Priority Communication

Biological Psychiatry

# A Mitochondrial Health Index Sensitive to Mood and Caregiving Stress

Martin Picard, Aric A. Prather, Eli Puterman, Alexanne Cuillerier, Michael Coccia, Kirstin Aschbacher, Yan Burelle, and Elissa S. Epel

## ABSTRACT

**BACKGROUND:** Chronic life stress, such as the stress of caregiving, can promote pathophysiology, but the underlying cellular mechanisms are not well understood. Chronic stress may induce recalibrations in mitochondria leading to changes either in mitochondrial content per cell, or in mitochondrial functional capacity (i.e., quality).

**METHODS:** Here we present a functional index of mitochondrial health (MHI) for human leukocytes that can distinguish between these two possibilities. The MHI integrates nuclear and mitochondrial DNA–encoded respiratory chain enzymatic activities and mitochondrial DNA copy number. We then use the MHI to test the hypothesis that daily emotional states and caregiving stress influence mitochondrial function by comparing healthy mothers of a child with an autism spectrum disorder (high-stress caregivers, $n$ = 46) with mothers of a neurotypical child (control group, $n$ = 45).

**RESULTS:** The MHI outperformed individual mitochondrial function measures. Elevated positive mood at night was associated with higher MHI, and nightly positive mood was also a mediator of the association between caregiving and MHI. Moreover, MHI was correlated to positive mood on the days preceding, but not following the blood draw, suggesting for the first time in humans that mitochondria may respond to proximate emotional states within days. Correspondingly, the caregiver group, which had higher perceived stress and lower positive and greater negative daily affect, exhibited lower MHI. This effect was not explained by a mismatch between nuclear and mitochondrial genomes.

**CONCLUSIONS:** Daily mood and chronic caregiving stress are associated with mitochondrial functional capacity. Mitochondrial health may represent a nexus between psychological stress and health.

*Keywords:* Chronic stress, Daily affect, Mind-body, Mitochondria, mtDNA copy number, Respiratory chain activity

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Chronic psychological stress is a common human experience that has harmful effects on health and well-being. Years of chronic adversity cause affective, cognitive, behavioral, and metabolic changes that accelerate aging and predispose to disease (1–4). Caring for a family member with a chronic condition is an example of a stressful life situation and in some contexts is an independent risk factor for adverse cardiovascular events (5), dementia (6), and earlier mortality (7). However, the cellular mechanisms that underlie these adverse effects remain unclear (2), and there are likely multiple mechanisms underlying stress-induced pathophysiology and cellular aging (8,9). One potential mechanism mediating these effects is mitochondrial dysfunction (10), because it is known that mitochondria play a role in adaptation to stress (11) and aging (12) in animal models. However, mitochondrial health has not been well examined in relation to psychological stress in humans (13), owing in part to the lack of adequate methods to functionally assess mitochondria and the need for comprehensive life stress assessments. To bridge this gap, we developed a novel index of mitochondrial health for blood immune cells and applied it to a maternal caregiving model of chronic stress where we tested its sensitivity to daily mood.

Mitochondrial health is emerging as a major determinant of healthy physiological regulation and resilience (14). Mitochondria are multifaceted organelles, and there are hundreds in each cell of the body. They are the cellular powerhouse, providing energy for most cellular functions and generating signals of adaptation that influence the cell's physiological response to stressors (11,15). Research in animal models has shown that mitochondrial defects cause profound alterations of stress response axes including the hypothalamic-pituitary-adrenal axis and sympathetic activation, which contribute to allostatic load and stress adaptation (11). In relation to disease, mitochondria also play a determinant role in cellular life and death (16), and they regulate disease-related processes that overlap with those of chronic stress pathophysiology (10), including inflammation, metabolic regulation, and cellular gene expression (17,18). Despite the important role of mitochondria in human health and potential association to stress and psychopathology (19–24), currently, methods available have not





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allowed assessments of mitochondrial health, or quality, in sizeable human cohorts.

There are different aspects of mitochondrial biology that must be taken into account to obtain an overall measure of mitochondrial health. Mitochondria contain multiple copies of their own genome known as the mitochondrial DNA (mtDNA). The mtDNA is critical for normal mitochondrial function because it encodes components of the respiratory chain complexes, where energy from food substrates and oxygen are transformed into adenosine triphosphate to power cellular processes (25). Two main causes of mitochondrial dysfunction are known: 1) reduction in the total amount of mitochondria—or mitochondrial content within cells; and 2) reduction in specific mitochondrial energy production capacity—or quality of each mitochondrion. For instance, individuals who inherit mitochondrial disease have a primary reduction in mitochondrial quality, but they can exhibit elevated mitochondrial content in blood leukocytes (26). As a result, mtDNA copy number (mtDNAcn) per cell can be increased as a compensatory mechanism for poor mitochondrial quality (26,27), and by itself mtDNAcn is not a reliable marker of mitochondrial content or quality. Empirically distinguishing between differences in mitochondrial content and quality is therefore essential to assess mitochondrial health and evaluate its relationship to aging, disease, and life stress exposure.

In this study, we tested the hypothesis that psychological states are associated with mitochondrial health. We first designed a mitochondrial health index (MHI) that mathematically integrates biochemical enzymatic activities and mtDNAcn into a single score, thus representing mitochondrial functional capacity on a per-mitochondrion basis. Next, we assessed the MHI in isolated peripheral blood mononuclear cells (PBMCs) in chronically stressed (caregiver) and control women and combined these data with psychosocial assessment of trait and daily emotional states, measured both prior to and after the collection of mitochondria (28). This enabled us to address two primary questions: 1) whether mitochondrial health was associated with perceived stress, negative affect, and daily mood; and 2) whether mitochondrial health was lower in caregivers. A mediation analysis demonstrated that the effect of caregiver status on mitochondrial health is partially mediated by lower daily positive mood. Overall, our results demonstrate that daily mood influences mitochondrial function, which may contribute to lower leukocyte mitochondrial health in chronically stressed caregivers.

