorus magnetic resonance spectroscopy. Resting state metabolic parameters differed significantly between HIV-infected subjects (HIV+) and HIV-uninfected controls (HIV-): ADP/ATP (adenosine diphosphate/ATP) ratio (**a**), phosphorylation potential (**b**), and pH (**c**) (n = 23 each; ADP/ATP, p = 0.001; phosphorylation potential, p = 0.003; pH, p = 0.002). In contrast, the rate of ATP re-synthesis (estimated as $\tau_{½}$ ADP) following exertion was not significantly impaired in HIV-infected subjects compared with controls (p = 0.09) (**d**).

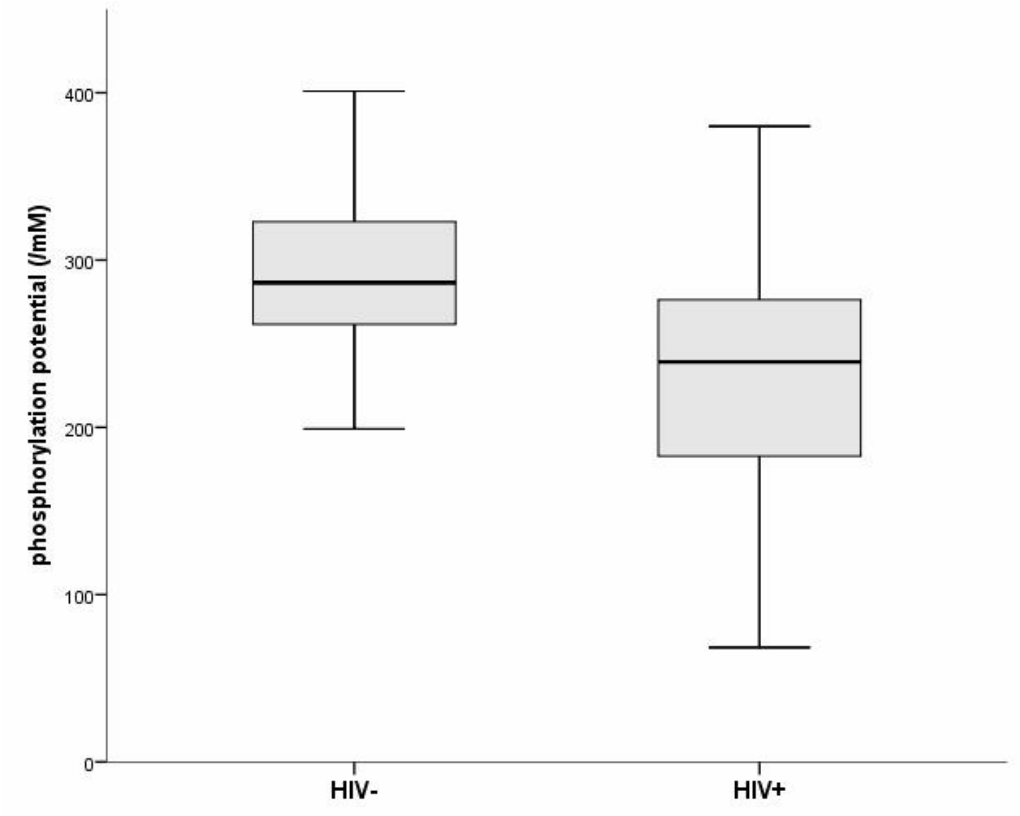
(a)



(b)



(c)



(d)

