Muscle mitochondrial function and contemporary anti-retroviral therapy

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1. Clinical characteristics

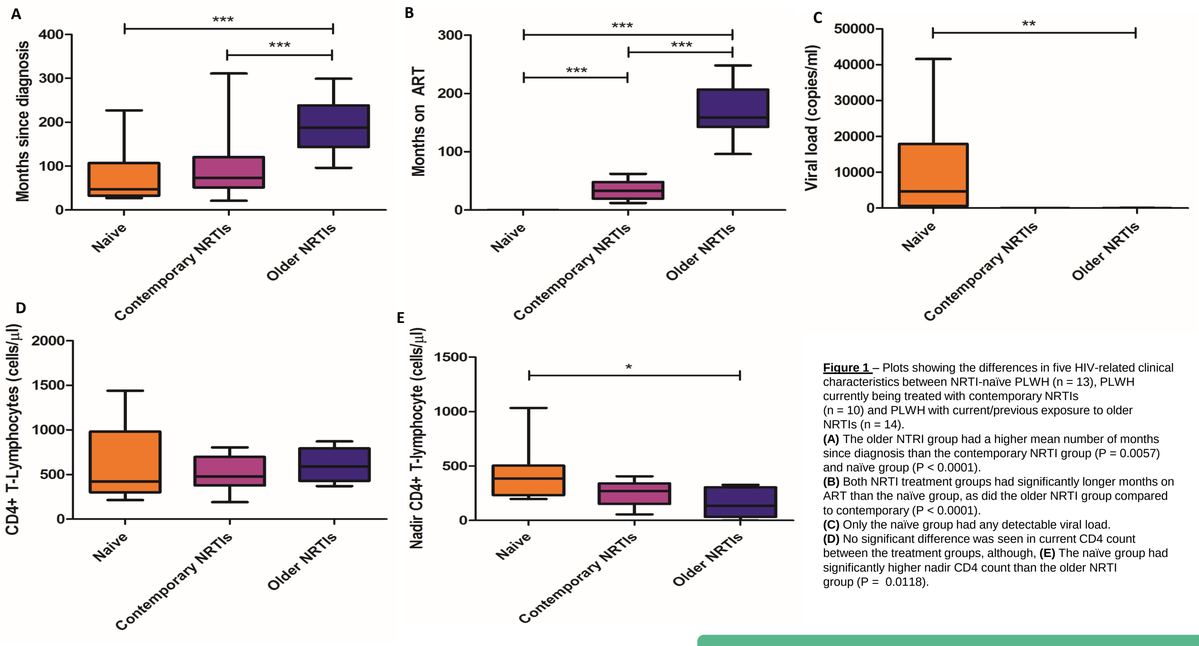

Figure 1 – Plots showing the differences in five HIV-related clinical characteristics between NRTI-naïve PLWH (n = 13), PLWH currently being treated with contemporary NRTIs (n = 10) and PLWH with current/previous exposure to older NRTIs (n = 14). **(A)** The older NTRI group had a higher mean number of months since diagnosis than the contemporary NRTI group (P = 0.0057) and naïve group (P < 0.0001). **(B)** Both NRTI treatment groups had significantly longer months on ART than the naïve group, as did the older NRTI group compared to contemporary (P < 0.0001). **(C)** Only the naïve group had any detectable viral load. **(D)** No significant difference was seen in current CD4 count between the treatment groups, although, **(E)** The naïve group had significantly higher nadir CD4 count than the older NRTI group (P = 0.0118).

	Naive	Older NRTIs	Contemporary NRTIs
n	13	14	10
Age (y)	36.9 ± 10.6	57.7 ± 8.7	48.4 ± 13.3
Months since HIV diagnosis	74 ± 58	193 ± 60	100 ± 86
Months on treatment	0	171 ± 42	34 ± 16
CD4 lymphocyte count (cells/µL)	634.7 ± 431	613.2 ± 179.1	512 ± 200.7
Nadir CD4 lymphocyte count (cells/µL)	414.6 ± 228.1	163.5 ± 132.2	249.6 ± 114.2
Viral load (copies/mL)	11533.1	<40	<40

Table 1 – HIV-related clinical characteristics of the subject population (values where stated are mean ± SD).

