Age-related and heteroplasmy-related variation in human mtDNA copy number

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Abstract

The mitochondrial (mt) genome is present in several copies in human cells, and intra-individual variation in mtDNA sequences is known as heteroplasmy. A recent study found that heteroplasmies were highly tissue-specific, site-specific, and allele-specific, suggesting that positive selection is acting on such heteroplasmies; however the functional implications have not been explored. This study investigates variation in mtDNA copy numbers (mtCN) in 12 different tissues obtained at autopsy from 152 individuals (ranging in age from 3 days to 96 years). Three different methods to estimate mtCN were compared: shotgun sequencing, capture-enriched sequencing and droplet digital PCR (ddPCR). The highest precision in mtCN estimation was achieved using shotgun sequencing data. However, capture-enrichment data provide reliable estimates of relative (albeit not absolute) mtCNs. Comparisons of mtCN from different tissues of the same individual revealed that mtCNs in different tissues are, with few exceptions, uncorrelated. Hence, each tissue of an individual seems to regulate mtCN in a tissue-related rather than an individual-dependent manner. Skeletal muscle (SM) samples showed an age-related decrease in mtCN that was especially pronounced in males, while there was an age-related increase in mtCN for liver (LIV) samples. MtCN in SM samples was significantly negatively correlated with both the total number of heteroplasmic sites and with minor allele frequency (MAF) at two heteroplasmic sites, 408 and 16327. Heteroplasmies at both sites are highly specific for SM, occur in more than 40 % of the individuals older than 50 years (with MAF up to 28.2 %), and are part of functional elements that regulate mtDNA replication. We hypothesize that positive selection acting on these heteroplasmic sites is reducing mtCN in SM of older individuals.

Introduction

Mitochondria are the central cellular structures for energy production. While most human mt proteins are encoded in the nuclear genome and imported to the mitochondrion following translation, mitochondria also contain their own genome [1]. In addition to tRNA and rRNA genes, the mt genome harbors 13 genes encoding proteins of the respiratory chain. The human mt genome is usually present in several copies per mitochondrion and the total number of mtDNA copies per cell varies greatly between different individuals and different tissues of the same individual [2, 3]. For example, myocardial muscle cells contain on average more than 6,000 copies per cell [4], while leukocytes have around 350 mtDNA copies per cell [5].

Replication and degradation of mtDNA are coupled in order to keep mtDNA levels constant in a cell [3]. Changes in mtCN have been correlated with several diseases and factors that influence mtCN have been described [3, 6]. These are for example replication regulating proteins such as the single-stranded-DNA-binding protein mtSSB, which is stimulating mitochondrial DNA polymerase ɣ and therefore increases replication rate [6]. In addition, overexpression of mitochondrial RNA polymerase and its mitochondrial transcription factor A (TFAM) enhance replication by increased synthesis of primers for replication [6]. A reduction of TFAM in heterozygous knockout mice showed a strong reduction in mtCN and a total knockout resulted in a total lack of mtDNA and lethality [7].

However, it is still not known how inter-individual differences arise in mtCN, to what extent such inter-individual differences in mtCN are correlated across different tissues of an individual, and what effect these natural differences might have on healthy individuals. Several studies have examined changes in mtCN with age; while some detected a decrease of copy number with increasing age in healthy individuals [8-10],

others failed to identify age-dependent changes in copy number [4, 11] or showed an increase for certain tissues [12]. Thus, there is currently much uncertainty concerning the role of age and other factors on mtCN.

Copy number is usually estimated by quantitative PCR methods [4, 10], although some studies have utilized shotgun sequencing data [9, 13-15]. However, comparisons of different methods for estimating mtCN are rarely done [9] and moreover most studies have focused on a single tissue. Here, we analyze mtCN in 12 different tissues that were obtained at autopsy from 152 individuals. We compared three different methods for estimating mtCN: ddPCR [16]; shotgun sequencing; and capture-enriched sequencing. Furthermore, we examined the influence of various features such as age, haplogroup and sex on mtCN.

We also inquired whether mtCN can be linked to mtDNA heteroplasmy, i.e. intra-individual variability in the mtDNA sequence. All samples in this study were investigated previously for heteroplasmy [17]. This previous study revealed that low-frequency heteroplasmies accumulate in all tissues investigated in healthy humans during aging. Furthermore, evidence was found for tissue-specific positive selection for certain heteroplasmic sites [17]. These particular sites are found exclusively in the control region of the mitochondrial genome, which is essential for replication and transcription initiation and regulation [18, 19]. It therefore seems likely that heteroplasmic mutations in the control region could have an influence on mtCN, and indeed we report here that two heteroplasmic sites that are common in skeletal muscle are associated with a decrease in mtCN.

Materials and Methods

Tissue collection and DNA extraction

Twelve different tissues (blood: BL; cerebellum: CEL; cerebrum: CER; cortex: CO; kidney: KI; large intestine: LI; liver: LIV myocardial muscle: MM; ovary: OV; small intestine: SI; skin: SK; and skeletal muscle: SM) were obtained at autopsy from 152 human individuals (85 males, 67 females) and DNA was extracted as previously described [17]. The collection of samples and the experimental procedures were approved by the Ethics Commissions of the Rheinische Friedrich Wilhelm University Medical Faculty and of the University of Leipzig Medical Faculty.

MtDNA quantification using shotgun sequencing data and capture enriched data

Libraries for sequencing were prepared as previously described [17]. Shotgun sequencing (without mtDNA enrichment) was performed on an Illumina HiSeq platform using 95 base pair, paired-end reads. Either 611 libraries (with samples from BL, SM, LIV and MM tissue; referred to as shotgun sequencing run 1) or 309 individual DNA libraries (with BL and SM tissue samples; referred to as shotgun sequencing run 2) were pooled. Reads of at least 50 bp length were mapped against the human reference genome 19 (hg19) using BWA [20] without filtering for high mapping quality. All reads that aligned successfully, i.e. both mates of a pair mapped to the same chromosome and within a distance to each other of at most 3 standard deviations of the insert size distribution, were analyzed and the length of their insert sizes summed up per chromosome. For each chromosome the number of mappable bases was determined by removing any poly-N stretch in the hg19 (> 5 consecutive Ns) and counting the number of remaining bases. The number of aligned bases of each

chromosome was divided by the number of mappable bases on this chromosome to obtain the coverage per chromosome and sample.

Samples were subsequently removed from the data set when either of the following criteria was fulfilled: 1) the number of bases aligning to mtDNA was ≤ 60% of the total mtDNA length; 2) the logarithmic normalized SD of the autosomal coverage was identified by a Grubbs test [21] as an outlier.

For each tissue the standard deviation of the coverage of each autosome was determined over all remaining samples. Chromosomes that were identified as an outlier by a Grubbs test based on their SD in 50% of the tissues were not considered in the mtCN calculation. The mtCN was determined by the following formula [9]:

$$mtCN = \frac{2 * mtDNA\ coverage}{\frac{1}{22}\sum_{i=1}^{22} autosomal\ coverage} \quad (1)$$

In order to account for the difference in absolute mtCN per tissue, the SDs were normalized by the mean autosomal coverage of a sample.

In a previous study, Illumina sequencing had been performed after capture-enrichment for mtDNA [17]. For mtCN estimation from capture-enriched sequencing, data were processed as described above for shotgun sequencing data.

MtDNA quantification using ddPCR

The mtCN from BL and SM samples was determined using ddPCR [16], a quantitative PCR method that allows an absolute measurement of the number of target DNA molecules. A 20 µl PCR mix was prepared and dispersed into up to 20,000 droplets. After amplification, droplets that included template DNA were identified by fluorescent dyes and counted. Specific primers (S1 Table) for amplification of mt and nDNA

regions were modified according to [10]. For determination of mtCN in BL, mtDNA and nDNA were amplified in the same multiplex reaction using HEX- and FAM-labeled probes for n and mtDNA, respectively. Reactions were performed in ddPCR Supermix including primers, probes and 1 ng template according to manufacturer's instructions. Due to the high mtCN in SM, determination of nDNA and mtDNA in SM samples was performed in two separate reactions with 10 ng template for nDNA and 20 pg template for mtDNA and either nuclear or mt specific primers in EvaGreen Supermix. To control for differences between DNA preparations, at least 12 DNA samples per tissue type were extracted twice and the mtCN of different preparations was compared. MtCN was calculated after correction for dilution factors by

$$mtCN = \frac{2 * mtDNA\ counts}{nDNA\ counts} \quad (2)$$

For each sample the arithmetic mean and the standard deviation (SD) of mtCN was calculated over all replicates. To account for the separate ddPCR reactions for nDNA and mtDNA measurement per SM sample, the SDs were averaged over the individual SDs of the SM samples. To compare the SDs between BL samples and SM samples, the SD was normalized by the mean mtCN of each sample. Both the arithmetic mean and the SD were subsequently averaged over all samples of a tissue type.

Estimating the likelihood of detecting NUMTs

In order to estimate the likelihood that recent mtDNA insertions into the nuclear genome (NUMTs) could artificially increase the mtCN estimates from sequencing data, we analyzed the length distribution of identified NUMTs from a NUMT database [22] and compared it to the insert size distribution in our data set. When paired-end reads overlapped, the length of the merged read was defined as the insert size [23]. If reads

were too far apart to be merged, the length of the region flanked by the two reads was defined as insert size. A read from a NUMT can only be aligned falsely with high quality to mtDNA when the read does not contain a flanking nuclear DNA signature of at least a few high-quality bases. For each insert size, all NUMTs longer than the insert were extracted from the published list as those inserts could potentially fully fall in a NUMT region. The probability of false alignment to the human mtDNA sequence was then estimated, taking into account the number of putative read insert positions within a NUMT, the number of NUMTs in the human genome that were larger than each read insert size, and the average coverage of all autosomes in each sequencing run, as follows:

$$probability\ p = \sum_{i=1}^{M} \frac{i * \sum_{j=1}^{N} j * c}{l},$$

$$with\ i = insert\ size, M = maximal\ insert\ size,$$

$$j = numt\ length, N = max.NUMT\ length$$

$$c = average\ coverage\ autosomes$$

$$l = length\ of\ the\ mappable\ genome$$

(3)

Correlation of mtCN with individual parameters and with heteroplasmy

The mtCN values for each tissue were log-transformed to produce a normal distribution of the residuals for comparison with other parameters, e.g. age, sex, haplogroup, etc. An underlying normal distribution of the residuals is a requirement for many statistical tests (including the linear regression models used here) and was therefore formally tested for every tissue using a Shapiro-Wilk test. In order to guarantee a normal distribution, one extreme LIV-outlier had to be excluded from the shotgun sequencing data sets prior to further investigations. Linear regression and

Pearson correlation analysis as implemented in R [24] was performed and the resulting p-values were corrected for multiple testing using the Benjamini-Hochberg method [25]. Partial regression analysis was used to investigate the combined effect of age and heteroplasmy levels on mtCN [26]. When performing linear regression analysis between MAF of specific heteroplasmies and mtCN, only heteroplasmies that were present in at least 10 individuals were considered. We tested for correlations of mtCN with single heteroplasmic sites, pairs of sites occurring in the same individual, and triplets of heteroplasmic sites, using linear regression and Pearson correlation analysis as described above.