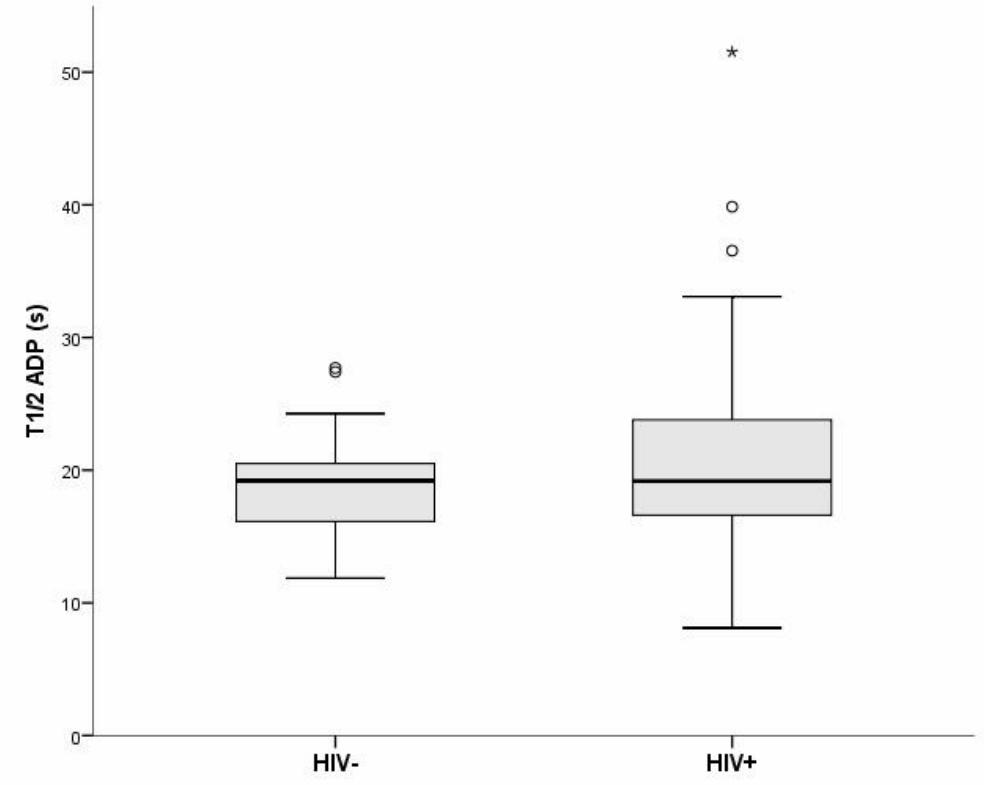
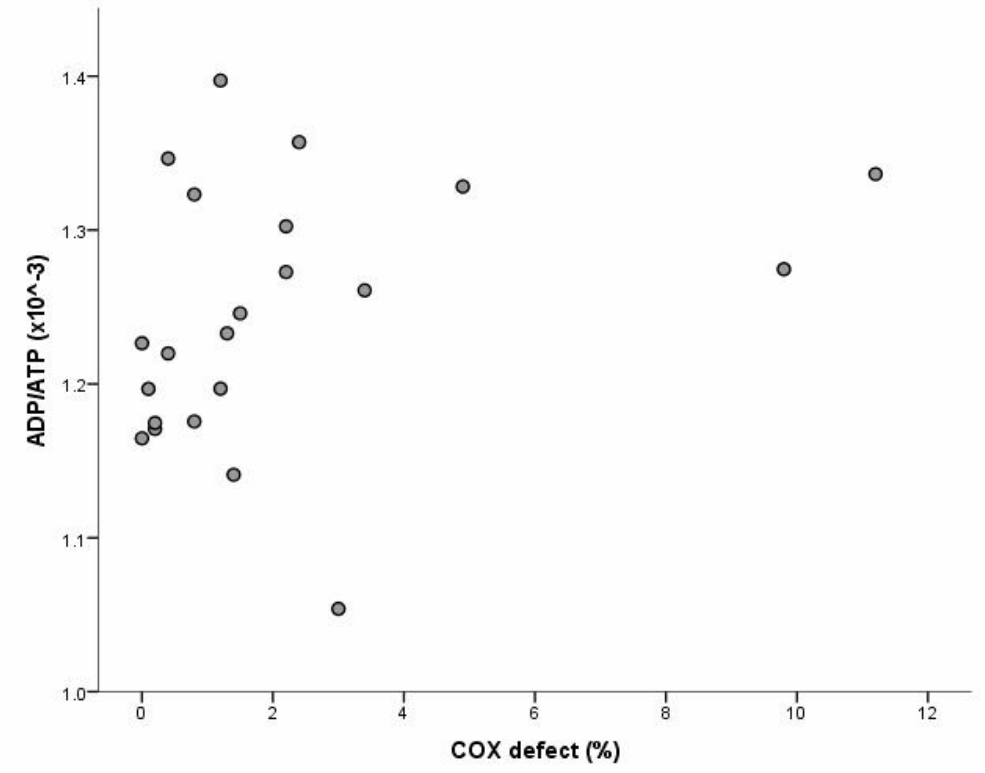


Figure 2. Relationship of phosphorus magnetic resonance spectroscopy and muscle histochemistry. Resting state ADP/ATP ratio showed moderate correlation with the percentage frequency of COX deficient muscle fibers in treated HIV-infected subjects (Kendall's $\tau = 0.34$, $p = 0.034$) (**a**), whereas the rate of ATP re-synthesis following exertion (estimated as $\tau_{½}$ ADP) did not (Kendall's $\tau = 0.04$) (**b**).

