A Mitochondrial Health Index Sensitive to Mood and Caregiving Stress

Supplemental Information

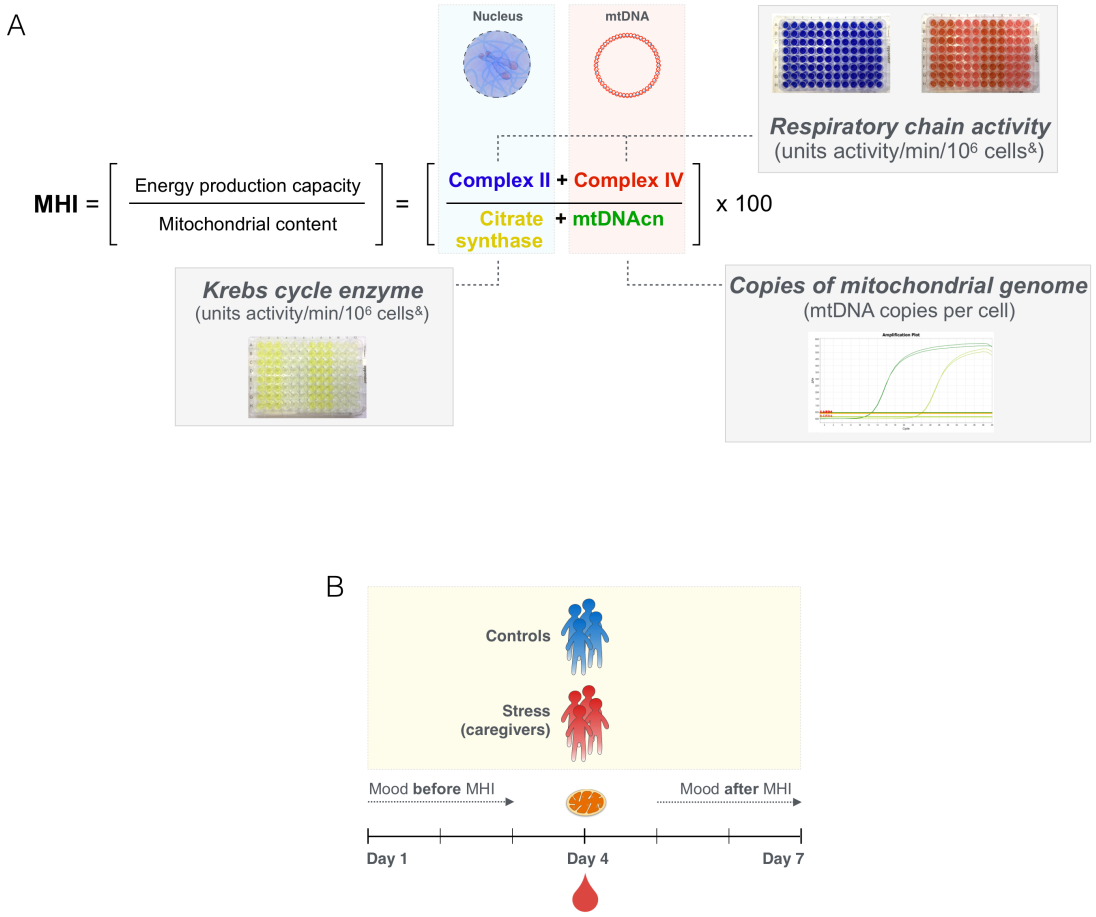
Supplemental FIGURES S1 - S6.

Supplemental TABLES S1 - S3.

Supplemental METHODS AND MATERIALS: Additional methodological details about the study cohort, recruitment procedures, the blood draw procedures, the complete description of experimental procedures used to measure the mitochondrial enzymatic activities and mtDNA copy number, mitochondrial protein content, daily mood measures, and data analysis.