Results

Reproducibility of mtCN estimation

For all analyses, tissue samples that had been collected at autopsy from 152 individuals and 12 different tissues in a previous study [17] were used. MtCN was estimated using shotgun sequencing, capture-enrichment sequencing, and ddPCR. The first two approaches are based on sequencing data and mtCN is estimated by dividing the number of bases that map to the mtDNA by the number of bases mapped to the nuclear genome. In order to estimate reproducibility, variation in coverage across the different autosomes was measured and chromosomes with high variation (as identified by the Grubbs outlier test) were removed. The mtCNs were calculated from shotgun sequencing data for BL, LIV, MM and SM samples. Chromosomes 16 and 19 were excluded from the mtCN calculation in this data set as the SD of their coverage was enhanced in at least 50 % of the tissues according to the Grubbs test (S1 Fig). In addition, for all 12 tissues from all individuals, mtCNs were estimated using capture-enriched sequencing data. Here, sequencing libraries had been enriched for mtDNA prior to sequencing [17], and so mtCN estimates will be elevated in the capture-enriched data; however, relative mtCN estimates might be the same, and so allow for comparisons between individuals. According to the Grubbs outlier test, SD values for the coverage of chromosomes 1, 16 and 19 were enhanced and therefore these chromosomes were not included in mtCN calculations from capture-enriched sequences (S1 Fig).

MtDNA insertions into the nuclear genome (NUMTs) could potentially artificially increase mtCNs calculated from sequencing data if reads deriving from recently-inserted NUMTs are falsely aligned to mtDNA. With currently used alignment tools, NUMTS that have been in the nuclear genome for a long time are identified as they

have accumulated mutations that distinguish them from authentic mtDNA sequences. Only NUMTs that have inserted into the nuclear genome rather recently have not had enough time for distinguishing mutations to occur. As we are therefore not able to directly identify reads arising from recent NUMTs, we instead estimate the probability of incorrectly attributing a NUMT read to the mtDNA genome, using the length distribution of a previously published list of identified NUMTs [22]. For each read insert length, all NUMTs longer than that length were identified as NUMTS from which reads could be generated that could be fully placed within the NUMT. The chance that a read from a NUMT was falsely aligned to the mtDNA genome, given the observed read distribution, was ≤0.06%, and therefore NUMTS were considered to have a negligible impact on mtCN estimation.

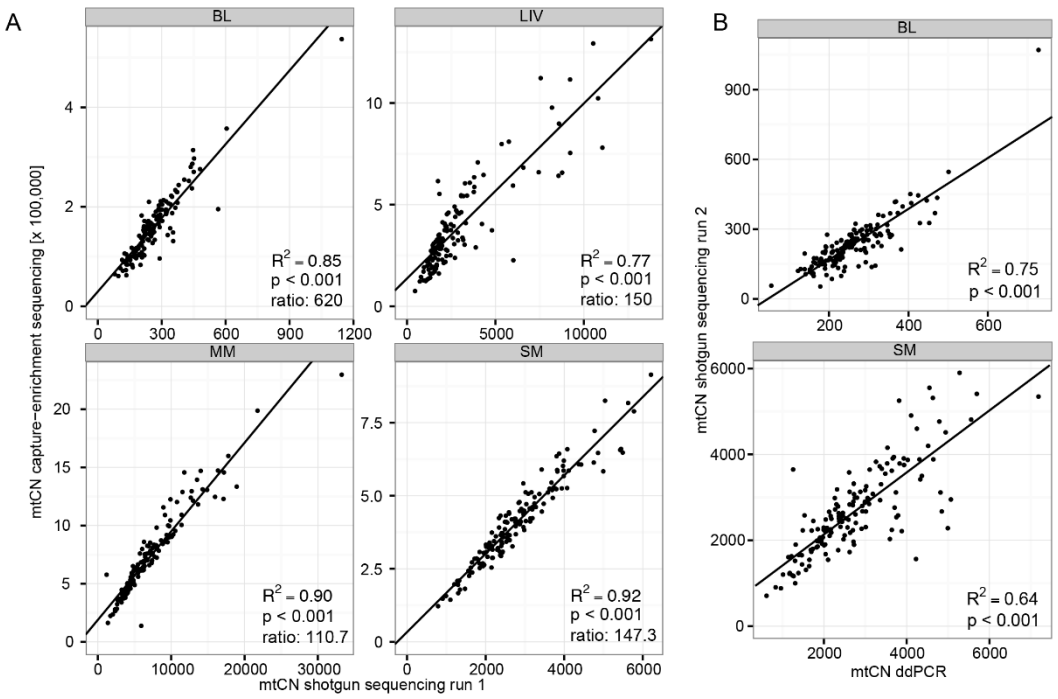

Fig 1: Comparison of mtCN estimates obtained with different methods. Outliers excluded. R^2 is the proportion of the variance explained in a linear regression analysis and p is the significance level of the Pearson correlation. (A) MtCN estimates from shotgun sequencing for four tissues (BL, LIV, MM, and SM) vs. those obtained after capture-enrichment. "Ratio" is the amount of enrichment after capture-enrichment, calculated as the average mtCN obtained from capture-enrichment sequencing divided by the average mtCN obtained from shotgun sequencing. (B) MtCN estimates from ddPCR for BL and SM vs. those obtained by shotgun sequencing.

We next evaluated the reproducibility of the estimated mtCNs for the two sequencing methods. To estimate standard deviations (SDs) of the methods, intra-individual differences in the chromosomal coverage were evaluated, and the resulting SD estimates were averaged over each tissue and normalized by the average mtCN for the tissue. The shotgun sequencing method returned normalized SD values for chromosomal coverage of <3.2 %, while the SDs of mtCN estimates from capture-enrichment were between 8.6 and 18.2 % (average: 12.6%) (Table 1), indicating more variation in chromosomal coverage of the capture-enriched data (S1 Fig). As both shotgun and capture-enrichment data were available for four tissues (BL, SM, LIV, and MM), we further investigated the reliability of mtCN estimates from capture-enrichment by testing if mtCN estimates from both methods were correlated. While there was a greater enrichment in mtCN for BL than for the other tissues, for all four tissues there was a significant correlation between mtCN values estimated via shotgun sequencing vs. capture-enriched sequencing (Fig 1A). Hence, mtCN estimates based on capture-enrichment sequencing data were considered suitable and used for further investigation into factors influencing mtCNs.

In a third approach, mtCN was estimated by ddPCR in SM and BL, with each sample measured at least 3 times and the SD calculated over all replicates per sample. Using ddPCR the normalized SD was 10-13% for replicates (Table 1). The estimated mtCN values from ddPCR vs. shotgun sequencing of the same samples were significantly correlated (BL samples: Pearson correlation $p<0.001$, $R^2=0.75$; SM samples: $p=$ $p<0.001$, $R^2=0.64$, Fig 1B) and the mean mtCN values were quite similar (BL: mean mtCN from shotgun sequencing = 257 and from ddPCR = 260; SM: mean mtCN from shotgun sequencing = 2788 and from ddPCR = 2744; Table 1).

Table 1: Variation in mtCN estimates from different methods. For each combination of method and tissue, the sample size and mean mtCN estimate across individuals are given. For the sequence-based methods, the mean SD across chromosomes is given (SD-chromosomes); for the shotgun and ddPCR data from BL and SM tissue the mean SD across replicates for all individuals is given (SD-replicates). All SD values were normalized by the mean mtCN for each tissue.

Method	Tissue	Sample size	Mean	SD-chromosomes	SD-replicates
shotgun	BL	148	257	0.025	0.085
shotgun	SM	151	2788	0.026	0.032
shotgun	LIV	137	2873	0.032	
shotgun	MM	150	7023	0.026	
capture-enrichment	BL	140	159348	0.086	
capture-enrichment	SM	149	408534	0.118	
capture-enrichment	LIV	150	394297	0.132	
capture-enrichment	MM	149	725243	0.182	
capture-enrichment	CEL	150	404773	0.121	
capture-enrichment	CER	142	360418	0.116	
capture-enrichment	CO	152	591684	0.149	
capture-enrichment	KI	151	393972	0.135	
capture-enrichment	LI	150	387283	0.130	
capture-enrichment	OV	47	217524	0.096	
capture-enrichment	SI	149	419861	0.154	
capture-enrichment	SK	152	254660	0.098	
ddPCR	BL	150	260		0.134
ddPCR	SM	151	2744		0.101

To further evaluate the error rates in qPCR vs. shotgun sequencing, the libraries for the BL and SM samples were sequenced a second time and the mtCNs compared (S2 Fig). For both tissues mtCNs could be determined with high reproducibility from shotgun sequencing, with R^2 values of 0.92 and 0.99 for BL and SM samples respectively. The average SD was ≤8.5% and therefore lower than the SD for ddPCR; however, it should be noted that the shotgun sequencing replication was carried out on the same libraries, and hence did not include variation from library preparation or other experimental procedures. As with the ddPCR experiments, the SD for mtCN in SM was lower than that for BL, indicating a higher reproducibility for the tissue with higher mtCN.