(a)



(b)

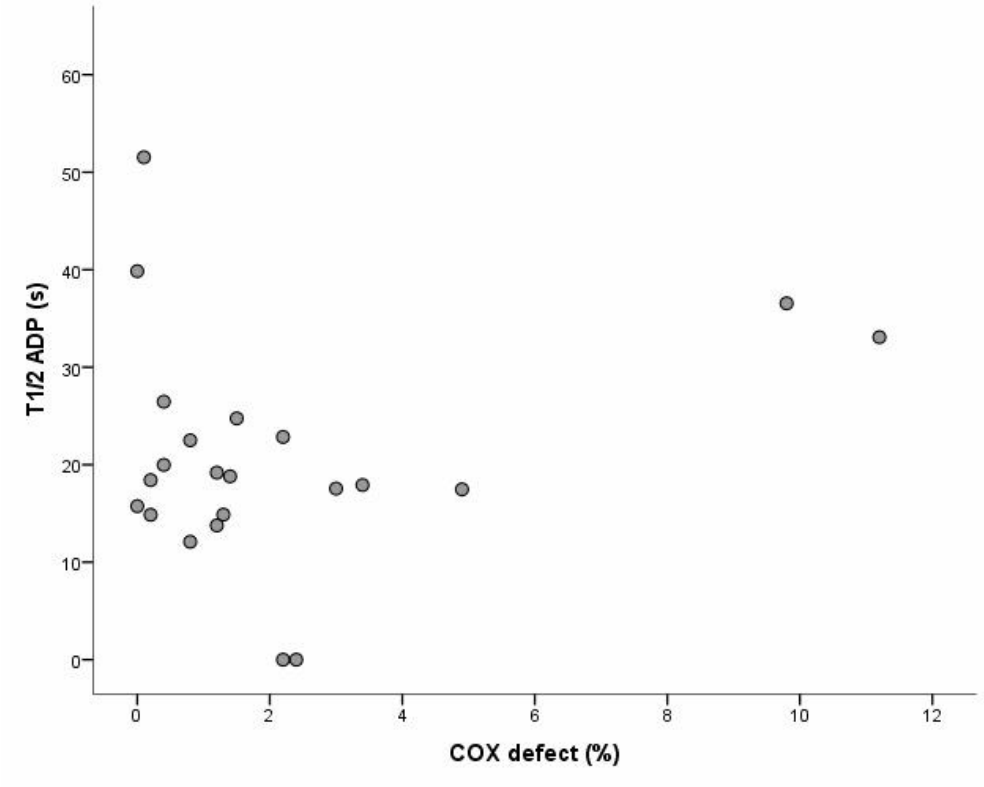


Table 1. Characteristics of HIV-infected subjects.