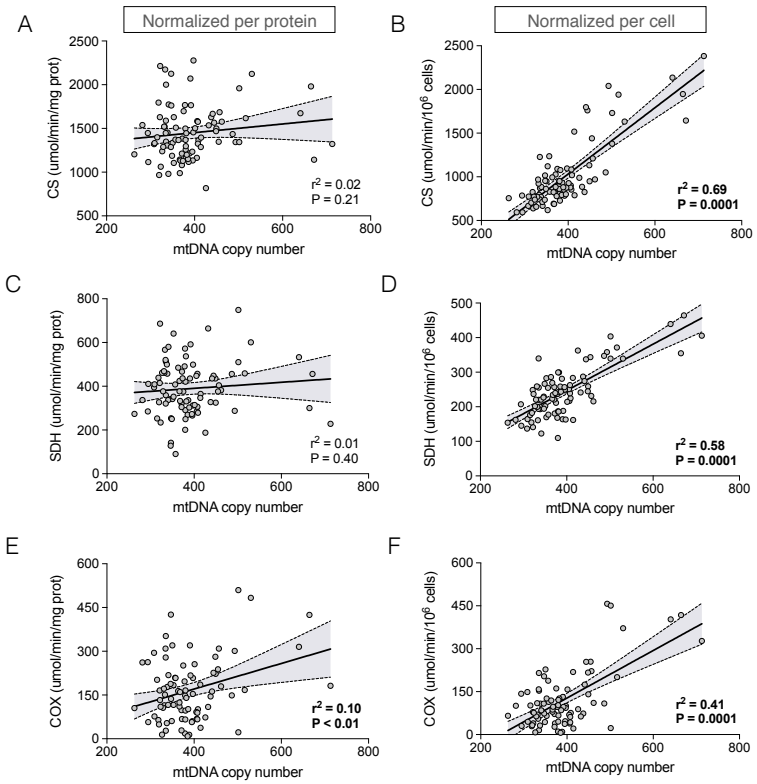
Supplemental RESULTS AND DISCUSSION: Detailed discussion mitochondrial proteins analysis, the mediation model, post-hoc analysis of freezer storage time and MHI.

Figure S1



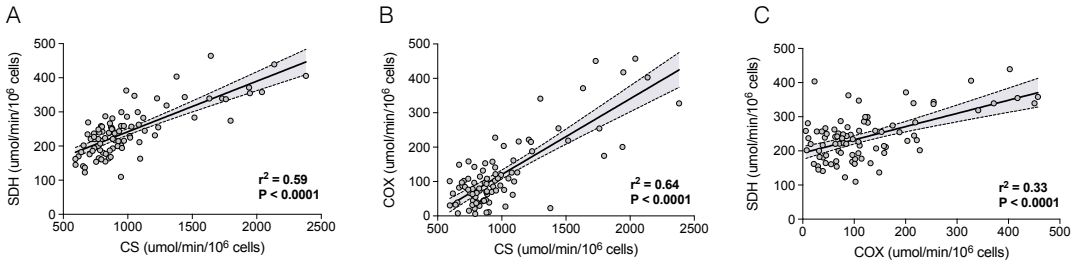
The Mitochondrial Health Index (MHI) and study design. (**A**) Simplified MHI formula with energy production capacity at the numerator and mitochondrial content at the denominator, and detailed version of the MHI equation with each component and their units outlined. For calculations, raw values for each component is mean-centered, then values for Complex II (SDH) and Complex IV (COX) are added as the numerator, and citrate synthase and mtDNAcn are added at the denominator. The quotient is then multiplied by 100. See supplemental calculation sheet (.xlsx) for detailed example. (**B**) Study design including blood sampling and mitochondrial health assessment at Day 4, with daily assessments of daily mood three days pre-, and post-blood collection. &: Cell count is adjusted based on by qPCR for a single-copy gene (B2M).

Figure S2



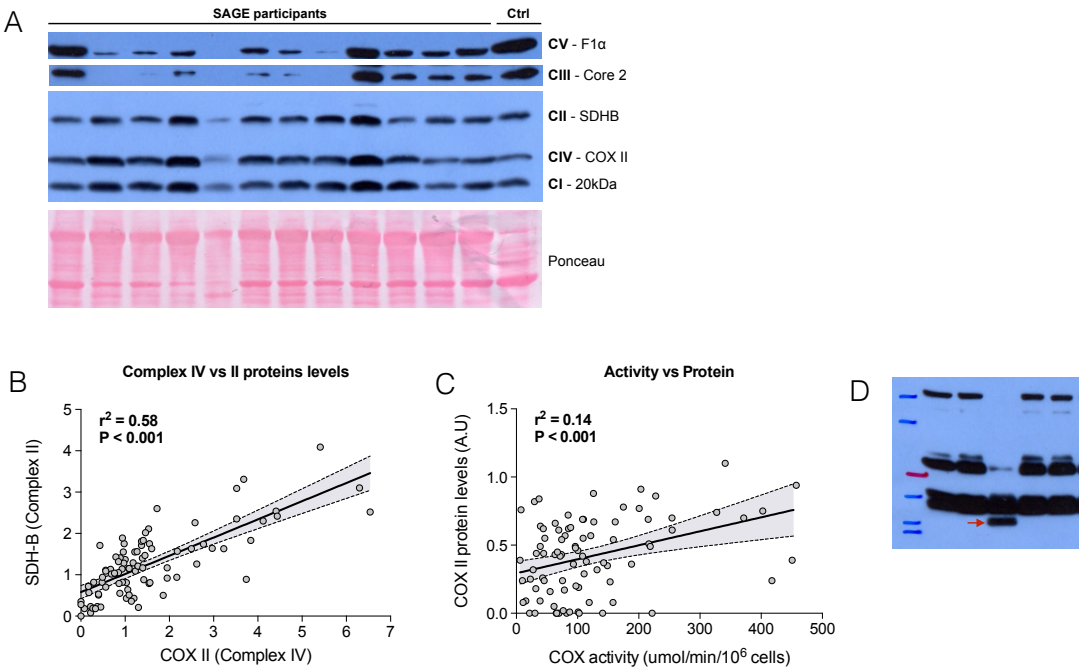
Normalization of mitochondrial enzymatic activities on a per cell basis improves internal consistency of MHI parameters and mtDNA copy number. (**A**) Correlations between mtDNA copy number and mitochondrial content marker citrate synthase (CS) activity normalized per protein, or (**B**) per cell by qPCR. (**C**, **D**) Same comparison between mtDNA copy number and succinate dehydrogenase (SDH), and (**E**, **F**) cytochrome c oxidase (COX) activities. Graphs show Pearson linear regressions with 95% confidence intervals (dotted lines with shaded areas); n = 89-91.