Extreme outliers

Remarkably, two extreme outliers in mtCN were identified. One SM sample from a 71 year old male who died of cardiac death showed a 137-fold higher mtCN (shotgun sequencing) than the average of all other SM samples (S3 Fig) and a more than 60-fold higher mtCN than the sample with the 2nd highest mtCN. Results from capture-enrichment (32-fold higher than the average, 14-fold higher than the second highest mtCN) and ddPCR (80-fold higher than average, 30-fold higher mtCN than the second highest) showed a similar tendency. In addition, one LIV sample from a 72 year old male who died of multiple organ failure showed a 20-fold higher mtCN than the tissue average in shotgun sequencing (8-fold in capture-enrichment). The mtCN estimates for other tissues in these two individuals are all within the average range for that tissue, indicating that these extreme outliers are tissue-specific.

In other tissues, the highest mtCN value was maximum 4-fold higher than the average in shotgun sequencing (6-fold in capture enrichment), showing that extreme mtCN outliers are rare in the data set.

Inter-individual differences in mtCN and correlations of mtCN with age, sex and haplogroup

For each method, we tested if mtCN estimates were correlated between pairs of tissues within an individual (Fig 2). All p-values were corrected for multiple testing using Benjamini-Hochberg correction [25]. In the capture-enriched data, positive correlations were identified between SI and LI ($r=0.31$, $p<0.001$), between CO and CER ($r=0.33$, $p<0.001$) and of KI with CER, CO, SI and SM. In addition, mtCN in SK was negatively correlated with that in SI, LI and in LIV. In the shotgun sequencing data there was a negative correlation of mtCN in SM vs. LIV (Pearson's $r=-0.23$, $p=0.042$,

Fig 2), but this was not observed in the capture-enriched data (Fig 2). Overall, we did not observe any regular pattern in inter-individual variation in mtCN among different tissues (S4 Fig).

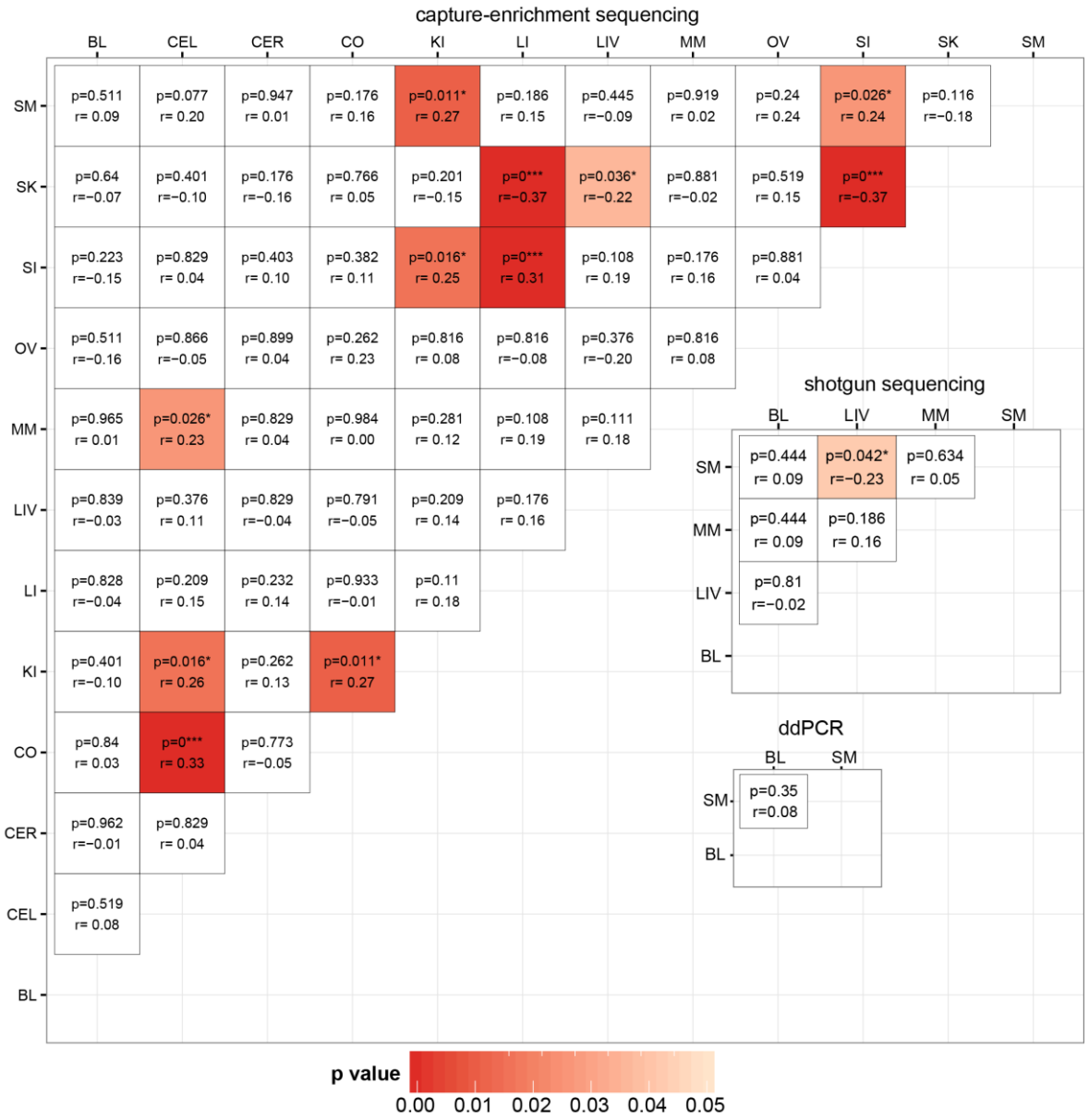

Fig 2: Correlation analysis of mtCN estimates from different tissues by three different methods. Corrected level of significance (p) and the Pearson correlation coefficient (r) are given in each field; fields are colored by p-value according to the scale.

We also investigated associations between mtCN and age, sex, or haplogroup. There is a strong age-related decrease of mtCN in SM (r=-0.25, p=0.016 in shotgun sequencing, Fig 3A, S2 Table). Remarkably, this association holds only for males (r=-

0.35, p=0.008 in males compared to r=-0.15, p=0.442 in females; S2 Table). Similar correlation coefficients were obtained for mtCN estimates from capture sequencing and ddPCR, although the p-values tend to be higher (S2 Table). In LIV, mtCN shows an increase with age that is significant in capture enrichment data (r=0.27, p=0.022) and approaches significance in shotgun sequence data (r=0.20, p=0.088; S2 Table). This increase was mainly due to a subset of individuals (Fig 3A) with higher mtCNs. When looking at samples with mtCN≥4,500, these showed a stronger correlation of mtCN with age (r=0.64, p=0.016) than the group mtCN≤4,500 (r=0.19, p=0.048).

No solely sex-related associations with mtCN were identified for any tissue (Fig 3B, S2 Table) and there were no significant associations between mtCN and major haplogroup (Fig 3C, S3 Table).

Associations between mtCN and heteroplasmy

As all samples had been previously analyzed for heteroplasmy [17], we tested for associations between mtCN and the total number of heteroplasmic positions as well as the MAF at specific heteroplasmic positions. SM exhibited a highly significant negative correlation between mtCN and the total number of heteroplasmies for all three methods (r=-0.27 to -0.31, all p≤0.002; Fig 4, S4 Table). BL and LIV also exhibited significant negative correlations between the total number of heteroplasmies and mtCN, but not for all of the methods (S4 Table). When comparing the influence of age and the total number of heteroplasmic sites in SM by partial regression, we found that mtCN was best explained by age (p<0.05), although the number of heteroplasmies also showed a slight correlation with mtCN that was significant at least for shotgun sequencing (p=0.049, Fig 4). In LIV, changes in mtCN were significantly explained by age (p<0.05, Fig 4), whereas BL showed a higher partial regression of

mtCN with heteroplasmy than with age (Fig 4). The number of heteroplasmic sites has been shown to significantly increase with age [17] and our data indicate that correlations of mtCN with the total number of heteroplasmies are explained mainly by age rather than the increase in the number of heteroplasmic sites.