Subject	Age (yrs)	Gender	Duration of diagnosed HIV infection (mo)	Current CD4 count (cells/uL)	Current HIV VL (copies/mL)	Nadir CD4 count (cells/uL)	Duration of ART (mo)	Current cART	Lifetime ART	Biopsy COX defect (%)
1	55	M	96	503	<40	117	48	TDF FTC ATV/r	TDF FTC ATV/r	0.0%
2	71	M	119	448	<40	UK	119	TDF FTC EFV	ddI AZT 3TC EFV TDF FTC	3.0%
3	74	F	201	825	<40	UK	103	TDF FTC EFV	AZT ddi d4T SQV TDF 3TC EFV	0.4%
4	45	F	71	537	<40	10	70	TDF FTC NVP	AZT 3TC EFV TDF FTC NVP	0.1%
5	55	F	59	406	<40	112	22	TDF FTC AZT DRV/r	TDF FTC LPV/r AZT DRV/r	0.8%
6	63	M	76	783	<40	169	76	ABC 3TC EFV	AZT ABC 3TC EFV	0.0%
7	63	M	215	361	<40	UK	198	ABC 3TC NVP	AZT ddI d4T 3TC ddC IDV NVP ABC	2.2%
8	62	M	43	180	<40	56	42	TDF FTC NVP	TDF FTC NVP	0.2%
9	49	M	193	762	<40	120	193	TDF FTC ATV/r	AZT ddC ddI 3TC d4T SQV NVP IDV NFV ABC TDF LPV/r FTC ATV/r	1.3%
10	48	M	158	872	<40	10	151	TDF ABC NVP	AZT ddI d4T 3TC RTV NVP IDV ddC ABC ATV/r TDF	4.9%
11	60	F	146	666	<40	99	145	ABC 3TC EFV	d4T ABC 3TC EFV	0.2%
12	51	M	141	494	<40	151	140	AZT 3TC NVP	AZT 3TC NVP	1.4%
13	66	M	57	403	<40	287	12	TDF FTC EFV	TDF FTC EFV	11.2%
14	63	F	182	865	<40	300	154	TDF FTC EFV	d4T 3TC NVP NFV EFV AZT TDF FTC	1.2%
15	60	M	101	419	<40	UK	98	TDF FTC NVP	AZT 3TC EFV NVP TDF FTC	2.4%
16	61	M	262	422	<40	UK	160	ABC NVP LPV/r	SQV AZT ddC 3TC d4T ddI IDV ABC NVP NFV LPV/r	0.8%
17	54	M	66	603	<40	244	25	TDF FTC DRV/r	TDF FTC EFV DRV/r	3.4%
18	51	M	237	559	<40	327	165	TDF FTC EFV	AZT ddI RTV NFV TDF FTC EFV	1.5%
19	62	M	143	329	<40	163	55	TDF FTC DRV/r	AZT 3TC NVP FOS-APV RTV TDF FTC DRV/r	1.2%
20	53	M	UK	804	<40	301	48	TDF FTC EFV	TDF FTC EFV	NA
21	56	M	240	401	97	150	224	TDF FTC ETR DRV/r	AZT ddC SQV 3TC IDV d4T NVP ddI ABC LPV/r TDF ATV/r FOS-APV/r DRV/r MVC FTC	2.2%
22	45	M	165	592	<40	305	146	RAL ABC ATV/r	d4T 3TC NVP ddI IDV ABC ATV/r RAL	9.8%
23	57	M	145	435	<40	379	21	TDF FTC EFV	TDF FTC EFV	0.4%

VL, plasma HIV-1 RNA viral load; (c)ART, (combination) anti-retroviral therapy; AZT, zidovudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir; FTC, emtricitabine; EFV, efavirenz; NVP, nevirapine; ATV, atazanavir; DRV, darunavir; LPV, lopinavir; SQV, saquinavir; NFV, nelfinavir; IDV, indinavir; FOS-APV, fosamprenavir; RTV, ritonavir at therapeutic dose; /r, ritonavir at pharmacokinetic boosting dose; MVC, maraviroc; RAL, raltegravir; UK, unknown; NA, not available; COX, cytochrome c oxidase. COX data (but not MRS data) from 5 subjects has been previously described (Payne *et al.*, 2011).

Supplementary Table. Phosphorus magnetic resonance data.

	HIV (n = 23)		Control (n = 23)		
	mean	**SD**	**mean**	**SD**	**p-value**
Basal (Resting state)					
P_i/ATP	0.52	0.27	0.37	0.06	**0.010**
PCr/ATP	5.04	1.89	3.75	0.26	**0.004**
ADP/ATP ($x10^{-3}$)	1.24	0.08	1.16	0.05	**0.001**
Phosphorylation potential (mM^{-1})	227	86	292	53	**0.003**
pH	7.07	0.03	7.04	0.02	**0.002**
Post-exercise (Recovery)					
Initial PCr resynthesis (mM/min)	15.6	14.8	12.2	4.9	0.22
τ½ PCr (s)	30.2	13.6	27.1	8.3	0.31
τ½ ADP (s)	22.1	9.9	18.8	4.4	0.09
Q_{max}[ATP] (mM/min)	27.5	19.2	23.3	10.2	0.28
Minimum pH	6.98	0.14	7.00	0.03	0.48
Initial proton efflux (mM/min)	2.37	2.10	1.69	1.70	0.10

(P_i, inorganic phosphate; PCr, phosphocreatine; ADP, adenosine diphosphate; ATP, adenosine triphosphate.)

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HIV-associated fatigue in the era of highly active antiretroviral therapy: novel biological mechanisms?

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Abstract

Objective

The aim of the study was to determine the prevalence and risk factors for HIV-associated fatigue in the era of highly active antiretroviral therapy (HAART).