2. Multiplex immunofluorescence for assessing mitochondrial defects

- Multiplex immunofluorescence assay developed in our lab enables the quantification of mitochondrial respiratory chain complexes I and IV along with a mitochondrial mass marker and cell marker.
- Complex I (CI) was detected by using an antibody for accessory protein NDUFB8.
- Complex IV (CIV) was detected using antibody for mtDNA-encoded protein MTCO1.
- Mitochondrial mass was quantified using VDAC1 antibody for outer mitochondrial membrane channel porin, and laminin was used to label myofibres boundaries.
- Muscle fibres were classified into categories based on Z-scores of CI and CIV fluorescence intensity after normalisation against controls: 'severely deficient' (Z<-6SD); 'deficient' (Z between -3SD and -6SD) and 'normal' (Z>-3SD).

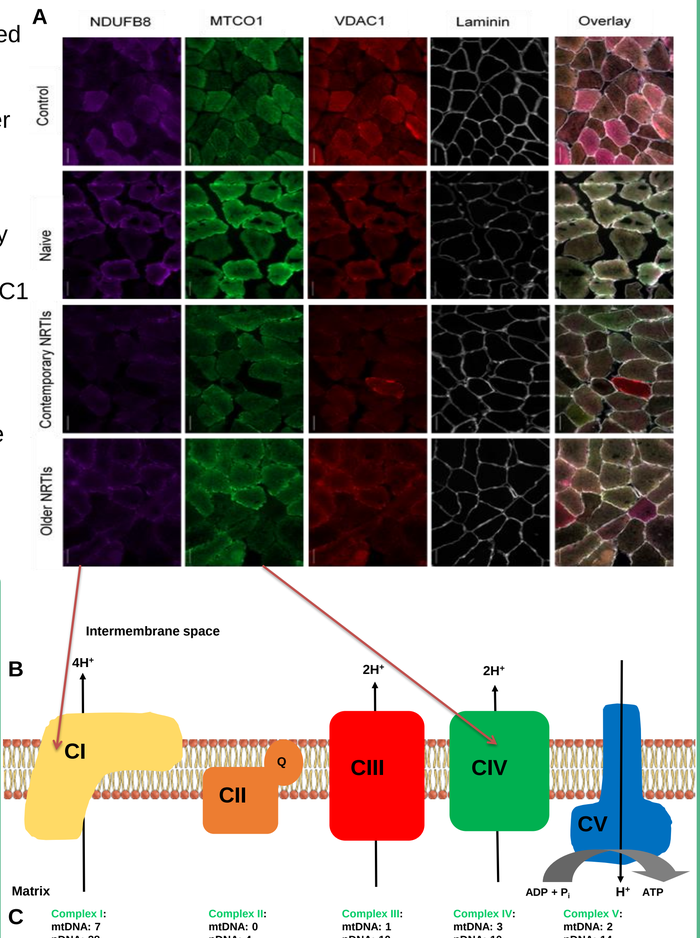

Figure 2 – **(A)** Fluorescence stained transverse skeletal muscle sections (x20). Multiplex immunofluorescence allows for the quantification of mitochondrial respiratory chain complex CI and CIV activity as well as mitochondrial mass. Sections from control (HIV-) patients, patients with no exposure to NRTIs (naïve), patients with exposure to contemporary NRTIs and patients with current/previous exposure to older NRTIs were stained for NDUFB8 (complex I) – purple; MTCO1 (complex IV) – green; VDAC (porin/mitochondrial mass) – red; laminin (myofibres boundary marker). **(B)** Overview of the oxidative phosphorylation process. Electrons are pumped through complexes I, III and IV, generating an electrochemical gradient that is harnessed by CV (ATP synthase) to drive the production of ATP. The arrows indicate which complexes are stained for by the multiplex immunofluorescence assay. **(C)** Indication of how many nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) encoded genes are required for the formation of each respective complex.