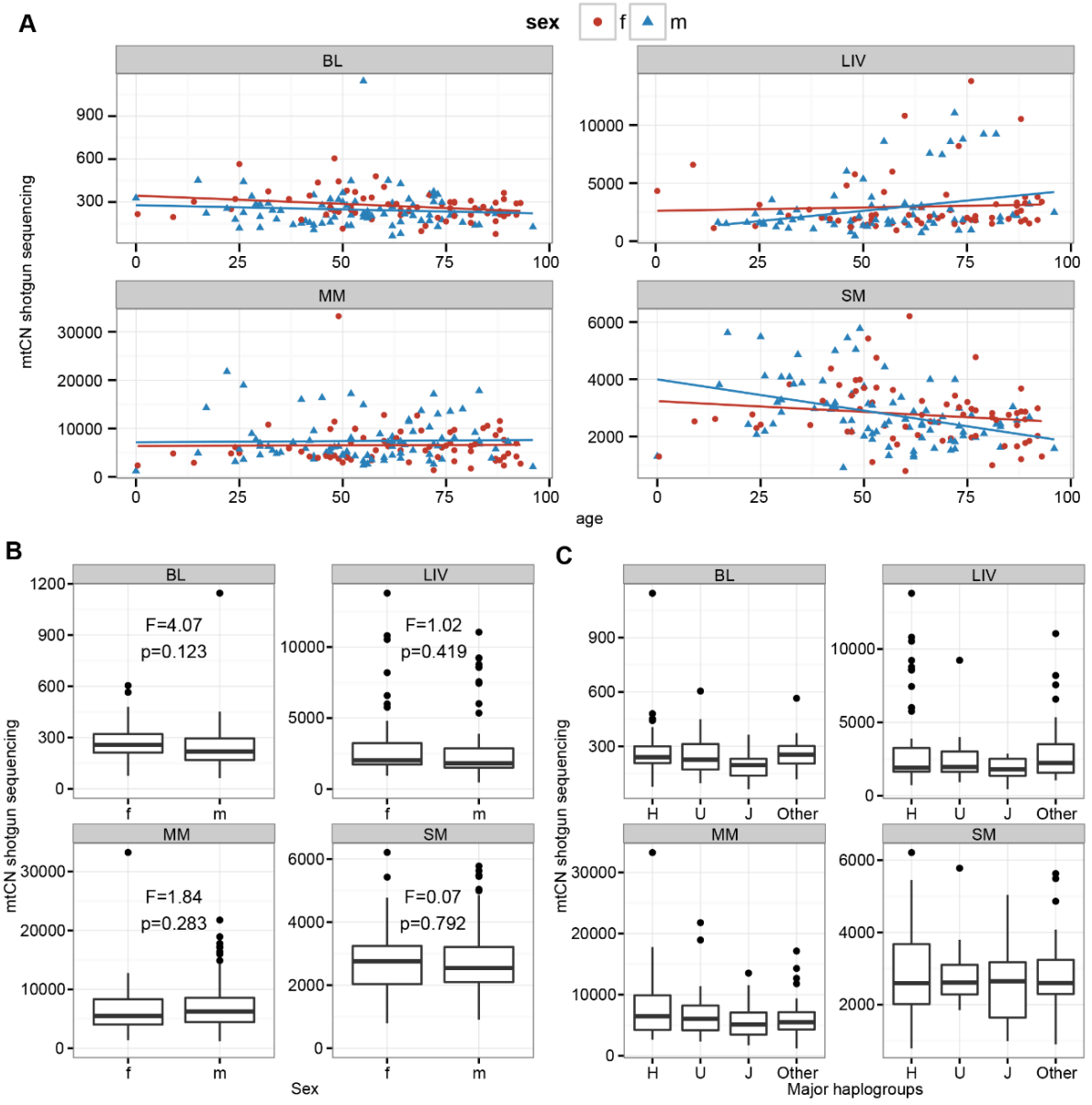

Fig 3: Correlation analysis of mtCN from shotgun sequencing with age, sex and haplogroup. Males (m) and females (f) are indicated. (A) Correlation of mtCN with age. (B) Correlation of mtCN with sex. F- and p-values are shown for each tissue. (C) Correlation analysis of mtCN with haplogroup, identified as H, J, U or other haplogroup.

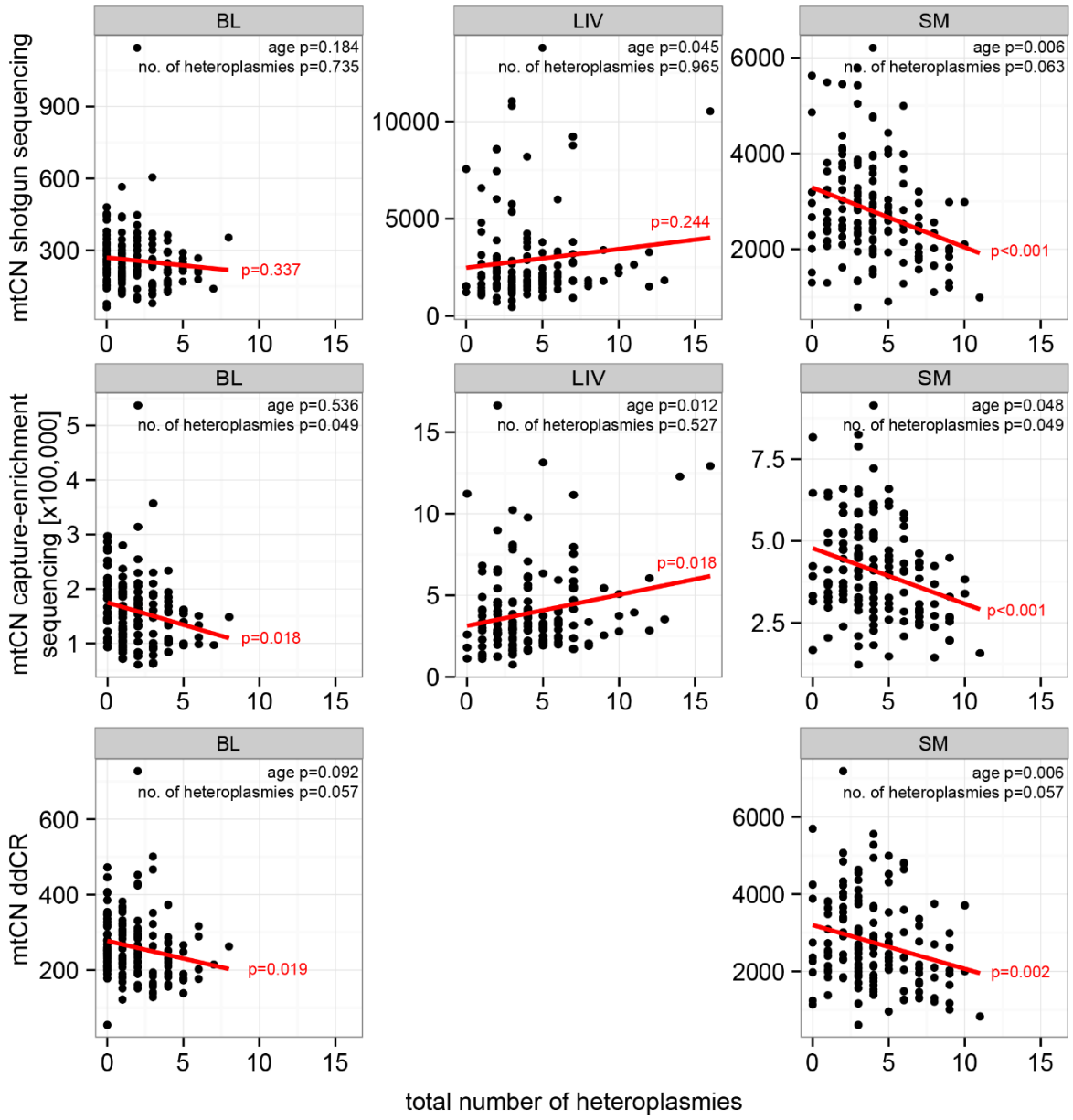

Fig 4: Correlation analysis of mtCN from shotgun sequencing with the number of heteroplasmic sites in BL, LIV and SM for shotgun sequencing, capture-enrichment and ddPCR. P-values of linear regression and regression line are given in red. P-values for partial regression of mtCN with age and the total number of heteroplasmies are shown in black.

With respect to associations between mtCN and the MAF at single heteroplasmic sites, analyses were limited to positions that were heteroplasmic in at least ten individuals for a tissue (S5 Table). After correction for multiple testing, sites 408 and 16327 each showed a significant association between mtCN and MAF in SM for shotgun sequence

data (p=0.014 for both sites, Fig 5). While position 408 is a T in any tissue, an A occurs at low (2-28.2 %) frequency in several individuals in SM. Site 16327 is usually a C, but some individuals have low frequencies of T at this position in SM (2-20 %). Other alleles were not found at either position [17]. As the MAF at these positions also increases with age [17], we applied partial regression to test whether age or heteroplasmy was more strongly associated with mtCN. While age was again significantly correlated with mtCN (p=0.001 for both sites), the MAF at both heteroplasmic sites also showed significant associations with mtCN (p=0.022 (408), p=0.02 (16327), Fig 5). The total number of heteroplasmies, however was not significantly associated with mtCN (p>0.05). This indicates that changes in mtCN in SM are associated with age, with the MAF at positions 16327 and 408 also explaining some of the change in mtCN.

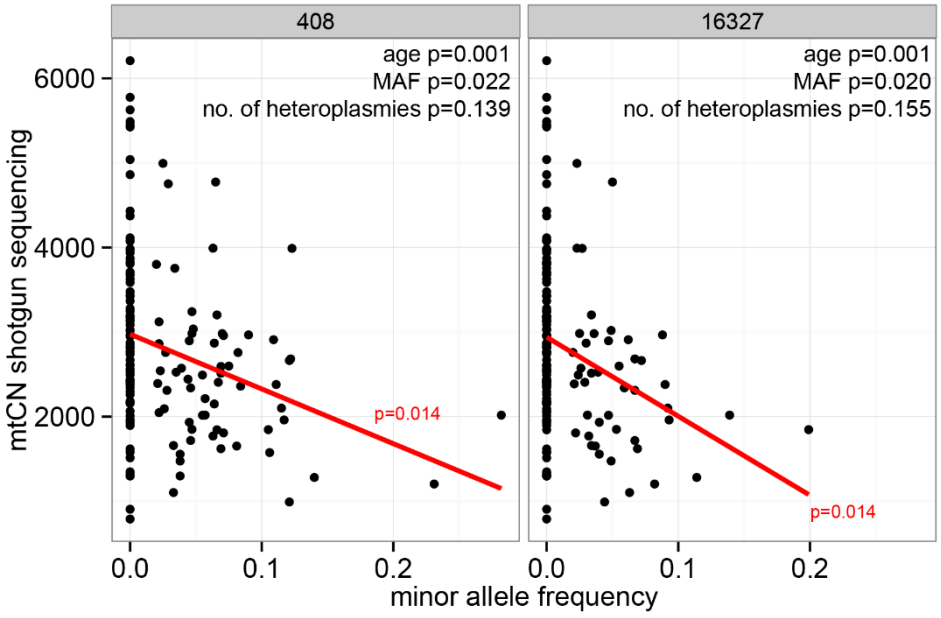

Fig 5: Correlation of mtCN with MAF at positions 408 and 16327 in SM. P-values of linear regression and regression line are given in red. P-values for partial regression of mtCN with age, MAF and the total number of heteroplasmies are shown in black.

No stronger correlations were found when testing the MAF of pairs or tripletons of heteroplasmic sites, indicating that heteroplasmy at two or three specific sites did not

describe variation in mtCN better than heteroplasmy at single sites. In sum, mtCN in SM differed dramatically from other tissues in exhibiting highly significant age, sex and heteroplasmy-dependent associations.