## METHODS AND MATERIALS

### Study Cohort

Data for the present study were derived from a larger longitudinal study focused on the effects of caregiving stress on immunological aging. Individuals were recruited from the San Francisco Bay Area and deemed eligible to participate if they were nonsmokers between the ages of 20 and 50 years and had to be the mother of at least one child between the ages of 2 and 16 years. To be characterized as a high-stress maternal caregiver, the participant had to care for a child diagnosed with autism spectrum disorder and report a score of $\geq$13 on the Perceived Stress Scale (PSS) (29). In contrast, low-stress maternal control subjects were characterized as caring for a neurologically typical child and a reported PSS score of $\leq$19. The PSS eligibility criteria were based on prior national norms (29,30). Structured Clinical Interviews for Diagnostic and Statistical Manual for Mental Disorders for Axis I Disorders were carried out during the eligibility period, and individuals with current psychiatric conditions, including bipolar disorder, posttraumatic stress disorder, and eating disorders, were excluded. Substance use disorders were also exclusionary. Depression was exclusionary among our control participants; however, this was not exclusionary for high-stress caregivers. At the time point at which measures of mitochondrial health were assessed, only 2 participants, both of whom were high-stress caregivers, met diagnostic criteria for depression. Anxiety disorders were not assessed by structured interview and were not exclusionary. Structured Clinical Interviews were readministered at subsequent study time points. This study was approved by the Institutional Review Board at the University of California, San Francisco, and written informed consent was obtained for each study participant.

### MHI Measurements

Platelet-free PBMCs were obtained by differential centrifugation and stored at $-80^{\circ}C$ until measurements were taken. All assays were performed on the same biological samples for each subject. Mitochondrial enzymes were selected based on four criteria: 1) they represent a known biological function (energy production capacity or mitochondrial content); 2) they are detectable in a microplate format for high throughput; 3) they have been shown to respond acutely to metabolic and biological stress; and 4) they are encoded by either the mitochondrial or nuclear genomes (see the Supplement for detailed information about each component). Thus, enzymatic activities were quantified spectrophotometrically for citrate synthase (CS), cytochrome c oxidase (COX, complex IV), and succinate dehydrogenase (SDH, complex II), and expressed per million cells. In parallel, mtDNA and nuclear DNA counts were measured by multiplex quantitative real-time polymerase chain reaction (qPCR) to normalize for cell number and calculate mtDNAcn. Finally, a composite measure, the MHI was calculated by mathematically integrating enzymatic activities and mtDNAcn, as described in Supplemental Figure S1 and in the Supplement, which includes a template MHI calculation file.

### Stress and Mood Measures

Psychological stress was measured using the 10-item PSS (29), depressive symptoms were assessed using the Inventory of Depressive Symptomatology (31), and symptoms of anxiety were measured using the State-Trait Anxiety Inventory (32). Daily mood was assessed over 7 consecutive days using a morning and nightly diary, which encompassed the biological sample collection on day 4. The nightly diary assessed the intensity of positive (e.g., joy, inspired, feeling in control) and negative (e.g., feeling stressed, sad, tired) affective states, which were derived from the modified Differential Emotions Scale (33). Participants also completed a morning diary on awakening that assessed positive and negative affective states as well as expectations on the day to come. Additional details regarding

nightly and morning diary measure are provided in the Supplemental Methods and Materials.

We examined mood on average over the week, and then, to take advantage of the daily data and explore the directionality of effect, examined mood either before or after the blood draw. Daily mood data were extracted and aggregated as pre–blood draw mood, averaging days 1 to 3, and as post–blood draw, averaging days 5 to 7. Thus, we performed analyses on a dual set of experience data to explore the temporal relationship between mood and MHI.

### Data Analysis

Analyses were carried out in four stages. First, we analyzed individual mitochondrial measures and their mathematical integration as MHI. Second, we employed linear regressions to examine across groups whether trait measures of distress or daily mood measured before or after blood draw were related to MHI. Third, analyses of covariance were performed to evaluate group differences between caregiver status (caregiver vs. control) on individual measures of mitochondrial activity (COX, SDH, and CS), mtDNAcn, and MHI. Finally, we performed mediation analysis to examine whether the caregiver difference in MHI was explained in part by differences in trait measures of distress or acute experience in daily affect. Correlation coefficients are computed as Pearson's *r*, unless noted otherwise. For partial least square discriminant analysis of MHI components, singular value decomposition was used to impute missing values (34) in Metaboanalyst 3.0 (http://www.metaboanalyst.ca/faces/docs/About.xhtml) (35), and data were mean centered and divided by the standard deviation of each variable to scale all variables. All other analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC).

For additional information about the study cohort, inclusion and exclusion criteria, questionnaires and daily mood measures, and complete experimental procedures for mitochondrial enzymatic assays, mtDNAcn, mitochondrial protein content, and data analysis, see the Supplement.

## RESULTS

### Demographics

The sample ($n$ = 91) was 43.4 ± 5.4 (mean ± SD) years old on average, with a body mass index of 25.9 ± 5.3, and 17.0 ± 1.8 years of education. There were no significant differences between the caregivers and control subjects in any demographic factors (Supplemental Table S1). Caregiver duration was on average 5.1 ± 3.0 years (range 1.1–13.9). Two caregivers met diagnostic criteria for major depression, and 9 caregivers and 1 control subject were taking antidepressants for mood. This study was not powered to test the association between depression and mitochondrial health, so no conclusion about this potential association can be drawn here. Nevertheless, a sensitivity analysis of the primary analysis excluding those taking antidepressants was conducted, showing no significant effect.

### Mitochondrial Measurements

For each participant, PBMCs were homogenized and the activities of SDH (complex II), COX (complex IV), and CS were measured (Figure 1A). The same lysate for each sample was subsequently used to measure mtDNAcn and to quantify cell number by qPCR (Supplemental Figure S1).

**mtDNAcn and Mitochondrial Content.** We first compared across the full study sample two markers of mitochondrial content—or the number of mitochondria per cell: CS enzymatic activity and mtDNAcn. Traditionally, mitochondrial enzymatic activity is normalized per total protein content (36), a procedure meant to correct for the total number of cells present in each sample. However, protein normalization does not take into account potential differences in cellular protein density or cell size. When applying this standard procedure, CS and mtDNAcn were not significantly correlated (Supplemental Figure S2A). We therefore applied qPCR to precisely adjust cell count in each cell lysate and used this metric to normalize enzymatic activity. This method uses a single copy nuclear gene *B2M* (β2-microglobulin) to determine relative cell number between samples and generate a correction factor that adjusts cell number per homogenate volume. When CS was normalized on a per-cell basis using this novel approach, the markers of mitochondrial content were significantly correlated and shared 69% of their variance (Supplemental Figure S2B), as expected.