Background

- Anti-retroviral therapy (ART) eliminates viral replication and restores immune function BUT it may be associated with premature molecular ageing.
- In particular, older nucleoside reverse-transcriptase inhibitors (NRTIs) cause dysregulation of mitochondrial maintenance, by inhibiting mitochondrial polymerase-γ leading to the clonal expansion of pre-existing mitochondrial DNA (mtDNA) mutations.
- Mitochondrial defects contribute to premature ageing in ART-treated patients, increasing frailty and the susceptibility to acquiring age-associated comorbidities

Aims

Using a cohort of 37 people living with HIV (PLWH) - 13 untreated; 10 treated with contemporary NRTIs (TDF, ABC, 3TC, FTC); 14 currently using contemporary NRTIs but previously treated with older NRTIs (AZT, ddC, ddI, d4T) - we aim to better characterise mitochondrial defects in skeletal muscle of PLWH and provide a link between age-associated mitochondrial defects and clinical HIV characteristics.

Methods

Tibialis anterior biopsies were obtained, in which a range of molecular assessments were performed on 10µm transverse sections. These include:

- COX/SDH immunohistochemistry (IHC).
- Multiplex immunofluorescence for mitochondrial mass and respiratory chain complexes I and IV, with automated analysis.

Summary

- **Patients exposed to older NRTIs have the highest levels of mitochondrial defects in skeletal muscle, despite no longer being treated with these medications.**
- **Surprisingly, patients exposed only to contemporary ART had intermediate levels of mitochondrial defects. Further work is needed to define the mechanisms behind this.**
- **Mitochondrial defects predominantly affected complex I, which could be of relevance for future novel therapeutic interventions.**

3. CI and CIV deficiency in NRTI treated individuals

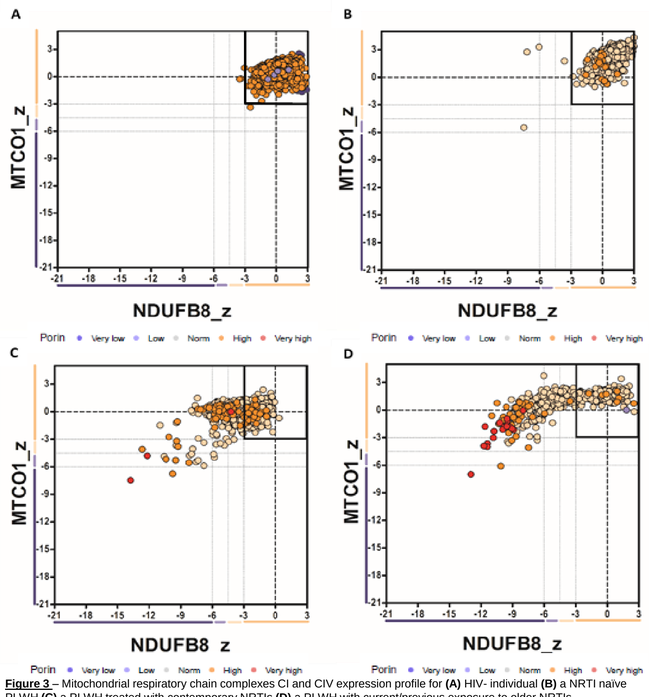

Figure 3 – Mitochondrial respiratory chain complexes CI and CIV expression profile for **(A)** HIV- individual **(B)** a NRTI naïve PLWH **(C)** a PLWH treated with contemporary NRTIs **(D)** a PLWH with current/previous exposure to older NRTIs.

Mitochondrial defect	ART group	Mean log_{10} defect (SD)	p value
CI (z<-3) 'Deficient'	Naive	-3.09 (1.31)	-
	Contemporary NRTI	-1.91 (1.46)	0.05
	Old NRTI	-1.93 (0.74)	0.01
CI (z<-6) 'Severely deficient'	Naive	-3.89 (0.39)	-
	Contemporary NRTI	-3.28 (0.96)	0.08
	Old NRTI	-2.68 (0.92)	<0.0001
CIV (z<-3) 'Deficient'	Naive	-3.24 (0.55)	-
	Contemporary NRTI	-3.13 (0.73)	NS
	Old NRTI	-2.52 (0.64)	0.004
CIV (z<-6) 'Severely deficient'	Naive	-4.00 (0.00)	-
	Contemporary NRTI	-3.83 (0.39)	NS
	Old NRTI	-3.57 (0.68)	0.04

Table 2 - Log transformed percentage of CI and CIV deficiency and severe deficiency in the three treatment groups.