Figure S3



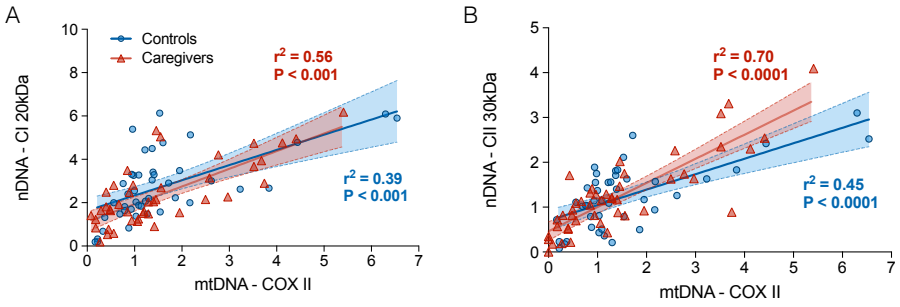
Inter-correlations of mitochondrial enzymatic activities. (**A**) Correlation between mitochondrial content marker citrate synthase (CS) and respiratory chain enzymatic activities of the nDNA-encoded succinate dehydrogenase (SDH) and (**B**) the mtDNA-encoded cytochrome c oxidase (COX). (**C**) Correlation between COX and SDH activities. Graphs show Pearson linear regressions with 95% confidence intervals (dotted lines with shaded areas).

Figure S4



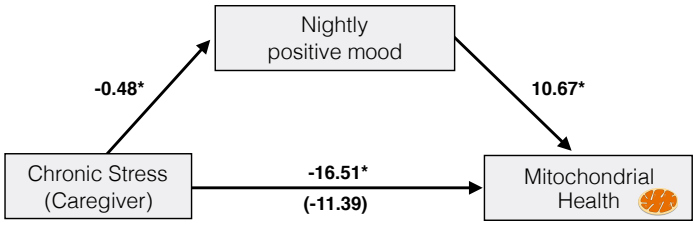
Analysis of mitochondrial respiratory chain proteins in PBMCs. (**A**) Representative Western blot for individual subunits from each of the mitochondrial respiratory chain complexes in human PBMCs. A reference control samples (Ctrl) is included for reference. (**B**) mtDNA-encoded COX II and nDNA-encoded SDH subunit B (SDH-B) protein levels are strongly correlated. (**C**) COX II subunit protein level is only weakly correlated with COX activity measured in the same sample. (**D**) Example of an individual lacking the typical SDHB band, but with an apparent sub-isoform (arrow). Four subjects showed this pattern. Graphs show Pearson linear regressions with 95% confidence intervals.

Figure S5



No evidence of mito-nuclear mismatch in caregivers. (**A**) Linear regression (Pearson) between mtDNA-encoded COX II and nDNA-encoded CI 20kDa or (**B**) SDHB subunits protein abundance measured by Western blotting. Shaded area denotes 95% confidence interval. Slopes are not significantly different between groups. Results are consistent with normal mito-nuclear coupling.

Figure S6



Daily positive mood partly mediates the effect of caregiver stress on mitochondrial health. Schematic of mediation model showing regression coefficients between caregiver status (predictor), MHI (outcome), and nightly positive mood (mediator). Age is included as a covariate. See Table 4 for model details.