Methods

A cross-sectional survey of 100 stable HIV-infected out-patients was carried out. Severity of fatigue was measured using the Fatigue Impact Scale (FIS). Symptoms of orthostatic intolerance (dysautonomia) were evaluated using the Orthostatic Grading Scale (OGS). Data for HIV-infected patients were compared with those for 166 uninfected controls and 74 patients with chronic fatigue syndrome (CFS) / myalgic encephalomyelitis (encephalopathy) (ME).

Results

Ninety-one per cent of HIV-infected patients were on HAART and 78% had suppressed plasma HIV viral load (≤ 40 HIV-1 RNA copies/mL). Fifty-one per cent of HIV-infected patients reported excessive symptomatic fatigue (FIS ≥ 40), and 28% reported severe fatigue symptoms (FIS ≥ 80). The mean FIS score among HIV-infected patients was 50.8 [standard deviation (SD) 41.9] compared with 13.0 (SD 17.6) in uninfected control subjects, and 92.9 (SD 29.0) in CFS patients ($P < 0.001$ for comparison of HIV-infected patients and uninfected controls). Among HIV-infected patients, fatigue severity was not significantly associated with current or nadir CD4 lymphocyte count, HIV plasma viral load, or whether on HAART. Prior dideoxynucleoside analogue (d-drug) exposure ($P = 0.016$) and the presence of clinical lipodystrophy syndrome ($P = 0.011$) were associated with fatigue. Additionally, fatigue severity correlated strongly with symptomatic orthostatic intolerance ($r = 0.65$; $P < 0.001$).

Conclusions

Fatigue is very common and often severe in HIV-infected out-patients, despite viral suppression and good immune function. In a subgroup of patients, prior d-drug exposure may contribute to fatigue, suggesting a metabolic basis. Dysautonomia may also drive fatigue associated with HIV infection, as in other chronic diseases, and CFS/ME, and should be further evaluated with the potential for a shared therapeutic approach.

Introduction

Fatigue was a very common symptom in HIV-infected persons in the pre-highly active antiretroviral therapy (HAART) era, when it appeared to be associated with advanced immunosuppression and clinical AIDS (Darko *et al.*, 1992). It is unclear to what extent fatigue remains a problem in the modern era.

Fatigue is a symptom which patients and physicians may respectively find difficult to articulate and manage (Woodward *et al.*, 1995). The pathological basis of fatigue is poorly understood; in particular, the contribution that biological, as opposed to psychological, factors may make has received little attention. However, recent research suggests that common mechanisms including dysautonomia (orthostatic intolerance) may plausibly drive fatigue in diverse chronic diseases (Freeman and Komaroff, 1997; Barendregt *et al.*, 1998; Rowe and Calkins, 1998; Flachenecker *et al.*, 2003; Newton *et al.*, 2007b; Jones *et al.*, 2009).

We therefore aimed to quantify the prevalence of excessive fatigue and its associated factors in a contemporary HIV-infected cohort.

Patients and methods

Participants

We evaluated fatigue and associated symptoms in consecutive unselected adult HIV-infected out-patients attending our regional infectious diseases unit. Demographic data, HIV surrogate markers, and antiretroviral treatment data were retrieved by case note review. Patients with another probable physical or psychiatric reason for fatigue symptoms were excluded, using the approach outlined in the Centers for Disease Control and Prevention (CDC) 1994 Fukuda criteria for the diagnosis of chronic fatigue syndrome / myalgic encephalomyelitis (encephalopathy) (CFS/ME) (Fukuda *et al.*, 1994). HIV-uninfected control subjects of comparable age from the same geographical area had been previously recruited through notices in local press and hospitals asking for volunteers to participate in research projects (no selection was made for presence or absence of fatigue). CFS/ME patients were receiving out-patient care in our institution and fulfilled the CDC 1994 Fukuda criteria.

Symptom assessment tools

We administered self-rating scales comprising the Fatigue Impact Scale (FIS) (Prince *et al.*, 2000) and the Orthostatic Grading Scale (OGS) (Schrezenmaier *et al.*, 2005). The FIS is a 40-item generic fatigue impact scale. A score ≥ 40 indicates excessive fatigue and ≥ 80 severe fatigue. The OGS reports symptoms of orthostatic intolerance resulting from orthostatic hypotension. It comprises five items (frequency of orthostatic symptoms, severity of orthostatic symptoms, conditions under which orthostatic symptoms occur, interference with activities of daily living, and standing time before experiencing orthostatic symptoms), each rated from 0 to 4, with the total score as the sum of the items. Studies have shown good correlation between OGS scores and conventional physiological measures of the autonomic nervous system (Schrezenmaier *et al.*, 2005). A score of ≥ 4 is considered consistent with at least moderate orthostatic intolerance and ≥ 9 consistent with a formal diagnosis of orthostatic hypotension. These tools have been validated for self-completion in a range of other fatigue-associated chronic diseases, such as primary biliary cirrhosis (PBC) (Prince *et al.*, 2000).