- CI deficiency and severe deficiency is significantly higher in both NRTI-treated groups compared to the NRTI-naïve group.
- No significant difference in CI deficiency and severe deficiency between NRTI-treatment groups.
- Subjects exposed to older NRTIs had significantly higher CIV deficiency (and severe deficiency) than NRTI-naïve subjects, unlike subjects in the contemporary NRTI group.

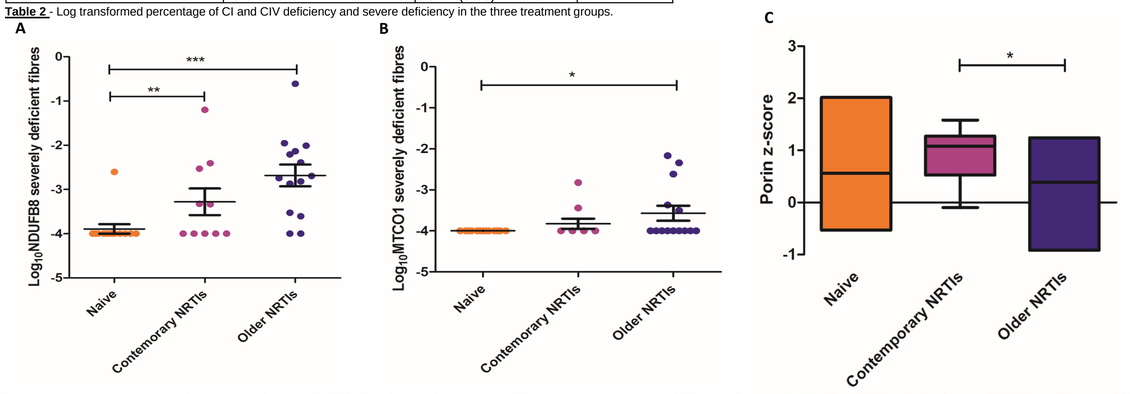

Figure 4 – **(A)** plot showing log transformed levels of severe NDUFB8 (CI) deficiency. Both the older NRTI (n = 14) and contemporary NRTI (n = 10) groups had significantly higher levels of severe CI deficiency (Z-score > -6SD) than the naïve group (n = 13). **(B)** plot showing log transformed levels of MTCO1 (CIV) severe deficiency. The older NTRI group but not the contemporary NRTI group had significantly higher levels than the naïve group. **(C)** plot showing porin Z-scores for the three groups. All three groups have a 'normal' (-2SD to +2SD) mean Z-score (naïve – 0.56; contemporary NRTI – 0.91; older NRTI – 0.39). All data was log transformed as to correct skew.

4. Correlation between mitochondrial deficiency and clinical characteristics

- Correlation between severe CI/CIV deficiency and COX defect. This validates the reliability of the multiplex assay as COX/SDH IHC is an established and comprehensively validated tool for assessing mitochondrial deficiency.
- Association between severe CI deficiency and months on ART, but not severe CIV deficiency.
- Association between severe CI deficiency and age, but not severe CIV deficiency and age.
- No association between mitochondrial deficiency and current CD4 count, nadir CD4 count or months since diagnosis.

