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Epigenetic aging and DNA methylation biomarker changes following ketamine treatment in patients with MDD and PTSD: a pilot study

Kristin L. Dawson[1,7], Athena May Jean M. Carangan[2,7], Jessica Klunder[3], Natalia Carreras-Gallo[2], Raghav Sehgal[4], Samantha Megilligan[1], Benjamin C. Askins[3], Nicole Perkins[3], Tavis L. Mendez[2], Ryan M. Smith[2], Matthew S. Dawson[2], Michael P. Mallin[2], Albert T. Higgins-Chen[5,6] and Varun B. Dwaraka[2]✉

Major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) are debilitating psychiatric conditions associated with poor health outcomes similarly observed in non-pathological aging. Ketamine is a dissociative anesthetic and NMDA receptor antagonist with demonstrated rapid reduction in symptoms associated with Treatment Resistant Depression (TRD) and PTSD. Ketamine's effects on biological aging have not been extensively studied among patients with moderate to severe symptoms of depression and/or trauma. To address this gap, this study looked at the changes in non-epigenetic measures, DNA methylation levels, immune cell composition, and biological age based on various epigenetic biomarkers of aging, of 20 participants at baseline and after completion of a course of six ketamine 0.5 mg/kg infusions in individuals with MDD or PTSD. As expected, depression and PTSD scores decreased in participants following ketamine infusion treatments as measured by the PHQ-9 and PCL-5. We observed a reduction in epigenetic age in the OMICmAge, GrimAge V2, and PhenoAge biomarkers. In order to better understand the changes in epigenetic age, we also looked at the underlying levels of various Epigenetic Biomarker Proxies (EBPs) and surrogate protein markers and found significant changes following ketamine treatment. The results are consistent with existing literature on ketamine's effects on different biomarkers. These results underline the ability of GrimAge V2, PhenoAge, and OMICmAge in particular, to capture signals associated with key clinical biomarkers, and add to the growing body of literature on ketamine's epigenetic mechanisms and their effect on biological aging. Clinical Trial Code. NCT05294835.

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INTRODUCTION

Major depressive disorder (MDD) is a debilitating condition characterized by changes in mood and affect persisting for at least two weeks [1]. Posttraumatic stress disorder (PTSD) occurs in patients who have undergone or seen a traumatic event, and presents as re-experiencing traumatic events, and intense and disturbing thoughts or feelings in afflicted subjects [1]. Studies have linked MDD with increased risk for functional impairments and premature mortality [2, 3], as well as a variety of diseases, including cardiovascular, cerebrovascular, and metabolic disorders [4, 5]. Similar to MDD, PTSD is generally associated with reduced healthspan, lower quality of life, and higher risk of early death [6–8].

In recent years, there has been growing interest in utilizing various aging-related biomarkers, such as telomere length, immune cell proportions, and DNA methylation (DNAm) levels [9] to quantify biological changes that occur when individuals age to help explain adverse health outcomes. Epigenetic clocks are commonly utilized biomarkers of aging based on DNAm. First-generation epigenetic clocks predict chronological age [10, 11]. Second-generation biomarkers of aging, such as the GrimAge, OMICmAge, and DNAmPhenoAge, instead measure clinical features associated with aging [12, 13]. OMICmAge, in particular, has shown strong associations with mortality and various chronic diseases, including depression [14]. Meanwhile, SystemsAge markers capture aging in 11 distinct physiological systems: blood, brain, heart, hormone, immune, inflammatory, kidney, liver, lung, metabolic, and musculoskeletal [15]. Finally, the third-generation biomarker of aging, DunedinPACE, predicts the rate of aging, rather than approximating biological age [16]. DNA methylation is not limited to aging clocks; it can also be used to predict metabolites, clinical measures, and proteins [14, 17], often demonstrating stronger associations with clinical outcomes and disease risk than their non-DNA methylation counterparts [18, 19].

Previous research has used these biomarkers to investigate the impact of MDD and PTSD on biological aging. MDD has been discovered to be linked to multiple biomarkers of DNAm age acceleration [20], as well as advanced DNAm age in blood [21].

[1]Expedition Mental Health 535 Wellington Way Suite 330, Lexington, KY, US. [2]TruDiagnostic, Inc., Lexington, KY, US. [3]Wild Health, Inc., Lexington, KY, US. [4]Program in Computational Biology and Bioinformatics, Yale University, New Haven, CT, USA. [5]Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA. [6]Department of Pathology, Yale University School of Medicine, New Haven, CT, USA. [7]These authors contributed equally: Kristin L. Dawson, Athena May Jean M. Carangan. ✉email: varun.dwaraka@trudiagnostic.com

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Epigenome-wide association studies have also demonstrated the involvement of differentially methylated genes in MDD in a variety of age-related biological processes, including metabolism and inflammatory response [22–24]. Additionally, individuals with MDD have been found to have higher transcriptomic age compared to healthy controls [25]. Likewise, PTSD is positively associated with epigenome-wide DNAm markers of increased cellular age, potentially mediated by single nucleotide polymorphisms (SNPs) in the Klotho longevity gene [26]. It is also reportedly linked with an enhanced pace of epigenetic aging over time, based on measurements from the Horvath and GrimAge clocks [27]. Studies have also found advanced DNAm age in the motor cortex of individuals with PTSD [28], as well as higher RNA age predictions compared to controls [29].