TABLES S1 - S3

Table S1. Descriptive statistics of sample by group

	Group	
	Caregivers (N = 46) Mean (S.D.)	**Controls** (N = 45) Mean (S.D.)
Age (years)	44.23 (5.69)	42.47 (4.96)
Body Mass Index (kg/m^2)	26.15 (4.85)	25.76 (5.77)
Education (years)	16.71 (1.59)	17.34 (2.05)
Antidepressant Use	n = 9	n = 1
Depression	n = 2	n = 0

Diagnosis of depression was based on SCID.

Table S2. Inter-correlations between components of mitochondrial function with overall Mitochondrial Health Index (MHI)

	COX	**SDH**	**mtDNAcn**	**MHI**	**Mean**	***SD***
CS	0.61***	0.74***	0.81***	0.48***	6.86	*0.31*
COX		0.36***	0.41***	0.84***	4.46	*0.90*
SDH			0.71***	0.48***	5.45	*0.29*
mtDNAcn				0.26*	5.95	*0.19*
MHI					**95.83**	***27.41***

CS: citrate synthase; COX: cytochrome c oxidase; SDH: succinate dehydrogenase; mtDNAcn: mitochondrial DNA copy number; MHI: mitochondrial health index. Values are log transformed for CS, COX, SDH, and mtDNAcn. n = 85-89, varies due to missing data. * $p < .05$, *** $p < .001$.

Table S3. Partial correlations between trait distress indices and mitochondrial health

Measure	**Mitochondrial Health Index (MHI)**
Concurrent Self-Report	
Depressive Symptoms (IDS Total)	-0.21+
Perceived Stress (PSS Total)	-0.06
Trait Anxiety (STAI Total)	-0.15

Note. + = p < .10. Partial correlations adjusted for Age.

SUPPLEMENTAL METHODS AND MATERIALS

Study Cohort. The current study was conducted as part of a larger study of the effects of chronic caregiving stress on markers of cellular aging. Participants were recruited via schools, parenting publications, social media, mailings and ads through child development centers in the San Francisco Bay Area and direct recruitment at the University of California, San Francisco Autism Clinic. Caregiver duration was calculated as the difference between the date of diagnosis of the child to date of current study time point.

All participants reported being premenopausal and in good general health with no major diseases, including no history of coronary heart disease, endocrine disorders, epilepsy, brain injury, autoimmune conditions, severe asthma or lung disease. Potential participants were excluded if they reported a history of cancer or had undergone chemotherapy or radiation in the past 10 years. Low-stress maternal controls with current major depression were excluded; however, given the high base rate of depression among high stress caregivers this was not exclusionary in high-stress maternal caregivers. All study participants were free from medications known to affect the immune and endocrine system. With the exception of antidepressant medication and oral contraceptives, all study participants were free from medications known to affect the immune and endocrine system, including cholesterol altering medication (e.g., statins), regular use of anti-anxiety medications (e.g., benzodiazepines), and corticosteroid medications. Use of anti-hypertensive medication, such as beta-blockers, was not exclusionary.

Participants were screened by self-report symptoms of infection prior to the blood draw and rescheduled if the participant reported experiencing an acute illness or showed evidence of a fever (>37.8°C). White blood cell count was also performed, which if elevated above 10,500/μL, is indicative of an acute infection. However, we did not observe any participants who met this criterion.

Participants were paid $110 at the conclusion of the baseline assessment. At nine months after recruitment, we drew blood to examine mitochondrial health in PBMCs, in a random subset of 91

participants. The sample size was limited by budget. Unless otherwise stated, all data reported here were collected at the 9-month time point.

Caregiver (CG) and Normative Control (NC) Groups. A priori groups were balanced within the total sample, and were similar in age (overall M=43.4 years, SD=5.4) and adiposity assessed by body mass index (BMI) (overall M=25.9, SD=5.3). Regular anti-depressant (AD) use was recorded for 9 Caregivers and 1 Control at the time of assessment. Within group descriptives are reported in Table S1.

Antidepressant use was not significantly correlated with MHI, but because more caregivers were taking antidepressants, we examined this potential confound in a sensitivity analysis by filtering out the ten participants who reported using antidepressants. This results showed the same pattern of significant results in MHI group differences (means age adjusted: 88.44 vs. 104.51, $t(72)= 2.52$, $P < .05$). Furthermore, we carried out Structured Clinical Interviews for Diagnostic and Statistical Manual for Mental Disorders for Axis I Disorders (SCID) to assess past history of depression which revealed that 31 participants (20 caregivers, 11 controls) had a past history of depression. MHI was unrelated to past history of depression status ($t(82) = 1.15$, $p = 0.26$). Potential participants were excluded if they reported substance abuse disorders, a history of cancer or had undergone chemotherapy or radiation in the past 10 years. Individuals with current psychiatric conditions, including bipolar disorder, post-traumatic stress disorder and eating disorders were also excluded. Low-stress maternal controls with current major depression were excluded; however, given the high base rate of depression among high stress caregivers this was not exclusionary in high-stress maternal caregivers. We re-administered the SCID at each of the study time points, including the 9-month time point to assess the current psychiatric conditions. At the 9-month time point, only 2 participants (both caregivers) met diagnostic criteria for major depression.

