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Reply to : Proofreading deficiency in mitochondrial DNA
polymerase does not affect total dNTP pools in mouse embryos

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2020-08

Hämäläinen , R H , Landoni , J C , Ahlqvist , K J , Goffart , S , Ryytty , S , Rahman , M O ,
Brilhante , V , Icaý , K , Hautaniemi , S , Wang , L , Laiho , M & Suomalainen , A 2020 , '
Reply to : Proofreading deficiency in mitochondrial DNA polymerase does not affect total
dNTP pools in mouse embryos ' , Nature metabolism , vol. 2 , no. 8 , pp. 676-677 . <https://doi.org/10.1038/s42255-020-0265-y>

<http://hdl.handle.net/10138/326256>

<https://doi.org/10.1038/s42255-020-0265-y>

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Reply to correspondence by Sharma S, Koolmeister C, Tran P, Nilsson AK, Larsson NG and Chabes A.

Progeric mitochondrial “mtDNA mutator” mice manifest nuclear genome instability

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Reply to Sharma S, Koolmeister C, Tran P, Nilsson AK, Larsson NG and Chabes A.

In their correspondence related to our recent article about the mechanism underlying the progeria phenotype in mitochondrial DNA (mtDNA) mutator mice, Sharma *et al.* raise technical concerns about our methodology for dNTP analysis. These mice have been considered as an important *in vivo* evidence for the link between mtDNA mutagenesis and ageing-like symptoms^{1,2}. Our evidence proposes that mtDNA mutagenesis is not the sole explanation of progeric features in these mice, and propose a unifying mechanism for mouse progeroid models based on nuclear DNA damage. Importantly, Sharma *et al.* do not challenge this conclusion.

The concern of Sharma *et al.* is directed at our quantification of cellular dNTP levels, yet the authors do not use similar study material for their comparison. Instead of using stem cells or analysing whole-cellular vs mitochondrial dNTP pools as we did in our article, they analysed whole E13.5 embryos from mutator, SAMHD1 homozygous knockout mice and wildtype mice. Using an HPLC-based method, they detect a dNTP concentration change between wildtype and SAMHD1-KO mice, but do not see a difference between wildtype and mutator embryos. Embryos are heterogeneous and organized systems of proliferating and differentiating cells, and it is known that dNTP pools are tightly regulated in a cell-type, developmental stage and cell cycle-dependent fashion, differing even among proliferating cell types^{4,5}. In the literature, dNTP analysis from mouse embryos is typically performed using mouse embryonic fibroblasts (MEFs), to increase the homogeneity of the sample. Indeed, we detected and reported similar changes in proliferative mutator MEFs as in stem cells. In conclusion, mutator mice have been robustly shown to have an adult somatic stem/proliferating progenitor cell defect^{6–9}. We studied this specific cell type and report remodelled dNTP pools, with imbalance between cytoplasmic and mitochondrial pools. We believe that to truthfully test our findings in two cellular compartments, in specific cell populations, both dNTP pools should be studied from a more homogeneous and relevant cell population.

Sharma *et al.* criticise our polymerase-based dNTP analysis methodology, yet do not use a similar method for comparison. Instead, they applied HPLC-based separation of total dNTP pools of the embryo coupled with UV detection. HPLC and polymerase assays have been compared with each other before, as they have been used for the past 30-40 years for nucleotide analyses¹⁰. The polymerase-based assays originally suffered from NTP incorporation when the Klenow fragment was utilised a decade ago, but as described by Ferraro *et al.*¹¹. However, this issue can be circumvented by using a highly specific *Taq*-

polymerase, as we did in our study, and which provides a specificity similar to HPLC but with an increased sensitivity for the analysis of low dNTP concentrations¹¹. The dNTP levels we report are in the same range as reported by others in the literatures⁵. As for an internal standard, we have here included evidence on the performance of the polymerase-based method measuring especially low dNTP concentrations from murine muscle with and without standard spiking (Table 1).

Our methodology was developed to minimise the number of processing steps to increase accuracy¹². Every handling step added to an extraction protocol reduces the quantitative properties of an assay, and rapid processing is especially important for unstable metabolites such as dNTPs. HPLC analysis requires a multi-step chemical processing of nucleotides. Our method is preceded by a one-step extraction method with – 20°C cold methanol (widely used in quantitative metabolite analysis) and all reactions and washing steps are performed in a single well in solid-phase format, minimising the loss of sample. Standards and replicates are run in parallel to the samples in a multi-plate format, minimising variability and ensuring linearity. Furthermore, we have observed measurable differences from overnight storage of snap-frozen tissues in – 80°C, so we emphasize the importance of samples to be compared are collected in parallel, and storage time minimized. These aspects were not evident from the Sharma *et al.* response.