We then repeated this analysis for enzymes of the respiratory chain, SDH and COX, two mitochondrial respiratory chain components that contribute to energy production capacity. Again, when normalized to protein, mtDNAcn was not correlated with SDH nor COX activities but was significantly correlated when expressed per cell using our novel approach (Supplemental Figure S2C–F). These results establish that normalization on a per-cell basis is a superior approach to assess mitochondrial functional capacity in human PBMCs.

**Mitochondrial Content Versus Specific Mitochondrial Function.** Mitochondrial content indexed by CS (37) was then compared with respiratory chain activity for SDH and COX. As expected, interindividual differences in mitochondrial content accounted for a substantial portion of the variance in total SDH (59%) and COX (64%) activities (Supplemental Figure S3A, B). Comparatively, SDH and COX activities were more weakly correlated to each other and showed only 33% overlap in variance (Supplemental Figure S3C). This indicated that the activities of both enzymes are likely regulated through largely independent mechanisms, consistent with the fact that SDH is entirely encoded by the nucleus, and that COX is partially encoded by mtDNA. Thus, SDH and COX must contribute mostly independent information about overall mitochondrial health. Similar findings were observed with measurements of mitochondrial respiratory chain proteins (Supplemental Figure S4).

### Development of the MHI

To develop a composite measure of mitochondrial health, we integrated four functional parameters in a simple equation with two numerators and two denominators, which equally represent the nuclear and mitochondrial genomes. Respiratory chain activity (SDH and COX) are mean-centered and added as the numerator, and markers of mitochondrial content (CS and

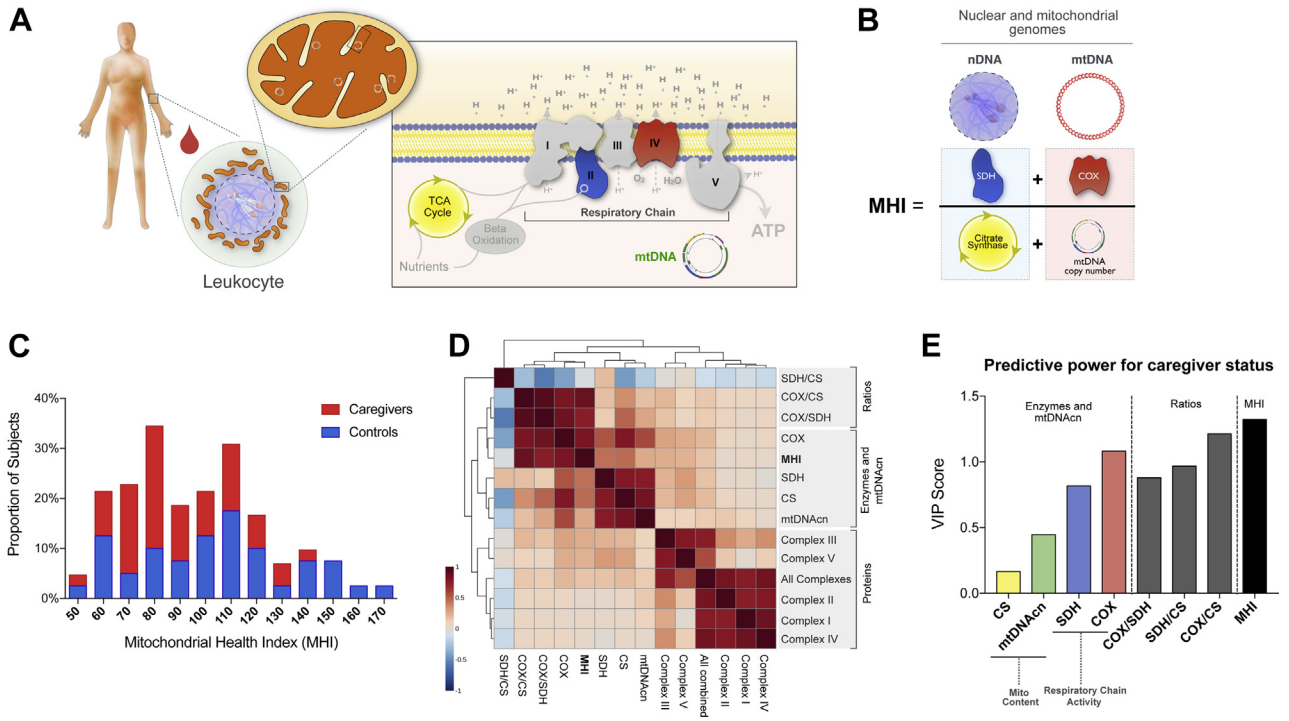


**Figure 1.** Mitochondrial health index (MHI) and mitochondrial profiling in human leukocytes. **(A)** Schematic of a human peripheral blood leukocyte and its mitochondria. The internal components of the mitochondrial respiratory chain (complexes I–V), the tricarboxilic acid ([TCA], also Krebs) cycle, and the mitochondrial genome (mtDNA) are shown in the inset with the inner mitochondrial membrane. **(B)** Mathematical integration of two nuclear DNA (nDNA)-encoded components (left), and mtDNA-related components (right) into the MHI. **(C)** Frequency distribution of MHI in the study sample. See Table 3 for statistics. **(D)** Correlation matrix generated by unsupervised clustering between enzymatic activities, calculated ratios, mtDNA copy number (mtDNAcn), and respiratory chain complexes protein levels. Three major clusters of correlated variables are highlighted. **(E)** Results from partial least square discriminant analysis model showing rank-ordered variables based on their variable importance in projection (VIP) scores for the first component of the full model; $n$ = 89 to 91 for all. VIP values >1 are considered significant. ATP, adenosine triphosphate; COX, cytochrome c oxidase; CS, citrate synthase; Mito, mitochondrial; SDH, succinate dehydrogenase.

mtDNAcn) are also mean-centered and added as the denominator. The quotient of both terms therefore yields a scalar index, the MHI, which reflects respiratory chain capacity per unit of mitochondrial content (see Figure 1B).

In our cohort, the calculated MHI was normally distributed (D'Agostino and Pearson normality test, $K^2$ = 4.25, $p > .10$), with a slight positive skew (skewness = 0.52, kurtosis = −0.34) (Figure 1C). A correlation matrix among all measured and calculated parameters of mitochondrial function showed that all enzyme activities, MHI, and mtDNAcn were most highly related (central cluster), whereas respiratory chain protein levels mostly behave independently from MHI (Figure 1D). Intercorrelations among the four mitochondrial parameters measured and the MHI index are shown in Supplemental Table S2, and the nonsignificant correlations with body mass index and age are presented in the Supplemental Results.