Discussion

Methods for mtCN determination

In this paper, mtCN from 12 different tissues was estimated using three different methods. Exact measurements of mtCN can be complicated in some tissues as intra-individual differences in mtCN of a single tissue occur. These differences arise from mosaic-like structures as those found especially in myocardial muscle [4] and one should therefore focus investigations of mtCN on rather homogenous tissues.

In ddPCR and shotgun sequencing experiments, SM samples showed smaller SDs for multiple measurements than BL samples. This could indicate that the precision of the two methods is increasing with higher mtCNs as small absolute measurement errors have less impact on the final results. In general, the shotgun sequencing method returned normalized SD values that were smaller than those for ddPCR. The high variance between measurements in ddPCR might arise from the several pipetting steps for mtDNA- or nDNA-specific primers as well as additional dilution steps between nDNA and mtDNA-determination. In Illumina® shotgun sequencing, the only step in which mtDNA and nDNA compete is the loading of DNA molecules into the nanowells of the flowcell. The distribution of reads across chromosomes indicates that for most chromosomes this happens randomly without any bias for certain sequences. Chromosomes that had to be excluded from the data set showed a large SD across all samples due to the assembly of reads with lower mapping quality. Considering the overall high reproducibility and the fact that shotgun sequencing allows high-throughput analysis of large sets of samples, this is the preferred method for mtCN determination; however, we emphasize that as we sequenced replicates of the same libraries, potential additional sources of variation in library preparation or other experimental procedures were not evaluated. We assumed that capture-enrichment

linearly increases the amount of mtDNA without saturation effects when estimating mtCN from sequencing after capture-enrichment. However, we observed that the enrichment process is non-linear, resulting in a stronger enrichment of mtDNA in samples with smaller mtCN. Samples with very high mtCN, like the SM-sample with a 100-fold increase in mtCN, already have a very high proportion of mtDNA prior to enrichment and will therefore be underestimated after enrichment. Samples with lower mtCN, like BL samples, have a low starting ratio that is strongly increased in the enrichment process leading to overestimated mtCNs after enrichment. Hence, enrichment most strongly influences the tails of the mtCN distribution; however, the overall high correlations between mtCN estimates from capture-enriched vs. shotgun data indicates that mtCN estimates by the former method do reliably reflect relative mtCN values.

Occurrence of massive mtCN outliers

We identified two samples from different individuals that had 20-fold (LIV) and a ≈100-fold (SM) higher mtCN compared to the tissue average. Similar results were obtained with the different methods for mtCN determination, indicating that these high mtCN are not method-dependent errors. To our knowledge no such increases in mtCN have been described before. While a decrease in copy number has been associated with several diseases, an excess of mtDNA proliferation is rare [3]. A strong increase of mtCN has been described when large parts of the coding region of the mtDNA are deleted [27] and steep increases in copy number occur during cell differentiation, such as an 1100-fold increase in mtCN between day 5 and 6 of differentiation of pluripotent embryonic stem cells [28]. As cells in the analyzed tissues were completely differentiated and no deletions were found in the sequences, both causes can be

excluded for our samples. The individuals we identified did not suffer from any diagnosed disease that might be expected to impact mtCN, and these extreme mtCNs occurred in just one tissue in each individual. Heteroplasmy, haplogroup or age do not explain these extreme outliers. As all enzymes required for mtDNA replication control are encoded in the nucleus [19], we speculate that mutations in nuclear-encoded genes might trigger this increase.

Regulation of mtCN in tissues

Following mtCN estimation, several parameters were tested for possible correlations with mtCN using linear regression and Pearson correlation test. The data from capture-enrichment returned the fewest significant results, which probably arises from Benjamini-Hochberg corrections for multiple testing as for capture-enrichment 11 or 12 tissues (ovarian tissue data could only be analyzed in females) were analyzed compared to four in shotgun sequencing and two in the ddPCR. As the F- and r-values, which were not corrected for multiple tests, were in the same range for all three methods, we conclude that the most striking correlations were identified regardless of the method used.

To our knowledge, few studies have investigated differences in relative mtCN between different tissues of an individual; however, one previous study did investigate mtCN in samples from brain, SM and MM from 50 individuals [11]. This study identified correlations of mtCN between most tissues. In our study, different tissues of the same individual showed a high variation in mtCN, not only for the total number of copies, but also for the relative number with respect to the tissue average. Positive correlations were mainly found for similar tissues, such as different parts of the brain or for large and small intestine. Interestingly, mtCN in skin had a strong negative correlation with

mtCN in both intestinal tissues (as well as liver). Skin and intestine are surface tissues with exposure to different microbiomes [29], and hence different environmental circumstances might be influencing mtCN. Overall, though, the variation in mtCN between different tissues of an individual is largely uncorrelated (Fig 2). Thus, it is not the case that individuals tend to have generally high or low mtCN values across all tissues, but rather mtCN varies in a tissue-specific fashion.

Haplogroup is one factor that might influence mtCN. A previous study comparing haplogroup H and J found that the haplogroup J defining mutation increased TFAM binding and as a consequence mtDNA replication and mtCN [30]. In our data set no significant differences in mtCN were detected for different haplogroups, including haplogroup J. While we used whole tissue samples from autopsy, Suissa *et al*. used cell cultures and *in vitro* transcription methods [30], and this difference may be responsible for the different outcomes. For example, opposite effects of DNA polymerase γ overexpression on mtCN were found in cell cultures vs. transgenic mice [6]; mtCN observations from cell cultures therefore may not reflect mtCN in tissues.

Age is another factor that could influence mtCN. Other studies have described age-dependent decreases in mtCN for BL in individuals older than 50 years [8, 10]. Although all three methods exhibited a negative correlation between age and mtCN in BL for individuals over 50, only for ddPCR did the correlation approach significance in our data set ($p=0.09$, S2 Table). In contrast, the age-dependent decrease in SM is very striking in our data, especially in males. This result contradicts other studies of mtCN in the same tissue [4, 31]. However, the study of Barthelemy *et al*. investigated samples from younger individuals (2-45 years, n=16), and no information on the sex distribution of the individuals was given in either study. The strong decrease in mtCN with age in males may be explained by the composition of muscle tissues. Males tend

to have a higher ratio of fast-contracting muscle fibers (type IIB) over slow fibers (type I) [32]. Slow muscle fibers are characterized by a high activity, strong coupling of the electron chain and therefore high oxygen capacity. To account for aging effects, slow fibers reduce the coupling of the electron chain, resulting in low reactive oxygen species and stable mitochondrial function in old age [33]. Fast contracting muscle fibers, on the other hand, have a short longevity and are susceptible to aging. They show an advancing transformation to slow-contracting muscle fibers during aging which is accompanied by a reduction of mtDNA content [32]. Due to these differences in muscle composition, effects on mtCN in males during aging might be stronger than in females.

Finally, we investigated the influence of heteroplasmy on mtCN. The total number of heteroplasmic sites was strongly negatively correlated with mtCN in SM in our data. As the number of heteroplasmic sites is strongly age-related [17] and mtCN in SM is also decreasing with age, the major predictor of mtCN remains unclear after linear regression analysis. We tried to account for this by partial regression, in which one factor is set constant while the effect of the other is investigated. For the total number of heteroplasmies, we found that age had a more significant correlation with mtCN, indicating that a non-specific enrichment of heteroplasmic sites is not the major effector for changes in mtCN.

Previous studies have found that sequence variations in the polycytosine tract at positions 16180-16195 and in a TFAM binding motif at position 295 [5, 30] in the mtDNA control region, are associated with changes in mtCN. Here, we found indications for similar effects of common low-frequency heteroplasmic sites (408 and 16327). Both heteroplasmies are present in more than 40 % of the individuals older than 50 years. Heteroplasmy at position 16327 is highly specific for SM (46 out of 151

individuals, with MAF up to 20 %) as it was not found in more than four individuals in any other tissue. Heteroplasmy at position 408 is even more common in SM (65/151, with MAF up to 28.2 %), but was also found in some individuals in CER (12/142) and CO (13/152, S5 Table) with very low MAF (≤1.1 %). Site 408 is adjacent to the transcription start site within the light strand promoter (S7 Fig), which initiates production of an RNA primer for heavy strand replication [19], while 16327 is located within the TAS-region (S7 Fig), which regulates D-loop formation. The ratio of D-loops over double stranded DNA has been shown to be crucial for mtCN control [34] and parts of the TAS region, including site 16327, have been identified as a putative binding site for unknown proteins [35]. As the occurrence of heteroplasmy arises in an allele-specific way, we propose that heteroplasmy at positions 408 or 16327 might lead to changes in replication regulation. The fact that MAF at 408 was not correlated with mtCN in CER and CO might be explained by the much lower MAF in these tissues. Intra-individual deleterious mutations in the mtDNA may persist due to a lack of negative selection [36] as they need to reach a critical frequency threshold in order to impact mitochondrial function [37], an effect that is also referred to as relaxation of functional constraints [38]. The fact that heteroplasmy at sites 408 and 16327 are almost exclusively found in SM, yet are very common with high MAFs, in combination with their location in replication controlling regions, make it very likely that there is positive selection for the specific minor alleles at these positions in SM [17]. Our results are supported by the finding that lower mtCN in BL is a supposed marker for longevity [8] and that reduced mitochondrial activity leads to reduced accumulation of reactive oxygen species and protects mitochondria against aging [39]. We suggest that a slight decrease in mtCN results in a slowdown of mitochondrial metabolism in order to reduce the production of aggressive reactive oxygen species and hence provides a

better cellular fitness during aging, in accordance with the “survival of the slowest” hypothesis [40]. However, the functional impact of reduced mtCN in aging SM tissue remains to be elucidated.

In conclusion, we found that SM exhibits several interesting properties with respect to mtCN and heteroplasmy, including age-related decreases in mtCN in males, and decreases in mtCN associated with increasing MAF at two heteroplasmic sites in the control region. Several additional questions remain. For example, why do the minor alleles at positively selected heteroplasmic sites remain at rather low levels and do not reach higher and therefore more detrimental frequencies in the cell during aging? One effect of aging is the reduction of mitochondrial fusion and fission events that allow an exchange of genetic material between mitochondria in young individuals [41]. It has been proposed that reduced fusion and fission prevent cells from spreading detrimental mitochondria throughout the cell [42] and therefore decelerate aging effects. This might also explain the persistence of low frequency of heteroplasmy in aging individuals.