These commonalities between MDD, PTSD, and biological aging bring to light the potential utility of antidepressants and other PTSD medications in protecting against accelerated biological aging. In particular, ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, has been found to induce marked improvement in depressive patients following intravenous administration, causing a drastic reduction in depressive symptoms after only three days of treatment [30]. Further studies have evaluated the effectiveness of ketamine and its enantiomers in monotherapy and adjunctive therapy in treating MDD or treatment-resistant depression (TRD) [31–35]. Research has also explained the prospective benefit of using ketamine on patients with PTSD, and its potential utility in reversing the synaptic abnormalities induced by PTSD [36–38]. In light of these, there is reason to believe that ketamine, as an antidepressant, can potentially reduce biological aging. Literature has demonstrated the ability of psychiatric medications, including antidepressants, to decelerate aging and extend lifespan in model organisms [39–41]. There is also evidence that conventional antidepressant use is linked to lower predicted brain age [42], and lessens mortality and disease risk in humans [43, 44]. We hypothesize that treatment with a novel, rapidly-acting antidepressant will reduce epigenetic aging in individuals with MDD and PTSD.

Nevertheless, there remains a dearth of information on the impact of ketamine on biological aging, as measured by molecular biomarkers and epigenetic aging biomarkers. Therefore, this study aims to comprehensively investigate the effect of a rapidly-acting agent, ketamine, on epigenetic aging in a cohort of patients diagnosed with MDD and/or PTSD whose symptoms did not remit with standard psychiatric treatment. We investigated the individuals' baseline DNA methylation levels, immune cell composition, and epigenetic age, and measured these parameters after ketamine administration. We predict that ketamine treatment will reduce epigenetic age and induce favorable changes in immune cell composition and methylation-based biomarkers of metabolism, clinical traits, and proteins, based on evidence that mental health can influence epigenetic aging [16]. This pilot study shall also provide insight on the biological processes and molecular functions that mediate the effects of ketamine in depression, PTSD, and epigenetic aging.

MATERIALS AND METHODS

Study participants and ketamine administration

To assess ketamine's effect on biological age in patients with MDD and PTSD, 20 participants were recruited by Wild Health, Inc., Lexington, KY in an outpatient clinic setting. Participants completed PHQ-9, PCL-5 and ACE questionnaires prior to a psychiatric evaluation by a double-board certified psychiatrist, in general Psychiatry and child & adolescence psychiatry, to confirm a diagnosis of MDD and/or PTSD. The duration of illness of MDD was at least 2 weeks, while the duration of illness for PTSD was at least 4 weeks. The study participants were predominantly caucasian. Self-reports by participants during that clinical evaluation confirmed an adequate previous trial of at least one standard antidepressant medication for the treatment of MDD and/or PTSD that did not result in remission of diagnosis. Inclusion criteria for participants with MDD required a PHQ-9 score greater than or equal to 15, signifying a moderately-severe to severe depressive mood episode. Participants with PTSD had a PCL-5 score $>=$ 33. Full inclusion and exclusion criteria are listed in Supplemental Appendix A. Participants received six subanesthetic ketamine infusions (0.5 mg/kg) over 2–3 weeks. Participants were required to have a driver available following each treatment and so participant schedule and driver availability determined the infusion dates within that window. Blood samples were collected at baseline and 10 days post-treatment, with 40 samples in total. One participant did not complete all six infusions due to an adverse event characterized by acute psychiatric symptoms leading to an ER visit. Samples were processed at LabCorp for clinical tests and TruDiagnostic Labs for DNA methylation analysis.

Clinical lab measurement collection and analysis

The following panels were taken for the clinical screening laboratory blood tests: Urine Drug Screen and Urine Pregnancy Test (females), Complete blood count (CBC), Comprehensive metabolic panel-14 (CMP-14), Folate (Serum), Free T3, Free T4, Free Testosterone, Vitamin B12, TSH (Thyroid Stimulating Hormone), C-Reactive Protein- Cardiac, Vitamin D- 25-Hydroxy, Apolipoprotein B, Hemoglobin A1c, Homocysteine, NMR LipoProfile + Lipids (Advanced Lipid Panel), Cortisol, and Insulin using clinically validated assays for baseline assessments; obtained by venipuncture for whole blood sampling at a LabCorp facility of the participant's choosing. Lab measurements of 16 patients were available for analysis comparing baseline and post-treatment measurements; the remaining individuals were unavailable due to logistical reasons. Blood was collected and processed at the LabCorp facility most convenient to the participant. EDTA tubes were used for collection, and individuals were fasting at the time of collection. Time of collection, however, was not standardized between baseline and trial collection.