**Comparison of Individual Measures Versus MHI.** To further assess how well the MHI can discriminate between caregivers and control subjects relative to each of its individual components, we performed a partial least square discriminant analysis with caregiver status as the independent variable. We extracted the variable importance in projection scores on the first and most discriminant component of the final model, where a higher score indicates greater power to discriminate between groups (Figure 1E). Scores showed that mitochondrial content (CS and mtDNAcn) were weakest, followed by SDH (nuclear encoded) and COX (mtDNA encoded). Calculated ratios, in which enzymatic activities are normalized to CS (i.e., mitochondrial content), had higher scores than individual components, corroborating the presence of a specific reduction of mitochondrial energy production machinery, rather than a lower mitochondrial content. Of all parameters, the MHI had the highest variable importance in projection score on the first component, confirming that the mathematical integration of individual MHI components results in a composite index with superior predictive potential in relation to caregiver status.

## Daily Mood and Mitochondrial Health

We then examined the relationship between daily mood and MHI. First, we averaged the mean of positive and negative mood measures across the full week to obtain a trait-like measure. In the evening, higher positive affect was significantly related to higher MHI ($r$ = .24, $p < .05$) (Figure 2A). In the morning, there was a trend ($p < .10$) for MHI to be related to greater positive affect ($r$ = .21) and lower negative affect ($r$ = −.19). Then, to understand whether mood had a greater effect on mitochondrial function when it was more proximate to the MHI measurement, we conducted two exploratory analyses examining the association

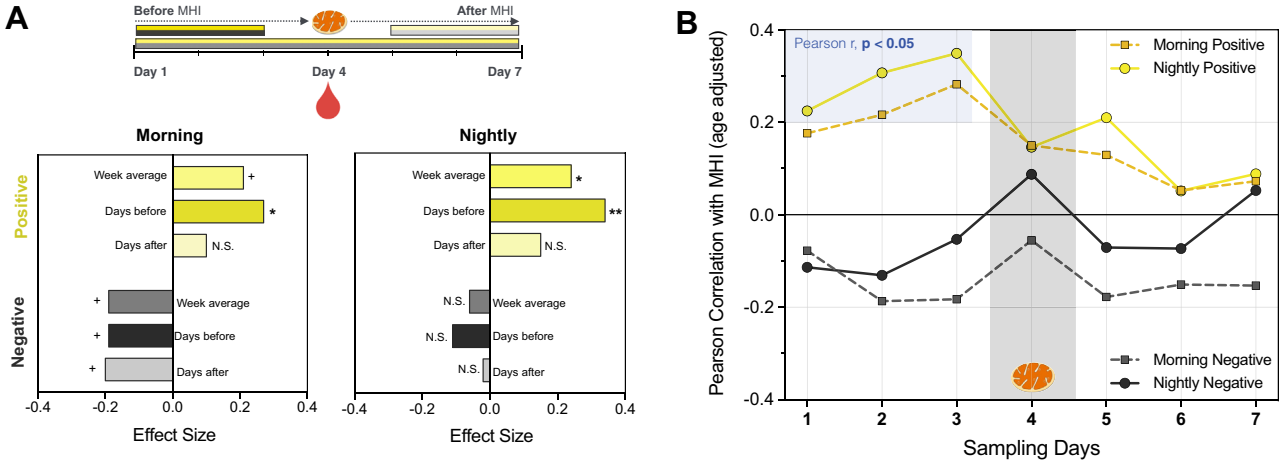


**Figure 2.** Exploratory analysis showing the strength of the association between daily emotional states and peripheral blood mononuclear cells' mitochondrial health index (MHI). **(A)** Combined effect size for the association between MHI and emotional states measured 1) across the week: Week average; 2) over 3 days preceding peripheral blood mononuclear cell collection: Days before; or 3) over 3 days after peripheral blood mononuclear cell collection: Days after. Note that effect sizes are larger for time points preceding blood draw, suggesting a directional relationship from mood to mitochondria. Mood was assessed in the morning and at night as described in the Supplemental Methods and Materials. $^{+}p < .10$; $^{*}p < .05$, $^{**}p < .01$; $n = 86$ to 89. N.S., not significant. **(B)** Individual Pearson's *r* correlation coefficients between daily measures of positive or negative mood and MHI measured from blood drawn at day 4; $n = 86$ to 89.

between MHI and daily mood both before and after biological sampling.

Mood was averaged for each individual over the 3 days before MHI was measured and was compared with the average of the 3 days after. For mood measures assessed before blood was collected, the cells were present in the body and thus exposed to any biological effects of emotional states. To the extent that mood varies daily within an individual, we expected to see stronger correlations with MHI in the "pre" blood draw mood than the "post" blood draw mood measures. Indeed, for mood measures before the biological sample was taken, we found that positive, but not negative, mood in the morning ($r = .27$, $p < .05$) and evening was significantly associated with higher MHI ($r = .31$, $p < .01$) (Figure 2A). In contrast, mood measured in the 3 days after sampling was not significantly associated with MHI ($p > .1$), suggesting a directional effect of mood on mitochondrial health.

This time-dependent association is further illustrated by plotting correlation coefficients between daily mood and MHI (measured at day 4) (Figure 2B). The strength of the association between positive mood and MHI appears to become stronger from day 1 to day 3 (only reaching significance for morning mood, on days 1, 2, and 3), reaching highest values (morning: $r = .28$, night: $r = .35$) the day immediately preceding blood draw. Thus, nightly mood on day 3 alone accounts for approximately 12.3% of the variance of MHI on day 4. None of the mood scores measured after the collection of PBMCs were correlated with MHI, and correlation coefficients appeared to regress toward zero for these time points (Figure 2B). The effect of negative mood was not significant when examined as separate days. This temporal association between positive mood and MHI suggests that the cells that were circulating during the experience of positive mood tended to have higher mitochondrial energy production capacity.

Lastly, we examined MHI in tertiles of positive and negative nightly mood across the whole sample. As shown in Figure 3, there was evidence for a dose response relationship, in the expected direction, between positive mood and higher MHI. Negative mood at night was also related to lower MHI. Individuals with the highest positive mood and/or lowest levels of negative mood had the highest MHI levels. The lowest and highest mood tertiles had MHI values that differed by 16% to 18%, which represent biologically meaningful differences in respiratory chain function.