Several sites in the control region show site-specific and allele-specific heteroplasmy in specific tissues, such as position 60 and 94 in LIV and KI or position 204 and 564 in MM [17], yet in this study only the heteroplasmy at sites 408 and 16327 in SM showed a correlation with mtCN. Elucidating the functional consequences of this heteroplasmy therefore remains a major challenge for further investigation.

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Supporting Information

S1 Fig: Average autosomal coverage and SD of coverage per tissue per chromosome (A) in shotgun sequencing data (B) in capture-enrichment sequencing data. Each bar indicates the 95% confidence interval of the chromosomal coverage. Red bars indicate significant outliers.

S2 Fig. Reproducibility of mtCN determination using shotgun sequencing data. BL and SM samples were sequenced in two independent sequencing runs of the same libraries. R^2 represents the coefficient of determination of a linear regression analysis adjusted for the degrees of freedom.

S3 Fig. MtCN estimated by shotgun sequencing vs. mtCN estimated after capture-enrichment sequencing, including outliers. One SM sample and one LIV sample (red) had very high mtCNs, and were excluded from the correlation analyses for violating the assumption of a normal distribution.

S4 Fig. Heat plots of relative mtCN of single individuals in BL, LIV, MM and SM estimated by shotgun sequencing. Vertical bars indicate single individuals, sorted from young (left) to old (right). Coloring of a vertical bar indicates the mtCN according to the scale on the right of each plot.

S5 Fig. Correlation analysis of estimated mtCN with age. Males (m) and females (f) are distinguished. (A) capture-enrichment. (B) ddPCR.

S6 Fig. Correlation analysis of mtCN with gender. Males (m) and females (f) are indicated. F- and p-values are specified for each tissue. (A) capture-enrichment. (B) ddPCR.

S7 Fig. Mitochondrial DNA control region with focus on important regulatory elements for replication. A short RNA primer is transcribed from the light strand promoter (LSP). Replication starts at OH (heavy strand origin of replication). Many replication events terminate in the TAS-region leading to release of a 7S DNA that stays attached to the D-loop region. Positions 408 and 16,327 are located within the LSP or TAS-region, respectively.

S1 Table. Primers and probes used for ddPCR mtCN estimation. Sequences are shown from 5' to 3'. 5' and 3' labeling of probes is indicated.

S2 Table. Correlation analysis of mtCN estimated by different methods with age and sex. MtCN in the specified tissue was tested for correlation with the indicated test parameter. F-value, correlation coefficient r and p-values (corrected for multiple testing) are given. Tests with significant results ($p<0.05$) are in red.

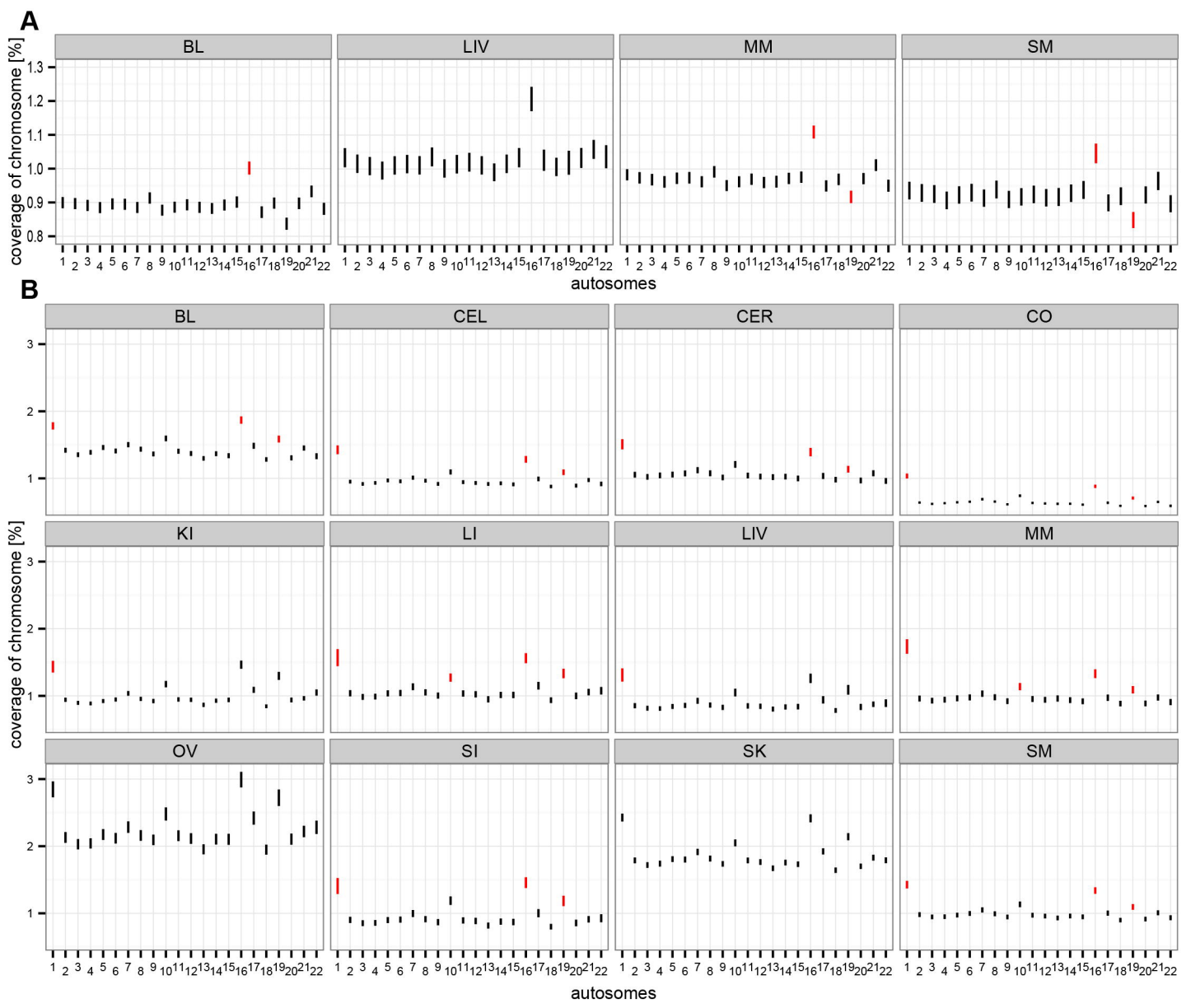
S3 Table. Correlation analysis of mtCN estimates from different methods with haplogroups. MtCN in samples of the major haplogroups H, J and U were compared to the residual sample set. If mtCNs were determined with different methods, results from all methods are given. F-value, Pearson correlation coefficient r and p-values (corrected for multiple testing) are given. No significant correlations were identified.

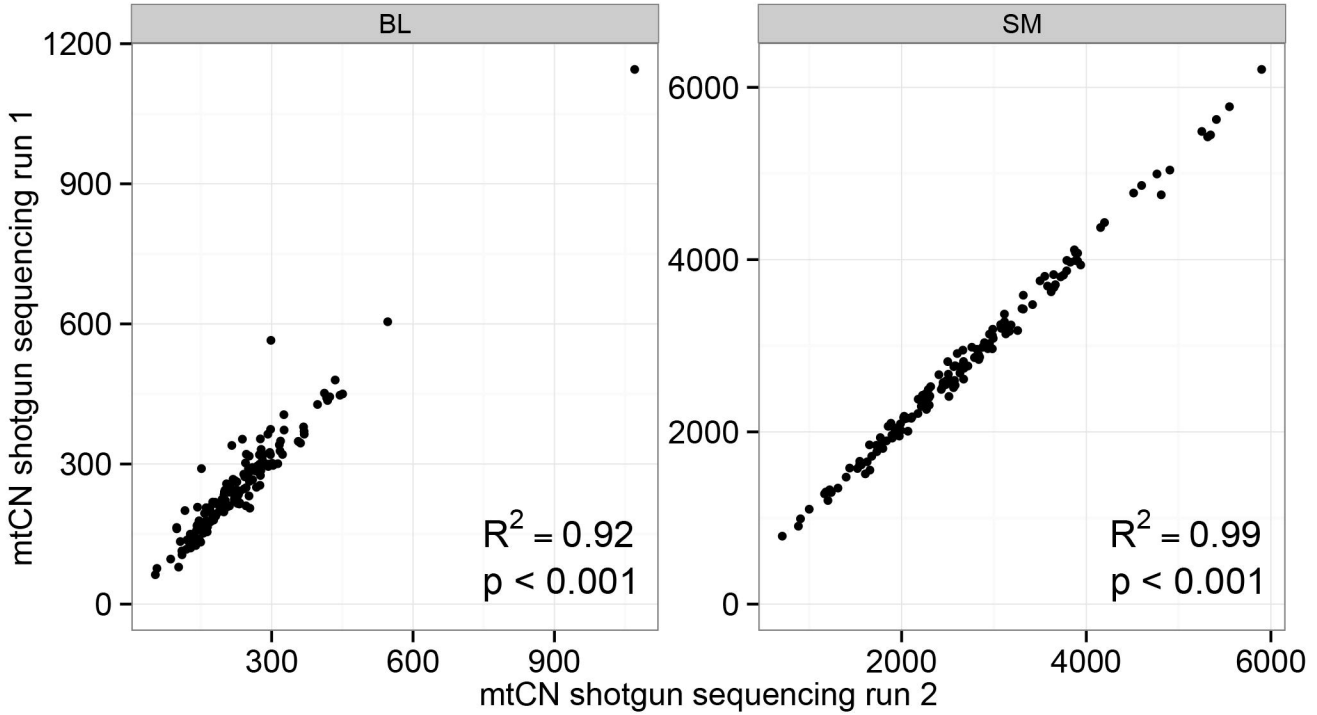
S4 Table. Correlation analysis of mtCN estimates from different methods with the total number of heteroplasmic sites per individual. The mean and maximum number of heteroplasmic sites per individual are given, along with the F-value, Pearson correlation coefficient r and p-values (corrected for multiple testing). Tests with significant results ($p<0.05$) are in red.