Statistical analyses

Comparisons of test scores between patient groups and by dichotomous variables were made using Student's *t*-test. Univariate relationships between FIS and continuous linear variables were assessed using Pearson correlation. Multivariate analysis was performed using linear regression. Statistical analyses were performed in SPSS 19 (IBM, Armonk, NY).

Results

Patient and control group characteristics

One hundred self-rating scales from HIV-infected patients were evaluated. Demographic, disease and treatment data were as follows: mean age 46.9 years (range 27–70 years); 64% male; median duration of diagnosed HIV infection 8.0 years (range 1–27 years); mean current CD4 lymphocyte count 520 cells/μL (range 151–1569 cells/μL); mean nadir CD4 count 194 cells/μL (range 11–928 cells/μL); 91% currently receiving HAART; 78% of total group with plasma HIV viral load ≤ 40 HIV-1

RNA copies/ml; 1% hepatitis C virus coinfected. A total of 166 HIV-uninfected control subjects were analysed. The group was well age-matched (mean 47.5 years; range 21–77 years), although it contained a lower proportion of men (33%). Seventy-four patients with CFS/ME were included in the analysis (mean age 54.0 years; range 24–80 years; 24% male).

Fatigue, HIV disease activity and HAART

Half of the HIV-infected patients (50 of 99; 51%) reported excessive fatigue (FIS $\geq$ 40), and 28 (28%) reported severe fatigue (FIS $\geq$ 80) (one rating scale was insufficiently complete for FIS analysis). Mean FIS scores were significantly higher in HIV-infected patients compared with uninfected control subjects [HIV-infected, 50.8 (SD 41.9); HIV-uninfected, 13.0 (SD 17.6); $P < 0.001$]. The highest FIS scores observed in HIV-infected patients were comparable with those seen in CFS/ME patients (Fig. 1a). The following demographic and disease factors showed no significant association with fatigue severity (FIS score) in the HIV-infected group: gender [mean FIS (SD): male patients, 54.6 (41.8); female patients, 44.6 (43.2); $P = 0.79$], age ($r = 0.04$; $P = 0.70$), current or nadir CD4 lymphocyte count (current, $r = 0.04$; $P = 0.70$; nadir, $r = 0.05$; $P = 0.66$), detectable plasma HIV viral load [mean FIS (SD): viral load $\leq$ 40 copies/ml, 50.0 (43.1); viral load > 40, 54.8 (40.0);$P = 0.64$], or whether on HAART [mean FIS (SD): on HAART, 50.3 (42.0); off HAART, 58.9 (47.0); $P = 0.56$] (Table 1). Duration of diagnosed HIV infection ($r = 0.26$; $P = 0.010$), current treatment with non-efavirenz-based HAART [mean FIS (SD): no EFV, 59.0 (44.2); EFV, 40.2 (37.4);$P = 0.036$], prior dideoxynucleoside analogue (d-drug) exposure [mean FIS (SD): d-drug, 68.9 (39.9); no d-drug, 43.8 (41.1); $P = 0.016$; Fig. 1b], and the presence of clinically ascertained lipodystrophy syndrome (LDS) [mean FIS (SD): LDS, 76.5 (40.5); no LDS, 46.4 (41.2); $P = 0.011$] were all significantly associated with FIS score on univariate analysis. There was a nonsignificant trend towards an association of fatigue severity with current protease inhibitor (PI) use [mean FIS (SD): PI, 62.0 (42.9); no PI, 45.4 (41.0); $P = 0.09$]. None of these factors remained independently significantly associated with FIS on multivariate analysis, as a result of strong co-segregation of these exposures.