To look at the differences between the participants' non-epigenetic measures, including clinical data from 60 laboratory tests and self-reported scores based on the PTSD Checklist for DSM-5 (PCL-5) and Patient Health Questionnaire-9 (PHQ-9) for MDD, we conducted a Wilcoxon signed-rank test for each set of scores in R. A p-value < 0.05 was used to denote significance.

DNA methylation assessment

Peripheral whole blood samples were obtained using the lancet and capillary method, and preserved through mixing with lysis buffer. 500 ng of DNA was extracted and subjected to bisulfite conversion using the EZ DNA Methylation Kit from Zymo Research, following the manufacturer's protocols. The converted samples were then randomly allocated to designated wells on the Infinium HumanMethylationEPIC 850k BeadChip, and then subsequently amplified, hybridized onto the array, and stained. Samples were then washed and imaged using the Illumina iScan SQ instrument to capture raw image intensities.

DNAm data was pre-processed using the Minfi package in R [45–48]. No outlier samples were identified using ENmix [49] and methylation values were normalized using the ssNoob method [50]. We utilized the k-nearest neighbors algorithm to impute missing CpG values and finally adopted a 12-cell immune deconvolution method to estimate cell type proportions [51].

DNA methylation biomarkers of aging and related measures

Using DNA methylation, we estimated the biological age of the participants by using the following epigenetic biomarkers of aging: DNAmPhenoAge [13], GrimAge [21], GrimAge v2 [52], and OMICmAge second-generation biomarkers, DNAmTL, which estimates telomere length, the third-generation DunedinPACE, and SystemsAge.

We used a custom R script available via GitHub (https://github.com/MorganLevineLab/PC-Clocks) to compute the principal component-based epigenetic clocks for the DNAmPhenoAge, GrimAge, GrimAge v2, and telomere length. For non-principal component-based DNAmPhenoAge epigenetic metrics, we used the methyAge function in the ENmix package in R. DunedinPACE was calculated using the PACEProjector function from the DunedinPACE package from GitHub (https://github.com/danbelsky/DunedinPACE). We calculated the SystemsAge using the authors' script [15], which will be incorporated into the methylCIPHER package upon publication (https://github.com/HigginsChenLab/methylCIPHER). In addition, epigenetic biomarker proxies (EBPs), which are surrogate DNA methylation based markers of clinical, metabolite, and protein estimates

Table 1. Characteristics of participants in the Ketamine trial.

Diagnosis group	Sex	N (Total 20)	Chronological age (mean ± SD)	PCL-5 baseline (mean ± SD)	PHQ-9 baseline (mean ± SD)
PTSD only	Female	4	37.16 ± 10.64	42.25 ± 7.89	8.25 ± 4.43
	Male	1	33.36	48.00	11.00
MDD only	Female	2	43.81 ± 4.75	23.00 ± 8.49	18.00
Comorbid MDD & PTSD	Female	9	43.38 ± 11.28	57.11 ± 8.24	19.22 ± 3.96
	Male	4	36.19 ± 20.15	53.50 ± 12.87	21.00 ± 2.83

Standard deviation (SD) and means are noted.

were calculated using models developed previously [14]. Additional protein estimates calculated from DNA methylation data were also collected by using the Marioni EpiScore predictors [17]; weights are available from the following Github: https://github.com/DanniGadd/EpiScores-for-protein-levels.

We computed the epigenetic age acceleration (EAA) of these metrics by fitting a regression model between each participant's chronological age and their corresponding epigenetic age estimates. EAA represents the residual from this model, where positive values indicate biological age advancement relative to chronological age. Because EAA reflects within-subject deviation, it does not require a separate control group and allows each participant to serve as their own baseline. In fact, using a non-twin control group in DNAm-based studies may introduce between-subject variability unrelated to treatment, potentially obscuring subtle biological effects. To control for potential batch effects, we also included in the regression model the first 3 principal components (PCs) calculated from control probes to the EAA calculation as adjustments, following the methodology prescribed by Joyce et al. [53]. Wilcoxon signed-rank tests were performed using EAA values between timepoints at a significance level of $p < 0.05$. Due to the number of tests conducted for the statistical analysis of clinical test epigenetic clocks, EBP, and Marioni Episcore markers, a multi-test correction was conducted using the "number of independent degrees of freedom" approach previously described. Briefly, principal components of each measure were calculated using prcomp() in R, and the minimum number of PC's which achieve at least 95% cumulative variance was used to adjust for Bonferroni correction [54, 55]. We then conducted a correlation analysis between the biomarkers of aging and all non-epigenetic measures to investigate if the changes in lab values and questionnaire responses are concordant with changes in the predictions of biological age. Finally, we compared baseline and post-treatment levels of DNA methylation surrogate markers for multiple proteins, metabolites, and clinical variables.