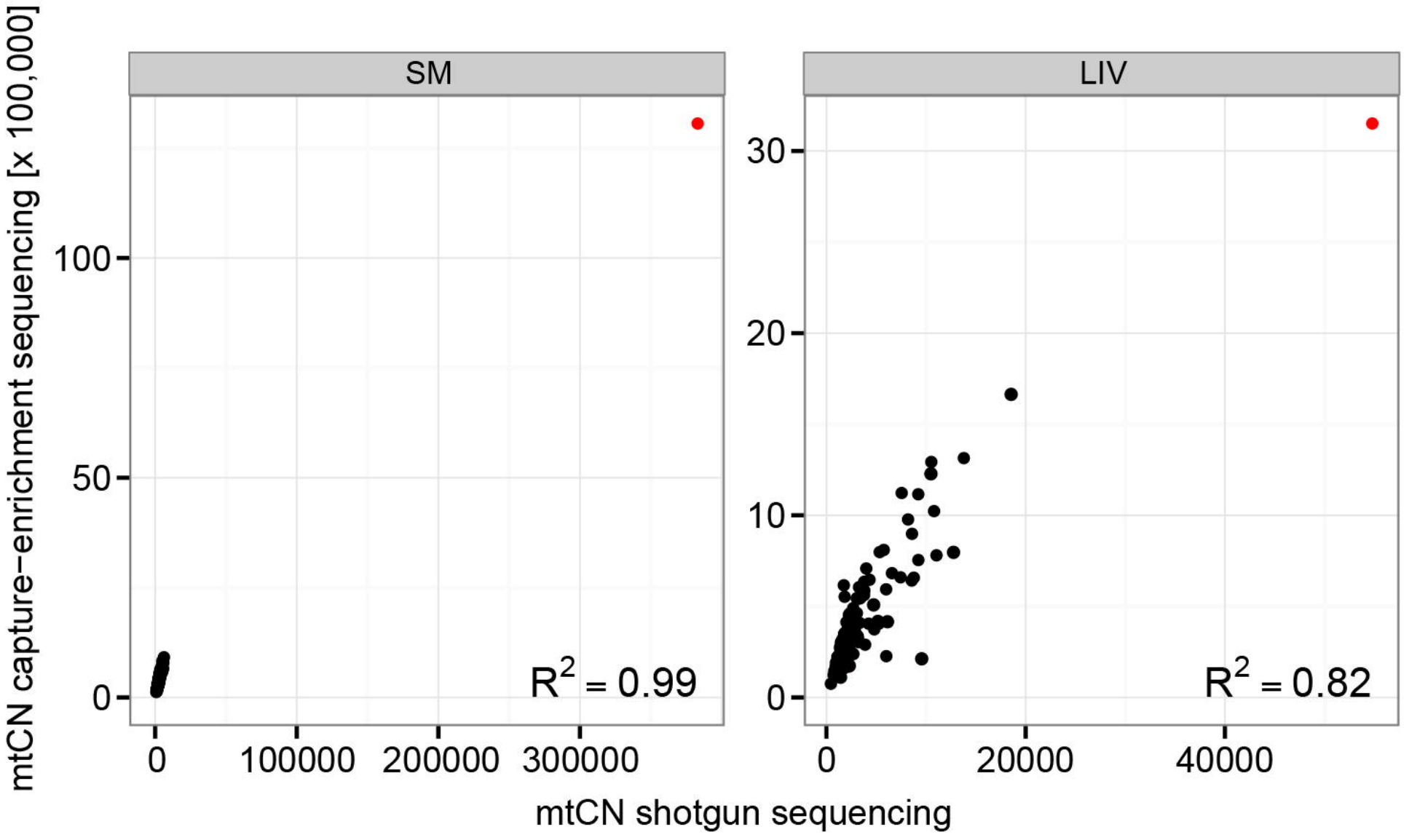
S5 Table. Complete list of heteroplasmic sites per tissue investigated for associations between mtCN and MAF. Only sites that were present in at least 10 individuals in a tissue, colored in red, were tested. The column “Sum” indicates the total number of sites that were tested in a tissue, while the row “Total” indicates the total number of tissues tested for each site.

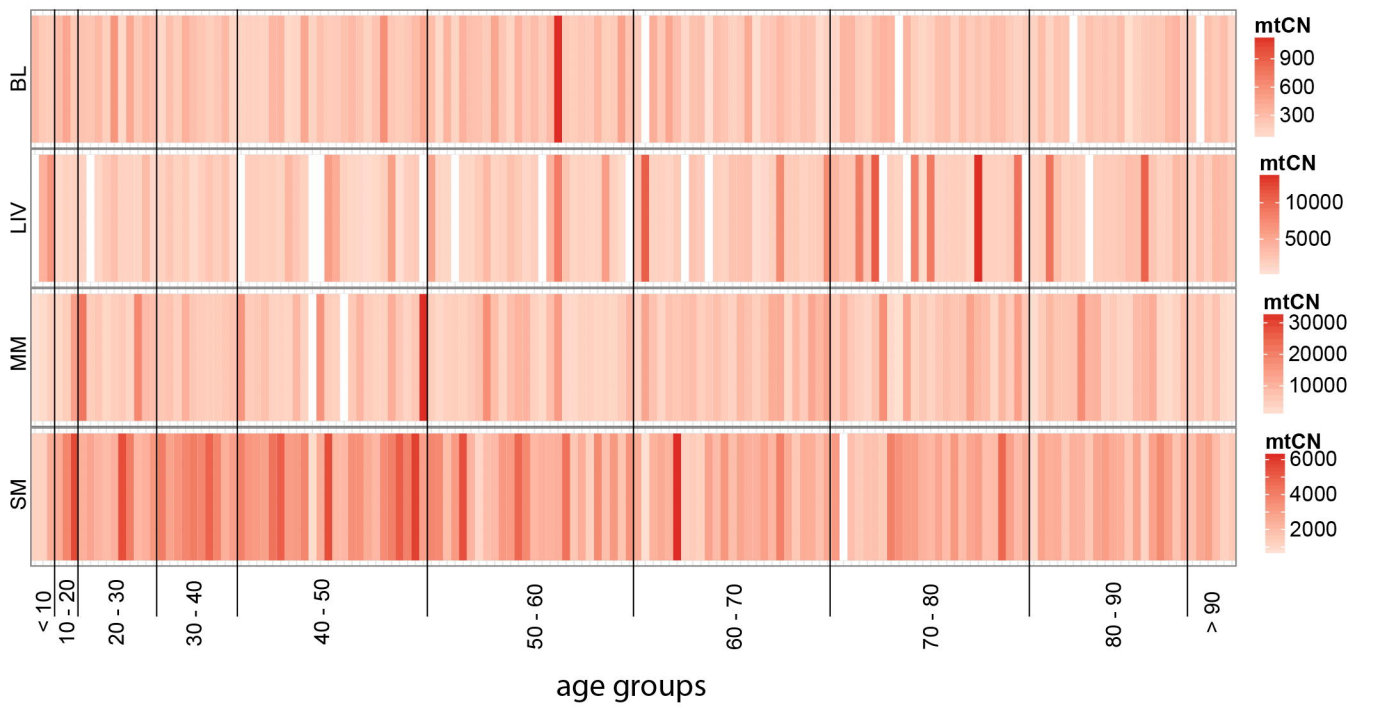
S6 Table. Linear regression and Pearson’s correlation analysis of MAF at heteroplasmic sites with mtCN estimates from ddPCR, shotgun sequencing and capture-enrichment sequencing. Tested sites were found in at least ten individuals in the indicated tissue. F-value, correlation coefficient r and p-values (corrected for multiple testing) are given. Sites with significant correlations with mtCN are marked in red.

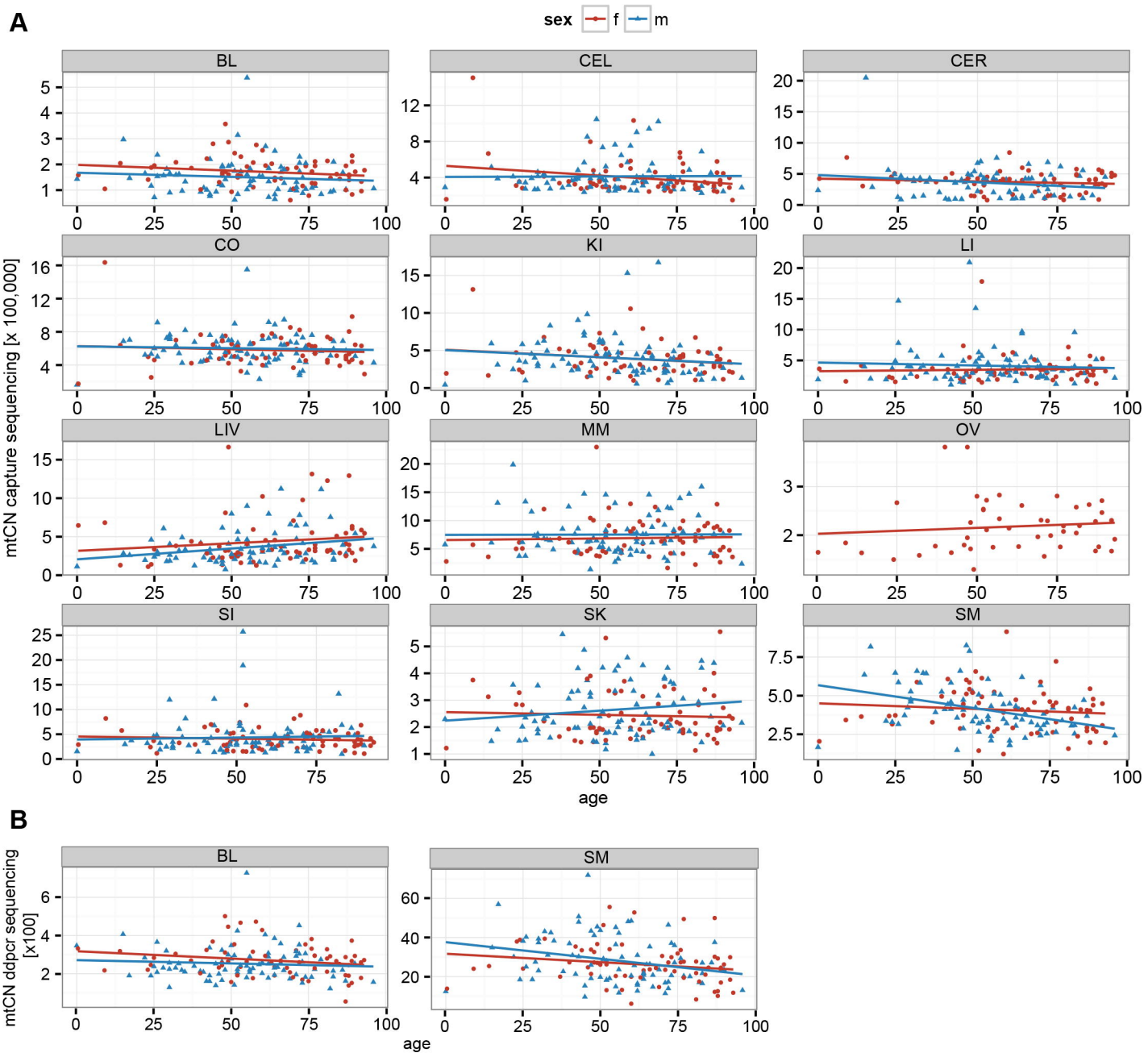
S7 Table. List of all individuals with age, sex, haplogroup and mtCN of each tissue estimated with the indicated method.