Daily Mood Measures and Analyses. Mood measures were collected at home using a daily diary in the morning and at night before bedtime. For nightly measures, the full modified version of the differential emotions scale (mDES) (1) was administered so all of the positive and negative items for the mDES were used. For morning measures, we used select items from the modified version of the mDES and custom items for positive expectation and worry were used. Prior to analyses, the items drawn from mDES (i.e., Stress, Control, Joy) were re-scaled to a Proportion of Maximum Possible (POMP) ranging in values from 0, 25, 50, 75, 100.

Items for morning positive mood included: i) I feel in control, coping well, on top of things; ii) I feel joyful, glad, happy; and iii) To what extent you are looking forward to versus dreading today's events? Items (i) and (ii) were ranked on a Likert scale: 1=Not at all, 2=A little bit, 3=Somewhat, 4=Moderately, 5=Extremely. Item (iii) was rated on a continuous visual analog scale anchored with “Really dreading today's events, Neutral, and Really looking forward to today's events”. The average of the three items was computed and used for analysis.

Items for morning negative mood included: i) I feel stressed, anxious, overwhelmed; and ii) To what extent you are worrying about how things are going to go today. Item (i) was ranked on a Likert scale: 1=Not at all, 2=A little bit, 3=Somewhat, 4=Moderately, 5=Extremely. Item (ii) was rated on a continuous visual analog scale anchored with “At ease, Neutral, Worrying a lot”. The average of the two items was computed and used for analysis. For nightly measures, all positive and negative items for the mDES (1) were used.

Measures of daily positive mood were positively correlated with MHI, but only when mood assessments preceded the MHI measure. Mood measured after blood draw, when the blood cells are no longer in the body, was not significantly correlated with MHI. This pattern existed despite the fact that mood was fairly stable in all participants across the week. Indeed, mood in the first half of the week strongly correlated with mood in the second half of the week. On average across participants, the correlation between positive morning mood on the first three days (pre) vs the last three days (post)

was moderately strong r = 0.59, and the pre-post correlation between negative morning mood was 0.58. Similarly, nightly mood was significantly correlated between the pre-and post-blood draw periods (positive mood, r = 0.60, negative mood, r = 0.65). One would expect these moderately strong inter-correlations of mood would reduce the probability of detecting pre-post differences in the mood-MHI association. Nevertheless, these results showed that only mood measured before MHI was significant, and that the strength of this association increased the more proximal it was to blood collection, indicating a potential directional effect of mood on mitochondria within days. This exciting finding is in need of replication.

Given the temporal association between mood and mitochondrial function, we reasoned that the differences in MHI between caregivers and controls could partially be explained by individual differences in daily mood. Indeed, caregivers have less positive and more negative daily mood (Table 2). To test this possibility, we ran a series of multiple regression models to test the indirect effects of caregiver status on MHI, via mood. Because we were testing in a post hoc fashion whether the already observed effect of mood before the draw mediated the relationship between stress group and MHI, we examined only positive mood and only in the pre-blood draw days.

Indices of Distress. The perceived stress scale-10 (2) is a standard 10-item questionnaire that assesses subjective perceptions of stress over the previous month. The scale has been normed in several large national surveys, and the average PSS scores among women was approximately 16 (3). Response options form a 5-point Likert scale ranging from 0 = never to 4 = very often. Notably, the overlap in PSS scores between the caregiver and control groups facilitated analyses of continuous stress measure irrespective of the caregiver status.

Depression. The inventory for depressive symptomatology (4) is a 30-item self-report scale that measures signs and symptoms of depression. All items are equally weighted and use scores on a 4-point Likert scale ranging from 0 to 3.

Anxiety. The State-Trait Anxiety Inventory (STAI) contains a 20-item self-report section used to assess trait-like anxiety (5). All items are equally weighted and scores on 4-point Likert scale ranging from 1 = almost never to 4 = almost always were used in analyses.