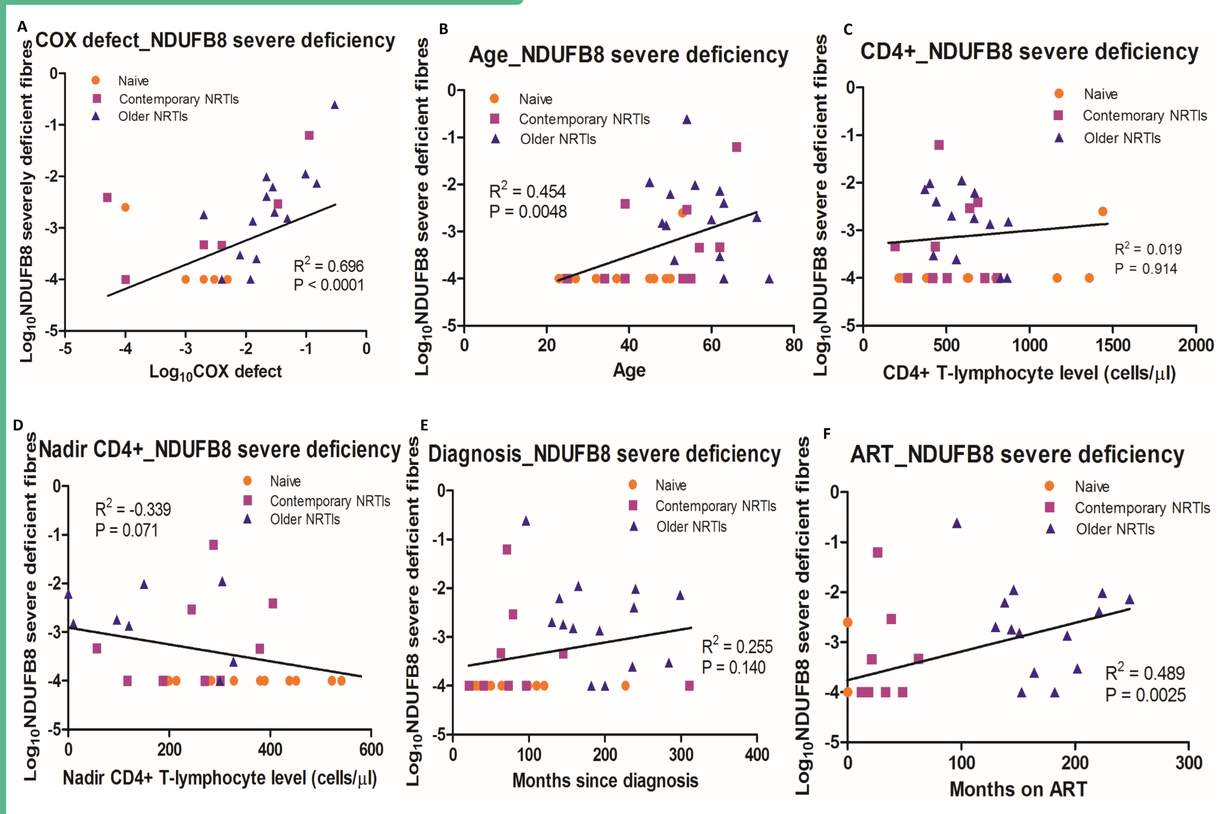

Figure 5 – Linear regression plots showing the correlation between severe CI (NDUFB8) deficiency and HIV-related clinical assessments. As severe NDUFB8 deficiency was more pronounced than severe MTCO1 deficiency, and due to the fact that severe CI and severe CIV deficiency have a significant correlation themselves (R2 = 0.680, P <0.0001), only plots for NDUFB8 are included. **(A)** COX defect level from COX/SHD IHC associated with severe NDUFB8 deficiency (R2 = 0.696, P <0.0001). **(B)** Severe NDUFB8 deficiency correlates to increasing age (R2 = 0.454, P = 0.0048). **(C)** Severe NDUFB8 deficiency has no association with current CD4 lymphocyte count (R2 = 0.019, P = 0.914) or **(D)** nadir CD4 lymphocyte count (R2 = -0.339, P = 0.071). **(E)** Months since diagnosis was not associated with severe NDFUB8 deficiency (R2 = 0.255, P = 0.140), although **(F)** months on ART was (R2 = 0.489, P = 0.0025).

Future work

Further characterisation of mitochondrial function and dynamics, which could include:

- Quantifying mtDNA and mtRNA levels in muscle fibres with mitochondrial defects;
- Quantifying oxidative stress/reactive oxygen species levels;
- Telomere and TFAM quantification;
- Characterising inflammatory markers and their gene expression;
- Multiplex immunofluorescence for complexes III and V.

References

1. Rocha MC *et al.* (2015) – A novel immunofluorescent assay to investigate oxidative phosphorylation deficiency in mitochondrial myopathy: understanding mechanisms and improving diagnosis. *Scientific Reports* **5.** 1-17.
2. Payne BAI *et al*. (2011) – Mitochondrial ageing is accelerated by anti-retroviral therapy through the clonal expansion of mtDNA mutations. *Nature Genetics* **43.** 806-810.
3. Payne BAI *et al*. (2015) - Clinical and pathological features of mitochondrial DNA deletion disease following anti-retroviral treatment. *JAMA Neurology* **72(5).** 603-605.