Fatigue and orthostatic intolerance

Symptoms of dysautonomia were common among HIV-infected patients, with 38 of 99 (38%) reporting significant orthostatic intolerance (OGS $\geq$ 4) and 12 (12.1%) with more severe symptoms, suggestive of orthostatic hypotension (OGS $\geq$ 9) (Schrezenmaier *et al.*, 2005). OGS and FIS scores showed a highly significant correlation ($r = 0.65$; $P < 0.001$; Fig. 1c). Mean OGS scores were significantly higher in HIV-infected patients compared with uninfected controls [3.57 (SD 3.70) *vs*. 1.25 (SD 1.47); $P < 0.001$], but lower than in CFS/ME patients [6.82 (SD 4.31); $P < 0.001$]. Both OGS ($P < 0.001$) and history of d-drug exposure ($P = 0.006$) remained significantly associated with FIS score in HIV-infected patients on multivariate analysis.

Discussion

Fatigue remains a very common and often severe symptom in HIV-infected patients. Prior to this study, very few of these patients had been identified clinically as having symptomatic fatigue and it is likely that this condition is significantly under-recognized. This surprisingly high level of fatigue was seen despite the vast majority of patients receiving suppressive HAART therapy, and no association was seen with routine markers of HIV disease (CD4 lymphocyte count or plasma viral load). This is in contrast to data from the pre-HAART era, where fatigue appeared to correlate with clinical disease stage, and suggests different aetiological factors may now be important (Darko *et al.*, 1992).

What may be driving HIV-associated fatigue in the HAART era? The pathogenesis of fatigue remains poorly understood, but is likely to be complex and involve both physiological and psychological factors. Our data suggest two novel physiological contributors. Firstly, fatigue severity was increased in patients with long-standing HIV infection, past d-drug exposure and LDS. As a result of the strong co-segregation of these factors, it was not possible to establish which factor was the most important predictor of fatigue in this group, but rather patients with these factors represent a subgroup of highly treatment-experienced HIV-infected patients. Given the established associations between nucleoside reverse transcriptase inhibitor (NRTI) exposure, LDS and acquired mitochondrial injury, it is certainly plausible that such patients show fatigue resulting from metabolic/mitochondrial dysfunction (Carr *et al.*, 2000; Zaera *et*

al., 2001; Payne *et al.*, 2011). Consistent with this notion, limited data from magnetic resonance spectroscopy (MRS) studies point to a role of muscle mitochondrial dysfunction in fatigue associated with CFS/ME and PBC (Hollingsworth *et al.*, 2010; Jones *et al.*, 2010). Secondly, we have shown that symptoms of dysautonomia (orthostatic intolerance) are independently associated with fatigue in HIV-infected patients. Recent evidence suggests that dysautonomia is a key biological driver of fatigue in several chronic diseases including multiple sclerosis (MS) and PBC, as well as CFS/ME (Freeman and Komaroff, 1997; Rowe and Calkins, 1998; Flachenecker *et al.*, 2003; Newton *et al.*, 2007a). Furthermore, fatigue is well described in vasovagal syncope (VVS), a primary dysautonomia syndrome (Legge *et al.*, 2008). Fuller understanding of the mediators of a causal relationship between fatigue and dysautonomia (for example, regulation of oxygen delivery to muscles) is the subject of ongoing research (Jones *et al.*, 2010).

None of the patients included in this study had untreated major depression, based on case note review, and explicit pathways exist in our unit for the management of depression. Nevertheless, it is well recognised that symptoms of depression are very common in HIV-infected patients and may be under-recognized (Asch *et al.*, 2003). Given the high prevalence of fatigue in this cohort in the absence of diagnosed depression, it seems unlikely that mood disorder is the prime mediator of fatigue in HIV-infected patients; however, as this is a correlative cross-sectional study it is not possible to draw firm conclusions regarding causality.

How might HIV-associated fatigue be approached clinically? The National Institute for Clinical Excellence (NICE) has recently recommended a model for assessment and treatment of CFS/ME in the UK (NICE, 2007). It is important that fatigue is more actively looked for in HIV-infected individuals, even when their HIV infection is effectively treated. Our data and those of others suggest that fatigue and associated symptomatology in CFS/ME is very similar to that seen in many chronic diseases including HIV infection (Jones *et al.*, 2009). Given our evolving understanding of fatigue associated with HIV infection and other chronic diseases, we suggest that a holistic symptom-based approach to management, similar to that proposed for CFS/ME, may therefore prove to be the most helpful (Jones *et al.*, 2008).

Figure 1.