Blood Draw. During the morning clinic visit, participants were fasted and had a morning blood draw. Peripheral blood mononuclear cells (PBMC) were isolated from 10 mL of whole blood using Ficoll Histopaque®-1077 (Sigma-Aldrich). Samples were layered on Ficoll and centrifuged at 25°C for 30 min at 800*g* without brake. The PBMC layer was recovered, washed twice (300g for 15 minutes; and 400g for 10 minutes) with phosphate buffered saline (PBS) supplemented with 1% BSA to remove platelets, and then treated with ACK (Ammonium-Chloride-Potassium) lysis buffer (Lonza Walkersville, Inc. #10-548E) to remove red blood cell contamination. The PBMC pellets were frozen at -80 degrees for up to four years, while the cohort was enrolled and completed their second visit, and shipped as a single batch for mitochondrial analyses. Half of PBMCs were homogenized and the activities of three mitochondrial enzymes and the other half was used to quantify protein abundance by Western blotting.

Rationale for Selecting MHI Components. The four MHI components were selected based on four criteria: i) represent a known biological function (energy production capacity or mitochondrial content), ii) detectable in a microplate format for high-throughput, iii) shown to respond acutely to metabolic and biological stress, iv) encoded by either the mitochondrial or nuclear genomes.

Supplemental Text – Table S4. Respiratory chain enzymes and citrate synthase

Complex #	Abbreviated name	Full name	Genome[1]	Enzyme[2]
Complex I	N/A	NADH dehydrogenase	~38 nDNA + 7 mtDNA	EC 1.6.5.3
Complex II	**SDH**	Succinate dehydrogenase	4 nDNA (0 mtDNA)	EC 1.3.5.1
Complex III	N/A	$CoQH_2$-cytochrome *c* reductase	10 nDNA + 1 mtDNA	EC 1.10.2.2
Complex IV	**COX**	Cytochrome c oxidase	10 nDNA + 3 mtDNA	EC 1.9.3.1
Complex V	ATP synthase	F_0F_1 ATP	14 nDNA + 2 mtDNA	EC 3.6.3.14
N/A	**CS**[3]	Citrate synthase	1 nDNA	EC 2.3.3.1

[1]: Number of protein subunits encoded by the nuclear (nDNA) and mitochondrial (mtDNA) genomes in each complex

[2]: International union of biochemistry and molecular biology (IUBMB) enzyme nomenclature with enzyme classification number

[3]: Not a respiratory chain enzyme, located in the mitochondrial matrix and used as a marker of mitochondrial content.

N/A: Not applicable

Mitochondrial Enzymatic Assays and mtDNA Copy Number. Five million PBMCs were homogenized in 0.4ml of extraction buffer containing 1mM EDTA and 50mM triethanolamine. To detect enzymatic activity, the absorbance of a specific reporter dye in each reaction was monitored over time, non-specific activity subtracted, and the final enzyme activity calculated from the first derivative and molar extinction coefficient for the reporter dye (6). Biochemical methods were adapted from previously published protocols for cells and tissues, with some modifications (6, 7). All measurements were performed in a 96-well plate format with the assay run in triplicates for each enzyme, along with a non-specific activity control.

Citrate synthase (CS) activity was measured by detecting the increase in absorbance at 412 nm at 30°C, in a reaction buffer (200 mM Tris, pH 7.4) containing acetyl-CoA 2 mM, 0.2 mM 5,5'- dithiobis-(2-nitrobenzoic acid) (DTNB), 0.35 mM oxaloacetic acid, and 0.1% Triton-x. Final CS activity was obtained by integrating OD^{412} change over 180 seconds, and by subtracting the non-specific activity measured in the absence of oxaloacetate. *Cytochrome c oxidase (COX, or Complex IV)* activity was

measured by detecting the decrease in absorbance at 550 nm at 30°C, in a 100mM potassium phosphate reaction buffer (pH 7.0) containing 0.1% n-dodecylmaltoside and 120uM of purified reduced cytochrome c. Final COX activity was obtained by integrating OD^{550} change over 120 seconds and by subtracting spontaneous cyt c oxidation without cell lysate. *Succinate dehydrogenase (SDH, or Complex II)* activity was measured by detecting the decrease in absorbance at 600 nm at 37°C, in potassium phosphate 100 mM reaction buffer (pH 7.0) containing 2 mM EDTA, 1mg/ml bovine serum albumin (BSA), 4 µM rotenone, 10 mM succinate, 0.24 µM potassium cyanide, 100 µM decylubuquinone, 100 µM DCIP, 200 µM ATP, 0.4 µM antimycin A. Final SDH activity was obtained by integrating OD^{600} change over 1.5-3 hours and by subtracting activity detected in the presence of malonate (5 mM), a specific inhibitor of SDH. The molar extinction coefficients used were 13.6 L mol^{-1} cm^{-1} for DTNB, 29.5 L mol^{-1} cm^{-1} for reduced cytochrome c, and 16.3 L mol^{-1} cm^{-1} for DCIP. Final enzyme activities are expressed as nmol/min/10^6 cells.