## Caregiver and Control Group Comparison

Caregivers reported significantly higher levels of perceived stress, depressive symptoms, and trait anxiety than control subjects (Table 1). Caregivers also showed lower levels of daily positive affect and higher negative affect over the week, both upon waking and at night (Table 2). Across the study sample, there were no significant associations between MHI and measures of distress (perceived stress, depressive symptoms, and anxiety) (Supplemental Table S3). There was only a trend suggesting higher levels of depressive symptoms with lower MHI, in the expected direction ($p = .10$). But there are relatively few individuals with high depression scores, and only two caregivers met criteria for major depression (none in the severe range), making this study not adequately powered to assess the association between depression and MHI.

To test the hypothesis that caregivers have lower mitochondrial health, we used standard analysis of covariance models to examine caregiver versus control group differences in enzymatic activities, mtDNAcn, and MHI. Table 3 displays age-adjusted group means across key mitochondrial outcomes. Individual components of the MHI yielded no statistically significant group differences on their own. However, the MHI was significantly lower in caregivers compared with control subjects, indicating lower mitochondrial respiratory chain activity per unit of mitochondria. This is also apparent in

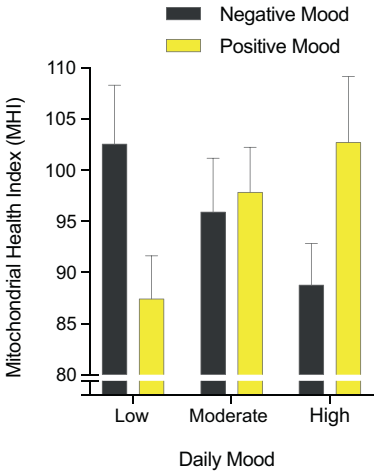


**Figure 3.** Cross-sectional association between mood and mitochondrial health index (MHI). Average MHI for tertiles of nightly negative and positive mood across the 3 days preceding mitochondrial measurements. Data are mean ± SEM; $n$ = 27 to 29 per tertile.

Figure 1C, where a greater proportion of caregivers have lower MHI, and only control subjects have high MHIs >150. This result illustrates the sensitivity of the composite MHI over individual components of the index in relation to caregiver stress and indicates lower mitochondrial quality, but not content, in caregivers.

**Does Daily Mood Mediate MHI in Caregivers?** Given the temporal association between mood and mitochondrial function, and the differences in mood between groups, we reasoned that the differences in MHI between caregivers and control subjects could partially be explained by daily mood. To test this possibility, we ran a series of multiple regression models to test the indirect effects of caregiver status on MHI, via mood from days preceding the blood draw.

As shown in Table 4, in the total effect model (38) caregiver group status is a significant predictor of MHI for both morning and nightly positive mood. This showed an indirect effect of nightly positive mood ($\beta = -5.12$, $p < .05$) (Supplemental Figure S6), suggesting that low positive nightly mood, before the blood draw, may be partly responsible for the lower MHI measured in caregivers relative to control subjects.

**Exploration of Reasons for the Group Differences.** One possible biological cause of lower MHI in caregivers is that they have an imbalance or mismatch between the amount of proteins synthesized by the mitochondrial and nuclear genomes, which is known to result from abnormal intracellular communication and influence the aging process (39,40). Comparing the balance of proteins levels for mtDNA-encoded COX II and nuclear-encoded complexes I and II subunits in control subjects and caregivers separately indicated that differences in MHI between caregivers and control subjects were unlikely due to altered communication between mitochondria and the cell nucleus (Supplemental Figure S5). The number of days since last menstrual period was also unrelated to MHI ($r = -.01$, $p = .96$).

As enzymatic activities could be affected by freezer storage time, we also evaluated duration in the freezer between sampling and measurements as a potential confounding variable. There was a significant correlation between storage time and MHI ($r = -.56$, $p < .001$), as well as with positive mood, perceived stress, anxiety, and depression (see the Supplement for analyses and discussion). This made it impossible to selectively covary out storage time across the whole sample without removing the effect of the group variable. But in the caregiver group, the correlation between positive mood and MHI remained significant and of similar effect size when adjusted for storage time compared with the unadjusted correlation that did not account for freezer time, indicating that the association between daily mood and MHI is invariant to freezer time. However, we cannot rule out the possibility that storage time may nevertheless have contributed to the group difference.

**Table 1. Comparison of Weekly Averages of Daily Mood in Caregivers and Control Subjects**

| | Group | | | | |
|---|---|---|---|---|---|
| | Caregivers | Control Subjects | $t$ | $df$ | Effect Size |
| Morning Mood | | | | | |
| Positive | 1.98 ± 0.53 | 2.43 ± 0.45 | 4.33[a,c] | 86 | 0.92 |
| Negative | 1.60 ± 0.56 | 1.19 ± 0.58 | 3.37[a,b] | 86 | 0.73 |
| Nightly Mood | | | | | |
| Positive | 1.72 ± 0.61 | 2.26 ± 0.50 | 4.60[a,c] | 89 | 0.97 |
| Negative | 0.61 ± 0.47 | 0.46 ± 0.31 | 1.89 | 89 | 0.37 |

Data are mean ± SD. Effect size was calculated as Cohen's $d$ from $t$ test.
[a]Statistically significant value.
[b]$p < .01$, two-tailed $t$ test.
[c]$p \leq .001$, two-tailed $t$ test.

## DISCUSSION

There are multiple pathways by which chronic stress can impact health. Here we developed a new way to quantify this relationship by combining biochemical and molecular mitochondrial measures in human blood leukocytes. The MHI approach integrates measures of both mitochondrial content and functional capacity assessed from the same samples, with a new procedure to normalize enzymatic activities on a per-cell basis. We first confirmed the sensitivity of this method and

**Table 2. Comparison of Mean Distress Scores in Caregivers and Control Subjects**

| | Group | | | | |
|---|---|---|---|---|---|
| | Caregivers | Control Subjects | $t$ | $df$ | Effect Size |
| Depression | 18.32 ± 8.32 | 10.83 ± 6.09 | 4.76[a,b] | 84 | 1.03 |
| Perceived Stress | 20.41 ± 5.74 | 14.61 ± 5.25 | 4.94[a,b] | 86 | 1.05 |
| Anxiety | 45.59 ± 10.83 | 37.16 ± 9.18 | 3.91[a,b] | 85 | 0.84 |

Values are mean ± SD. Effect size was calculated as Cohen's $d$ from $t$ tests.
[a]Statistically significant value.
[b]$p < .001$, two-tailed $t$ tests.