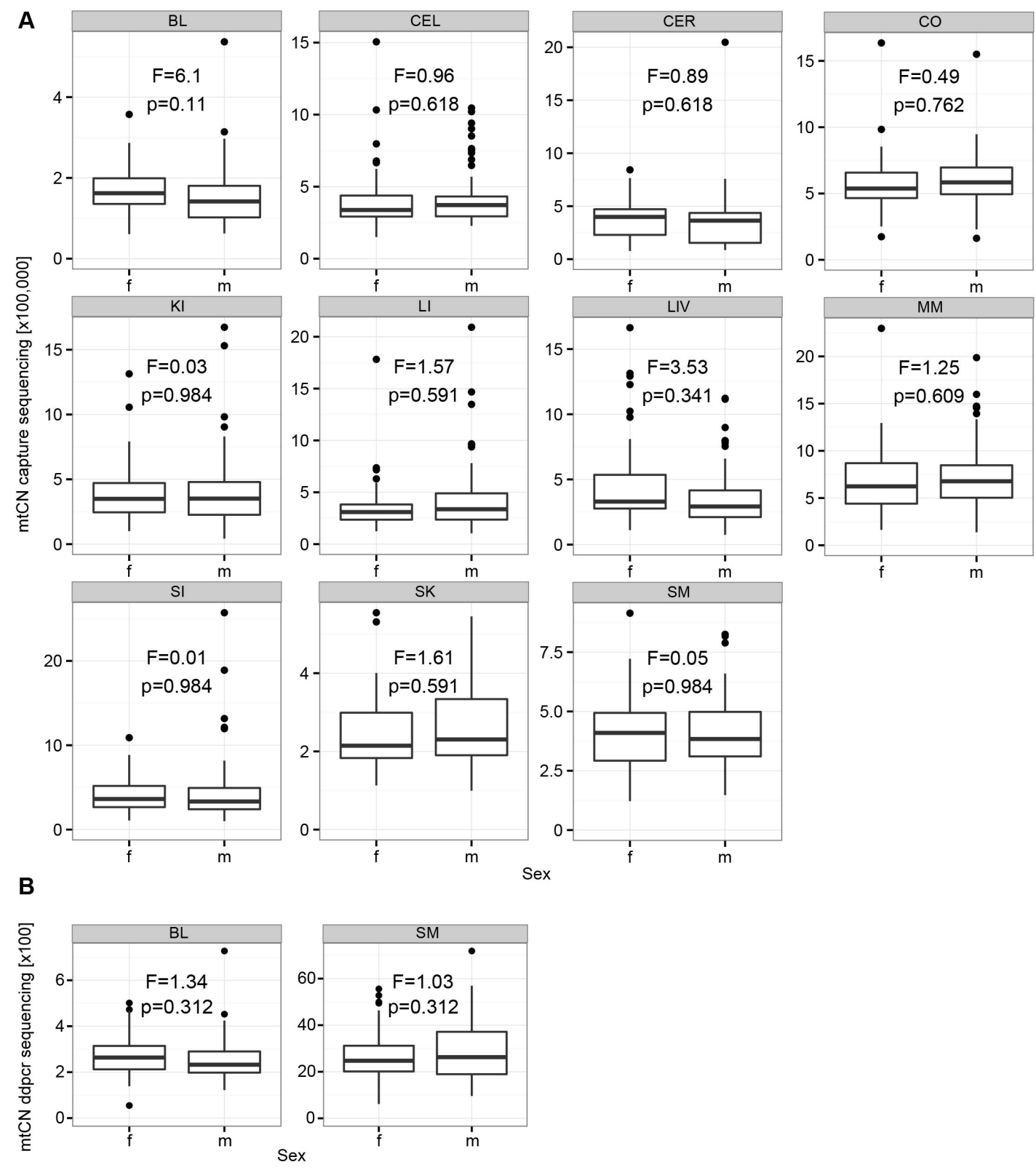

A
BL
LIV
MM
SM
coverage of chromosome [%]
1.3
1.2
1.1
1.0
0.9
0.8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
autosomes
B
BL
CEL
CER
CO
KI
LI
LIV
MM
OV
SI
SK
SM
coverage of chromosome [%]
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
autosomes

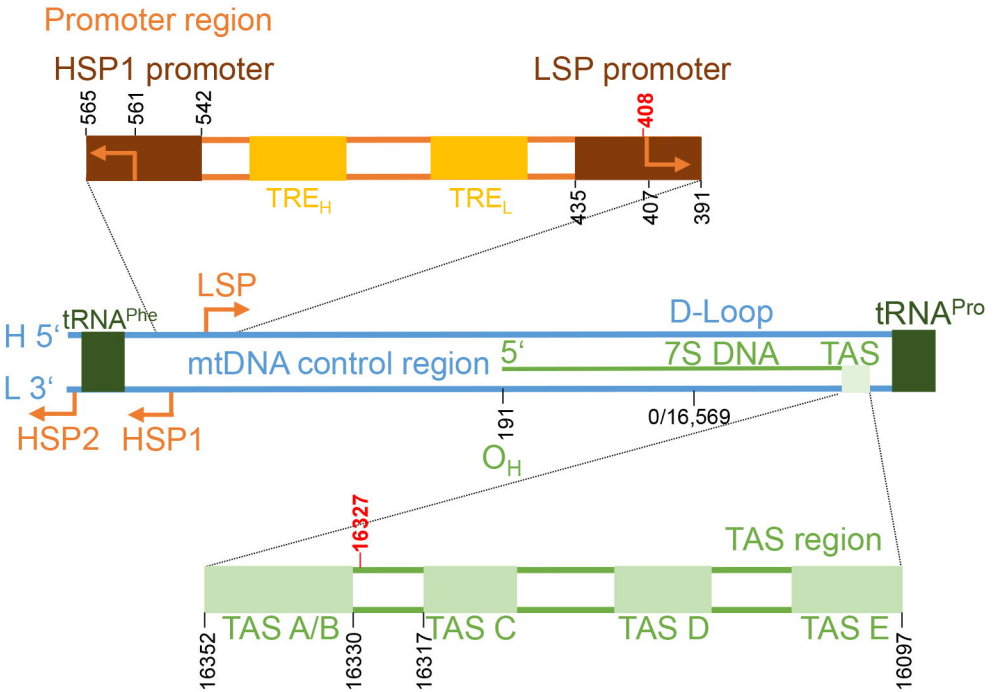

BL
SM
mtCN shotgun sequencing run 1
mtCN shotgun sequencing run 2
1200
900
600
300
0
6000
4000
2000
0
300
600
900
2000
4000
6000
$R^2 = 0.92$
$p < 0.001$
$R^2 = 0.99$
$p < 0.001$


SM
LIV
mtCN capture−enrichment sequencing [x 100,000]
mtCN shotgun sequencing
$R^2 = 0.99$
$R^2 = 0.82$
0
50
100
0
10
20
30
0
100000
200000
300000
0
20000
40000


BL
LIV
MM
SM
mtCN
900
600
300
mtCN
10000
5000
mtCN
30000
20000
10000
mtCN
6000
4000
2000
< 10
10 - 20
20 - 30
30 - 40
40 - 50
50 - 60
60 - 70
70 - 80
80 - 90
> 90
age groups


A
sex f m
BL
CEL
CER
CO
KI
LI
LIV
MM
OV
SI
SK
SM
mtCN capture sequencing [x 100,000]
age
B
BL
SM
mtCN ddpcr sequencing [x100]
age


A
BL
F=6.1
p=0.11
CEL
F=0.96
p=0.618
CER
F=0.89
p=0.618
CO
F=0.49
p=0.762
KI
F=0.03
p=0.984
LI
F=1.57
p=0.591
LIV
F=3.53
p=0.341
MM
F=1.25
p=0.609
SI
F=0.01
p=0.984
SK
F=1.61
p=0.591
SM
F=0.05
p=0.984
mtCN capture sequencing [x100,000]
f
m
Sex
B
BL
F=1.34
p=0.312
SM
F=1.03
p=0.312
mtCN ddpcr sequencing [x100]
f
m
Sex


Promoter region
HSP1 promoter
LSP promoter
565
561
542
408
TRE$_H$
TRE$_L$
435
407
391
LSP
H 5‘
tRNA^{Phe}
D-Loop
tRNA^{Pro}
mtDNA control region
5‘
7S DNA
TAS
L 3‘
HSP2
HSP1
191
0/16,569
O$_H$
16327
TAS region
TAS A/B
TAS C
TAS D
TAS E
16352
16330
16317
16097