On the same biological samples, two 10ul aliquots of lysate was transferred to 90ul of lysis buffer in two 96-well PCR plates. The lysis buffer contained 100mM Tris HCl pH 8.5, 0.5% Tween 20, and 200ug/ml proteinase K. Samples were lysed for 10 hours at 55°C, followed by heat inactivation for 10 minutes at 95°C, and used directly as template DNA for mtDNA copy number measurements.

mtDNA copy number was determined in triplicates, on two plates in parallel, using multiplex qPCR chemistry that simultaneously amplifies a mitochondrial (ND1) and a nuclear (RNAseP) amplicon to determine their relative abundance (8, 9). The sequences for the ND1 amplicon (IDT) are as follows: Forward primer (300nM), 5'CCCTAAAACCCGCCACATCT3'; Reverse primer (300nM): 5'GAGCGATGGTGAGAGCTAAGGT3'; and Probe (100nM): 5'FAM-CCATCACCCTCTACATCACCGCCC-TAMRA3'. The RNAseP assay is VIC-labeled and commercially available as a kit (Thermofisher Scientific #4403328). Taqman Universal Mastermix (Thermofisher #4304437) was used and the assay ran on a ViiA7 real-time PCR thermocycler. The average C.V. for mtDNA Cts was 1.3% (plate 1) and 1.2% (plate 2). Data was manually curated and in the cases where triplicates for a given sample yielded a standard deviation > 0.2, the divergent triplicate was removed.

Thereafter, the C.V. was 0.5% (plate 1) and 0.4% (plate 2). For nDNA the average C.V. was 0.5% (plate 1) and 0.3% (plate 2). After curating the C.V. were 0.4% (plate 1) and 0.3% (plate 2). mtDNA copy number was calculated as $mtDNAcn = [2^{(RNAseP\ Ct - ND1\ Ct)}] \times 2$, taking into account the diploid nature of the nuclear genome. Results from the two plates were averaged to yield final mtDNAcn values, expressed as the number of mtDNA copies per cell.

Respiratory chain activity was normalized in two ways: i) to protein concentration obtained by the bicinchoninic acid assay (BCA) method, and ii) to relative cell count obtained by qPCR for the RNAseP (nDNA) amplicon. As expected, correlation analyses between enzymatic activities and mtDNAcn showed that normalization to cell number was a more sensitive normalization procedure.

To compute the Mitochondrial Health Index (MHI), enzymatic activities and mtDNA copy number were z-scored so that each parameter has equal weight in the final equation. SDH and COX are added at the numerator, and CS and mtDNA copy number are added at the denominator (Fig. S1). The final MHI reflects mitochondrial energy production capacity per mitochondrial unit, on a per-cell basis.

For analyzes of individual functional parameters (CS, COX, SDH) and mtDNA copy number, variables were log transformed to reduce skewness; all values were within -/+ 4 standard deviation range. For all analyses, the MHI was analyzed using its original untransformed units.

Mitochondrial Protein Content. To measure the levels of respiratory chain complexes subunits, five million PBMCs were lysed in Ripa buffer containing 50mM Tris, 150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, and 0.5% Triton X-100, supplemented with protease and phosphatase inhibitors (Roche #11873580001). Samples were centrifuged at 13,000g (20 min, 4^{o}C) and the supernatant supplemented with laemmli to denature proteins. Protein concentration was then determined by the BCA method (Thermofisher #23225). Twenty micrograms of protein were loaded per well and resolved on 15% polyacrylamide gels. Proteins were then transferred to PVDF membranes (Immun-Blot #162-0177) reacted with an antibody cocktail recognizing a subunit for each of the five

OXPHOS complexes (Total OXPHOS human; Abcam #110411) used at a dilution 1:1000 in 5% fat free milk in TBST. All membranes were then developed in parallel with ECL Prime (GE #CA89168-782) and imaged on film (Diafilm #810). Exposure time was fixed for a given protein but ranged from 20 seconds to 3 minutes depending on the signal intensity for each of the five subunits. The density of immunoreactive bands was determined using ImageJ (NIH, version 1.47v) and results were normalised to Ponceau staining to ensure equal loading across lanes of a given gel/membrane. All samples were resolved on eight different gels/membranes and a reference sample, along with a molecular weight ladder, were loaded to each gel to ensure internal consistency across gels/membranes. Band intensity data was Z-scored and used without further transformation for all analyses.