**Table 3. Mitochondrial Enzymatic Activities, mtDNAcn, and MHI by Group**

| | Group | | | | |
|---|---|---|---|---|---|
| | Caregivers | Control Subjects | *t* | *df* | Effect Size |
| COX | 4.35 ± 0.13 | 4.57 ± 0.14 | 1.11 | 83 | 0.24 |
| SDH | 5.40 ± 0.04 | 5.50 ± 0.04 | 1.66 | 86 | 0.36 |
| CS | 6.84 ± 0.05 | 6.88 ± 0.05 | 0.51 | 86 | 0.11 |
| mtDNAcn | 5.94 ± 0.03 | 5.97 ± 0.03 | 0.75 | 85 | 0.16 |
| MHI | 88.06 ± 3.95 | 104.57 ± 4.20 | 2.84[a,b] | 82 | 0.63 |

Mean estimates ± SEM are age adjusted. Effect size was calculated as Cohen's *d* from *t* tests. Values are log transformed for CS, COX, SDH, and mtDNAcn. Mitochondrial health index (MHI) values are not transformed. Enzyme activities are expressed per million cells corrected by quantitative real-time polymerase chain reaction, and mtDNAcn as copies of mtDNA per cell.

COX, cytochrome c oxidase; CS, citrate synthase; mtDNA, mitochondrial DNA; mtDNAcn, mtDNA copy number; SDH, succinate dehydrogenase.

[a]Statistically significant value.

[b]$p < .01$, two-tailed *t* test.

tested the hypothesis that mood is associated with leukocyte mitochondrial health. Collectively, our findings demonstrate three main points: 1) the integrative MHI is an indicator of mitochondrial functional capacity with superior sensitivity to mood and chronic stress than individual mitochondrial measures; 2) chronic caregiving stress may be associated with lower mitochondrial respiratory chain capacity, but not mitochondrial content; and 3) daily mood may partially mediate the effects of caregiving stress on mitochondria.

Despite growing evidence that aging- and stress-related diseases involve mitochondrial dysfunction (18,41,42), this hypothesis has remained difficult to test in sizeable human cohorts. Recent studies have found that life history of depression and early life adversity are associated with greater mtDNAcn measured in whole blood (19,20). However, other studies have found a smaller, rather than greater, mtDNAcn with posttraumatic stress disorder (21), with depression (22),

**Table 4. Regression Models Testing Direct and Indirect Effects of Caregiver Group and Positive Mood on MHI**

| | Morning Positive Mood (*n* = 81) | Nightly Positive Mood (*n* = 85) |
|---|---|---|
| Total Effect | | |
| Caregiver group | −17.62[a,b] ± 6.02 | −16.51[a,b] ± 5.82 |
| Full Effects | | |
| Intercept | 52.37 ± 27.10 | 31.56 ± 26.4 |
| Caregiver group | −13.62[a,b] ± 6.53 | −11.39 ± 6.03 |
| Mood | 8.16 ± 5.39 | 10.67[a,b] ± 4.38 |
| Model $R^2$ | 0.13 | 0.16 |
| Indirect Effect (Bootstrapped, 95% CI) | | |
| Mood | −3.99 (−11.27, 0.82) | −5.12[a,b] (−11.61, −0.94) |

Values are mean ± SEM unstandardized B effects, representing units of mitochondrial health index (MHI), unless otherwise indicated. Daily mood measures were assessed before blood draw. Significance assessed using bias-corrected bootstrap confidence interval (CI). Age is a covariate in each model, not shown here.

[a]Statistically significant value.

[b]$p < .05$.

and in the elderly (43). In addition, early life trauma has also been linked to changes in cellular baseline oxygen consumption measured in cryopreserved whole cells (23). But such measurements could reflect differences in cellular energy demand rather than intrinsic differences in the actual functional capacity of mitochondria. Maximal mitochondrial energy production capacity is more directly quantified in fresh permeabilized cells (44) or, as in the current study, by the maximal rate of key enzymatic activities such as COX and SDH (45).

A limitation of studies measuring only mtDNAcn—not the activity or functional outputs from mitochondria—is that while potentially indicating mitochondrial recalibrations, the number of mtDNA molecules per cell is biologically relevant only to the extent that it enables respiratory chain function. It is therefore difficult to interpret when measured alone. Furthermore, measures in whole blood are confounded by cellular composition. Differences in platelet number substantially alter apparent mtDNAcn without affecting leukocyte mitochondrial function (46,47). Thus, the biological significance of variation in mtDNAcn alone is unclear, particularly when measured in whole blood. In general, laboratory methods currently available to assess the functionality of mitochondria typically require a substantial amount of fresh cells or tissues, involve specialized equipment, have low throughput, and do not readily allow researchers to distinguish between differences in mitochondrial content versus functional capacity.

The MHI addresses these limitations and represents specific mitochondrial energy production capacity on a per-mitochondrion basis—or mitochondrial quality. Here, parallel measurements of the MHI with daily mood provide evidence that mitochondria may respond to psychological stress and mood states. These results are consistent with animal studies on the effects of acute and chronic stress on mitochondrial structure and function (13). However, more research is required to replicate and extend the current findings in humans and to identify the interacting psychobiological mechanisms that may transduce chronic psychosocial stress into changes in mitochondrial health.

Some limitations of the current study must be noted. Though using mothers caring for a child with autism spectrum disorder is a well-established stress model, it could be confounded by an underlying predisposition to mitochondrial dysfunction. This limitation applies to the finding on group differences, but not to the findings relying on within-person differences in mood across the week, which demonstrates a temporal relationship whereby positive mood predicts 10% to 15% of the variance in next day MHI, a meaningful biological effect. Furthermore, although the four-component MHI is a substantial advance over single mitochondrial measurements, it paints only a partial picture of the entire spectrum of possible stress-induced mitochondrial recalibrations. The accumulation of stress-induced mitochondrial structural and functional recalibrations, or mitochondrial allostatic load (10), could manifest at multiple different levels. Furthermore, although mood was measured daily over 7 days, MHI was only measured at one time point, which limits our ability to infer causality. Another technical limitation of this study is the use of mixed leukocyte populations in PBMCs, as opposed to sorted leukocytes lineages, which may contain different mitochondrial phenotypes (48). Furthermore, the potential confound of freezer storage time in relation to the caregiver-control group difference underscores the need to replicate these findings and the

importance for future studies of leukocyte enzymatic activities and mitochondrial health to explicitly analyze storage time by clinical/experimental groups. Future studies should strive to comprehensively assess multiple facets of mitochondrial biology from homogenous leukocyte populations, in cohorts comprising both women and men.