Solid-phase minisequencing assays with radioactively labelled nucleotides have been widely used for DNA diagnosis and provide high sensitivity¹³. While both HPLC and DNA polymerase-based methodologies have their strengths and weaknesses, the polymerase-based methods are the state-of-the-art to analyse samples with low dNTP concentrations such as tissues and mitochondria^{11,14,15}. HPLC methods lack sensitivity and their required tissue material is higher, and therefore could not be used for the measurement of mitochondrial dNTP pools from cell culture samples, a fundamental measure in our report.

We thank Sharma *et al.* for noticing a typo in a figure of the paper, which has now been properly corrected. Regarding the intercellular differences in dNTP concentrations, the standard normalisation factor used is cell number, and the differences in cytosolic volume of the different cell types affect the absolute amount of nucleotides. Furthermore, as reported in the literature^{4,5,12}, cellular dNTP balance is tightly regulated in a cell-type and cell-cycle specific manner, varying according to nuclear and mitochondrial replication rates as well as other factors. Our results indicate consistent regulation of cellular dNTP compartments purely explained by the genotype of the cells. No systematically flawed methodology would enable such cell-type and genotype-specific significant observations to be made.

We wish to thank Sharma *et al.* for their interest in our data and the discussion they raised. However, as their results are produced using a different source material, analyzing total embryonal dNTP pools instead of those in specific cell compartments, and employing different methodology, we find no evidence that their results would question the quality of our original methodology or results. The effects of mtDNA replication on dNTP balance and genome stability are an extremely important topic and continued mechanistic studies are warranted. We look forward to future discoveries and are open to collaborate and share our materials for the generation of comparable data.

Author contributions

R.H.H, J.C.L, K.J.A, S.G, S.R, M.O.R, V.B, K.I, S.H, L.W, M.L, A.S wrote the manuscript. L.W performed experimental work and data analysis.

Competing interests

Authors declare no competing interests.

Methods

Nucleotides were extracted and analysed by using the Taq-polymerase-based method as previously described¹². Briefly, mouse quadriceps femoris muscle were mechanically homogenised and nucleotides were extracted with cold methanol. The extracts in the absence or presence of dNTP standards were added to a reaction mixture containing 40 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.25 µM of the specific primed oligonucleotide and 0.75 µM 3H-dTTP (for dATP, dCTP and dGTP measurement) or 3H-dATP (for dTTP measurement), and 0.30 unit of Taq DNA polymerase. The reaction mixtures were incubated at 48°C for 60 min, and then cooled down on ice. After brief centrifugation the mixture was spotted onto DE-81 filter paper and dried. The filters were washed three time in 5% Na₂HPO₄, once in water and one in 95% ethanol and the radioactivity was quantified by liquid scintillation counting. All animal experiments were approved by the National Animal Review Board and the Regional State Administrative Agency for Southern Finland.

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Table 1. dNTP levels in mouse tissue samples, measure in the absence and presence of standard dNTPs. The results are given as mean $\pm$ SD in fmol/mg tissue. QF, quadriceps femoris muscle. NS, no standard; St+ quantified against a standard dNTP. ND, not detectable.

Sample	Tissue weight (mg)	dATP		dTTP		dGTP		dCTP	
		NS	St+	NS	St+	NS	St+	NS	St+
QF male 1 + dNTP (6.2 fmol/mg)	95.7	ND	6.1 $\pm$ 0.2	49.8 $\pm$ 4.1	57.2 $\pm$ 1.7	43.8 $\pm$ 2.2	50.5 $\pm$ 2.0	16.4 $\pm$ 1.3	22.8 $\pm$ 1.5
QF male 2 + dNTP (4.8 fmol/mg)	125	ND	4.7 $\pm$ 0.2	36.3 $\pm$ 11,5	42.2 $\pm$ 1.9	12.0 $\pm$ 0.2	16.5 $\pm$ 0.5	7.0 $\pm$ 0.7	12.0 $\pm$ 1.0