Statistical Analyses and Missing Data. We collected biological samples from 91 participants total. A few samples had undetectable enzyme activities (n=5) or failed to yield adequate DNA amplification (n=3). For the main outcome, integrated MHI, data were available from 85 cases. For nightly diary entries, 80 participants completed entries for the entire week (i.e., 7 days), while some (n=11) provided partial data (range = 3-6 records). For missing data, analyses were performed allowing for listwise deletion of the cases above. All statistical analyses were performed in SAS 9.4.

SUPPLEMENTAL RESULTS AND DISCUSSION

Mitochondrial Respiratory Chain Protein Levels. Individual protein subunits for each of the five respiratory chain complexes I-V were then quantified by Western blotting (**Figure S4A**). We again evaluated the relationship between mtDNA-encoded and nuclear-encoded proteins: COX subunit 2 (COX II) and SDH subunit B (SDHB), respectively. They were strongly correlated, with 58% of shared variance (**Figure S4B**). However, there was little correlation between COX II protein abundance and COX enzymatic activity (**Figure S4C**), consistent with the fact that enzymatic activity is regulated by factors other than subunit protein abundance (such as complex assembly, post-translational

modifications, etc). By profiling respiratory chain proteins, we also identified individuals in the cohort (< 5-10%) with idiosyncratic respiratory chain protein patterns with missing subunits (**Figure S4D**), although the current sample size did not enable reliable interpretation of this observation.

Assessment of Covariates. We evaluated the correlation between measures of mitochondrial health and age, as well as potential covariates including body mass index (BMI), and regular antidepressant (AD) medication use. These covariates were not significantly correlated with any individual measures, or with the MHI, except for a weak association of BMI and SDH ($r = -.22$, $p < .05$). The correlations with MHI were as follows: age, $r = .12$, BMI, $r = -.02$, and regular antidepressant medication use, $r = -.10$, all non-significant. In this sample, even though there was a limited age span of twenty years, age is retained as a covariate in all subsequent analyses since it is theoretically a likely cause of poor mitochondrial health.

Mediation Model. The full effect model includes a simultaneous regression including both caregiver group and mood, and the covariate age. Because negative mood was not significantly associated with MHI (Figure 4), we focused our analyses on positive mood. Including positive mood in the model weakened the effect of caregiver group. The main effect of caregiver group was still significant for morning ($p = 0.040$), but it was no longer significant for nightly mood ($p = 0.063$). Nightly positive mood was a significant predictor of lower MHI with caregiver group. We then tested the indirect effect of positive mood on MHI in the adjusted model, with results reported in Table 4 and Figure S6. Since the distribution of the indirect effect based on the product of two coefficients was non-normal, we assessed significance using a bias-corrected bootstrap confidence intervals with 5,000 samples.

Freezer Storage Effect and Caregiver-Control Group Difference. After each participant's PBMCs were collected, they were stored and were only analyzed together once all the samples were collected

to preclude batch effects. As enzymatic activities could be affected by storage time, we evaluated duration in the freezer as a potential confounding variable. On average, caregivers were run 0.6 year earlier than controls, thus confounding caregiver status and freezer time. The caregiving group variable was correlated with storage time ($r = .65$) and the average storage duration time was significantly longer for caregivers (2.3 years) than controls (1.7 years, $t(89) = 7.96$, $p < 0.001$). Therefore, freezer storage time cannot be statistically covaried without removing most of the variance from group status.

As might be expected given the caregiver group difference in MHI and the caregiver group difference in storage time, freezer storage time and MHI were negatively correlated ($r = -0.56$, $p < 0.001$). Yet storage time among all participants was also correlated with positive mood ($r = -0.31$), perceived stress ($r = 0.35$) , anxiety ($r = 0.27$) and depression ($r = 0.44$. all $p < 0.05$). These correlations between psychological variables and storage time do not have face value, but rather reflect the association between caregiver group and storage time since caregivers also have significantly lower positive and higher negative affect.

Though the caregiver and control groups showed average differences in mood, they also overlap. The relation between mood and MHI can therefore be examined within each group separately to see if the MHI correlates with mood while covarying out the effect of freezer storage time. The correlation between MHI and positive mood in the caregiver group remained significant and of similar effect size when adjusted for storage time ($r = 0.30$, $p = 0.04$), compared to the unadjusted correlation that did not account for freezer time ($r = 0.29$, $p = 0.05$). However, this relationship was not significant for the control group alone, either adjusted for freezer time or raw, likely due to the limited range of values and sample size. Taken together, these results suggest that although the association between daily mood and MHI is not influenced by storage time, part of the group difference may be. Enzymatic activities may be more sensitive to storage time than static molecular markers and future studies should take this into account in their study design.

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