In summary, we present a novel scalable approach to quantify mitochondrial health and establish the extent of interindividual variability in human leukocyte mitochondrial function. This approach provides the first evidence of a directional effect of mood on mitochondrial function, which mediated in part the effect of caregiver status on MHI. Chronic caregiving stress was associated with lower mitochondrial energy production capacity, but not content. These results are consistent with the adverse effects of psychological stress on multiple physiological systems, with mitochondria representing a potential site for the biological embedding of chronic stress exposure.

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## ARTICLE INFORMATION

From the Department of Psychiatry (MP), Division of Behavioral Medicine; Department of Neurology (MP), H. Houston Merritt Center, Columbia Translational Neuroscience Initiative; and the Columbia Aging Center (MP), Columbia University Mailman School of Public Health, New York, New York; the Department of Psychiatry (AP, MC, ESE), and Department of Medicine (KA), Division of Cardiology, University of California–San Francisco, San Francisco, California; the School of Kinesiology (EP), Faculty of Education, University of British Columbia, Vancouver, British Columbia; and the Interdisciplinary School of Health Sciences (AC, YB), Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada.

Address correspondence to Martin Picard, Ph.D., Columbia University Medical Center, Division of Behavioral Medicine, Department of Psychiatry, 622 West 168th Street, PH1540N, New York, NY 10032; E-mail: martin.picard@columbia.edu.

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# A Mitochondrial Health Index Sensitive to Mood and Caregiving Stress

## *Supplemental Information*

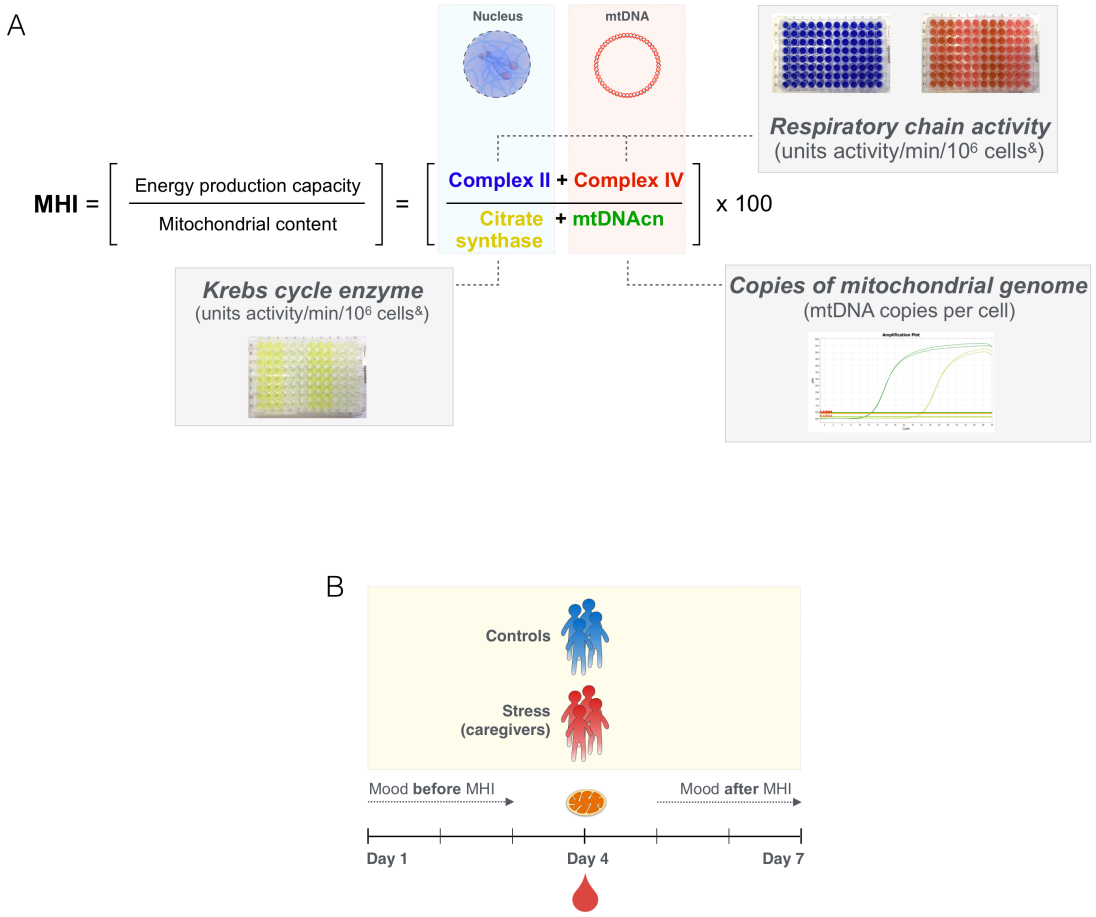
**Supplemental FIGURES S1 - S6**.

**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.

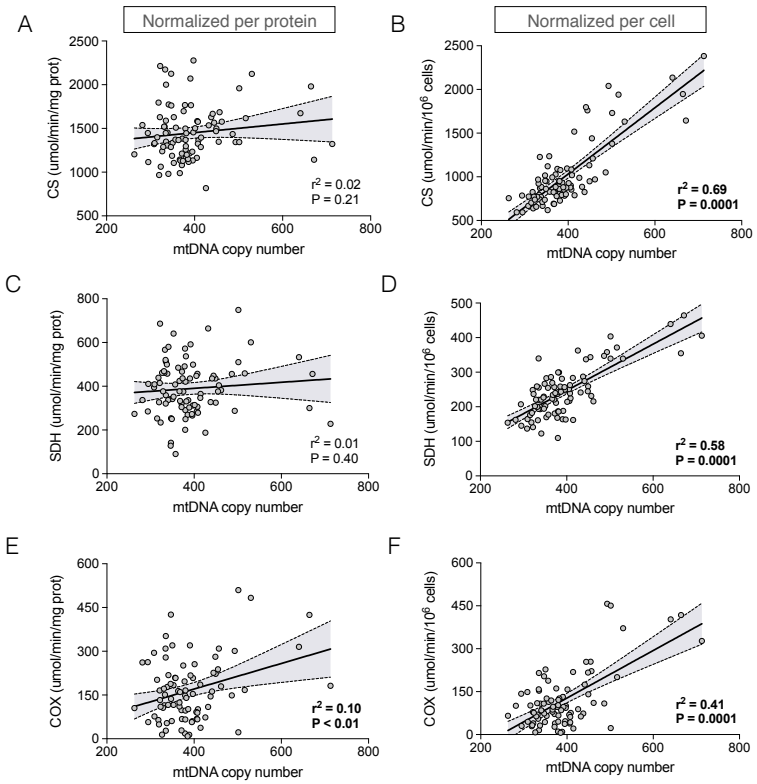
**Supplemental RESULTS AND DISCUSSION**: Detailed discussion mitochondrial proteins analysis, the mediation model, post-hoc analysis of freezer storage time and MHI.