(a) Distribution of Fatigue Impact Severity (FIS) scores in HIV-infected out-patients (HIV+), uninfected volunteers (HIV-), and chronic fatigue syndrome/myalgic encephalomyelitis (encephalopathy) (CFS/ME) out-patients. FIS ≥ 40 indicates excessive fatigue. (b) Distribution of FIS scores in HIV-infected patients according to the presence or absence of prior dideoxynucleoside analogue (d-drug) exposure. (c) Correlation between FIS and Orthostatic Grading Scale (OGS) scores in HIV-infected patients. OGS ≥ 4 indicates symptomatic orthostatic intolerance.

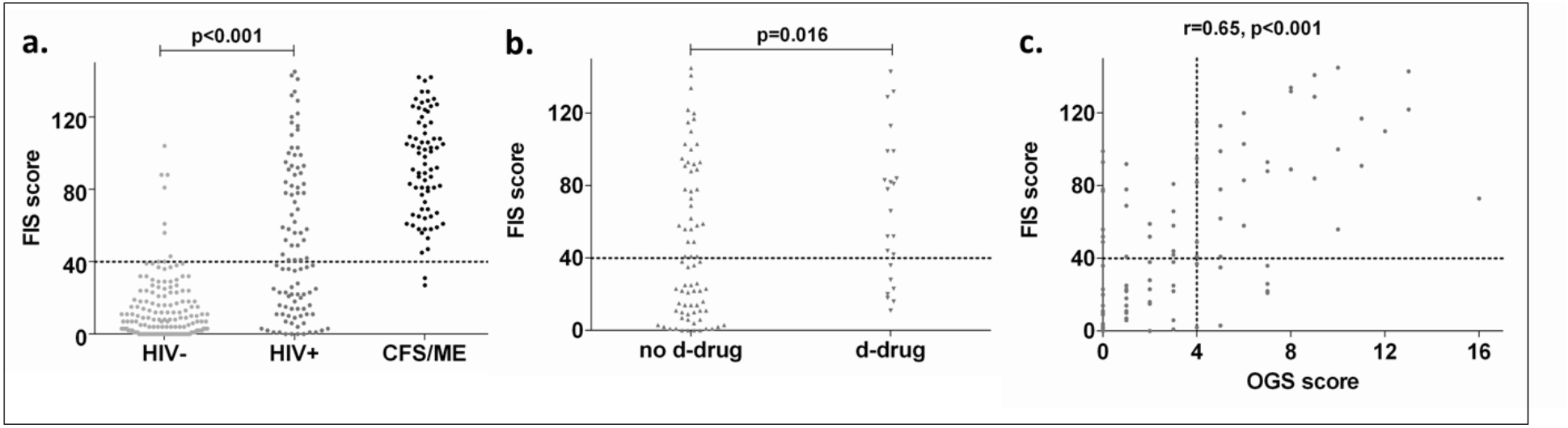

Table 1. Demographic, HIV and highly active antiretroviral therapy (HAART) factors associated with fatigue severity [Fatigue Impact Scale (FIS) score] in HIV-infected patients

Continuous variables		**Correlation, r, with FIS score**	**p value**
Age		0.04	0.70
Duration of diagnosed HIV infection		0.26	**0.010**
Current CD4 lymphocyte count		0.03	0.74
Nadir CD4 lymphocyte count		0.05	0.66
Binary variables		**Mean FIS (SD)**	**p value**
Gender	Male (n =63)	54.6 (41.8)	0.79
	Female (n =34)	44.6 (43.2)	
HIV VL	≤40 copies/ml (n =75)	50.0 (43.1)	0.64
	>40 copies/ml (n =22)	54.8 (40.0)	
Current HAART	On HAART (n =88)	50.3 (42.0)	0.56
	Off HAART (n =9)	58.9 (47.0)	
	No EFV (n = 47)	59.0 (44.2)	**0.036**
	EFV (n = 41)	40.2 (37.4)	
	Non PI-based (n = 62)	45.4 (41.0)	0.09
	PI-based (n = 26)	62.0 (42.9)	
Lifetime HAART	No d-drugs (n = 66)	43.8 (41.1)	**0.016**
	d-drugs (n = 22)	68.6 (39.9)	
Clinical LDS	No LDS (n = 82)	46.4 (41.2)	**0.011**
	LDS (n = 15)	76.5 (40.5)	

Significant p values are shown in bold. d-drugs, dideoxynucleoside analogues; EFV, efavirenz; LDS, lipodystrophy syndrome; PI, protease inhibitor; SD, standard deviation.

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