**Figure S1**



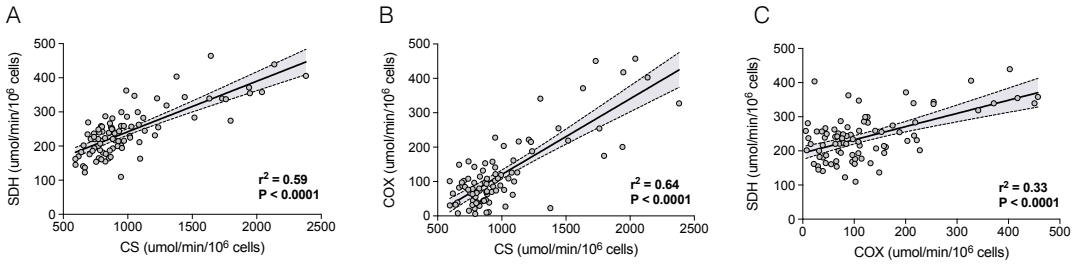

**The Mitochondrial Health Index (MHI) and study design.** (**A**) Simplified MHI formula with energy production capacity at the numerator and mitochondrial content at the denominator, and detailed version of the MHI equation with each component and their units outlined. For calculations, raw values 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**



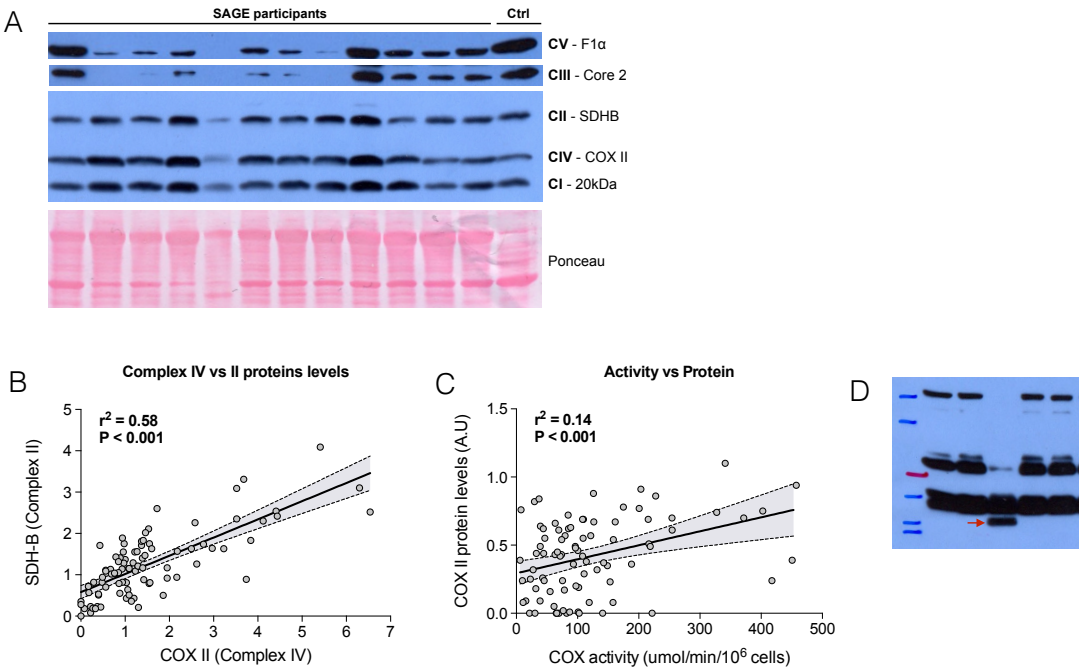

**Normalization of mitochondrial enzymatic activities on a per cell basis improves internal consistency of MHI parameters and mtDNA copy number.** (**A**) Correlations between mtDNA copy number and mitochondrial content marker citrate synthase (CS) activity normalized per protein, or (**B**) per cell 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**



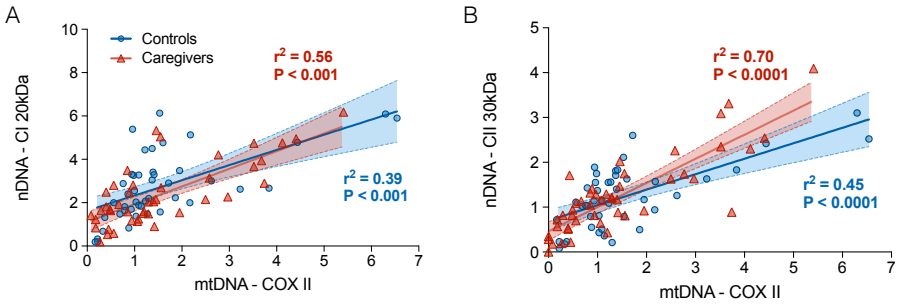

**Inter-correlations of mitochondrial enzymatic activities.** (**A**) Correlation between mitochondrial content marker citrate synthase (CS) and respiratory chain enzymatic activities of the nDNA-encoded succinate dehydrogenase (SDH) and (**B**) the mtDNA-encoded cytochrome c oxidase (COX). (**C**) Correlation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

**Figure S4**



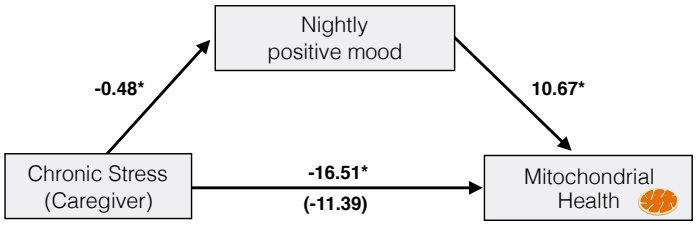

**Analysis of mitochondrial respiratory chain proteins in PBMCs.** (**A**) Representative Western blot for individual subunits from each of the mitochondrial respiratory chain complexes in human PBMCs. A reference control samples (Ctrl) is included for reference. (**B**) mtDNA-encoded COX II and nDNA-encoded 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.

## Supplemental References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