Sensitivity analysis with mixed-effects models

To evaluate whether changes in leukocyte composition influenced epigenetic age acceleration (EAA), we conducted linear mixed-effects (LME) models using the lme4 package in R. For each clock, EAA was modeled as a function of time (post-treatment vs. baseline), with a random intercept for participant ID. Two models were tested: Model 1 included sex, age, array principal components (PC1–PC3), and the two immune cell types that showed change following ketamine treatment (CD4 memory T-cells and neutrophils); Model 2 additionally included all 12 estimated immune cell fractions.

Effect size estimation

To quantify the magnitude of change in biomarker outcomes following ketamine treatment, we calculated Glass's Δ for each epigenetic biomarker proxy and Marioni EpiScore marker. Glass's Δ is a variation of the standard Cohen's *d* that is particularly well suited for paired pre/post designs in which the treatment may alter outcome variance. Rather than using a pooled standard deviation, Glass's Δ divides the mean difference by the standard deviation of the pre-treatment group only. This reduces sensitivity to post-treatment dispersion and provides a more stable and conservative effect size in small-sample studies.

$$\text{Glass's } \Delta = (X_{post} - X_{pre})/s_{pre}$$

where X_{pre} and X_{post} denote the group means at baseline and follow-up, respectively, and s_{pre} is the standard deviation of the baseline group.

This approach is widely recommended in intervention studies where treatment effects may impact both central tendency and variance.

Epigenome-wide association analysis

We used the limma package to perform an epigenome-wide association study (EWAS), comparing genome-wide CpG methylation between pre- and post-treatment [56]. Empirical Bayes regression models were adjusted for sex, age, 12 immune cell proportions, and three principal components. Moderated t-tests were used to assess for significance, and probes with an unadjusted p-value < 0.001 were considered significantly differentially methylated. While the false-discovery rate (FDR) was calculated, it was not used as a filter. LogFC values indicated hypermethylation (logFC > 0) or hypomethylation (logFC < 0) post-treatment. Enrichment analysis using rGREAT identified gene ontology (GO) terms associated with differentially methylated loci (DMLs) in promoter and enhancer regions.

RESULTS

This study analyzed clinical and epigenetic data from patients diagnosed with MDD and/or PTSD (n = 20) before and after administration of ketamine infusions over the time-course. Of the 20 participants, 65% (n = 13) had a comorbid diagnosis of MDD and PTSD, 10% (n = 2) were diagnosed only with MDD, and 25% (n = 5) had a diagnosis of only PTSD (Supplementary Fig. 1). Mean baseline scores in the PCL-5 and PHQ-9 are also described. Blood samples were taken to evaluate DNA methylation levels among the patients to calculate their epigenetic age, the immune subsets, and the DNA methylation levels pre- and post-ketamine treatment. Subjects were made up of 75% (n = 15) female and 25% (n = 5) male participants, with a mean chronological age of 41.78 and 35.62, respectively. Table 1 details the demographic information of patients recruited in this study.

Ketamine treatment results in a significant decline in PCL-5 and PHQ-9 scores, but not in other clinical variables

We utilized a Wilcoxon signed-rank test to compare the participant's scores (N = 20) before and after receiving the ketamine treatment course in the PCL-5 and PHQ-9. Results showed a significant decline in both PCL-5 (p = 0.00000381) and PHQ-9 (p = 0.0000949) scores after the participants received treatment with ketamine, with a median difference of 33 and 11 points, respectively. On the other hand, none of the laboratory tests (N = 16) exhibited a significant difference following ketamine administration (Supplementary Table 1).

Ketamine treatment reduces epigenetic age in the third generation OMICmAge biomarker in patients with MDD and/or PTSD

Given that MDD and PTSD are associated with increased morbidity and mortality, we investigated ketamine's effect on epigenetic biomarkers of aging most related to mortality risk. These included PhenoAge, PC PhenoAge, PC GrimAge, GrimAge v2, and OMICmAge; the DunedinPACE; and Systems Ages for blood, brain, heart, hormone, inflammation, immune, kidney, liver, lung, metabolic, musculoskeletal, and overall SystemsAge. We also

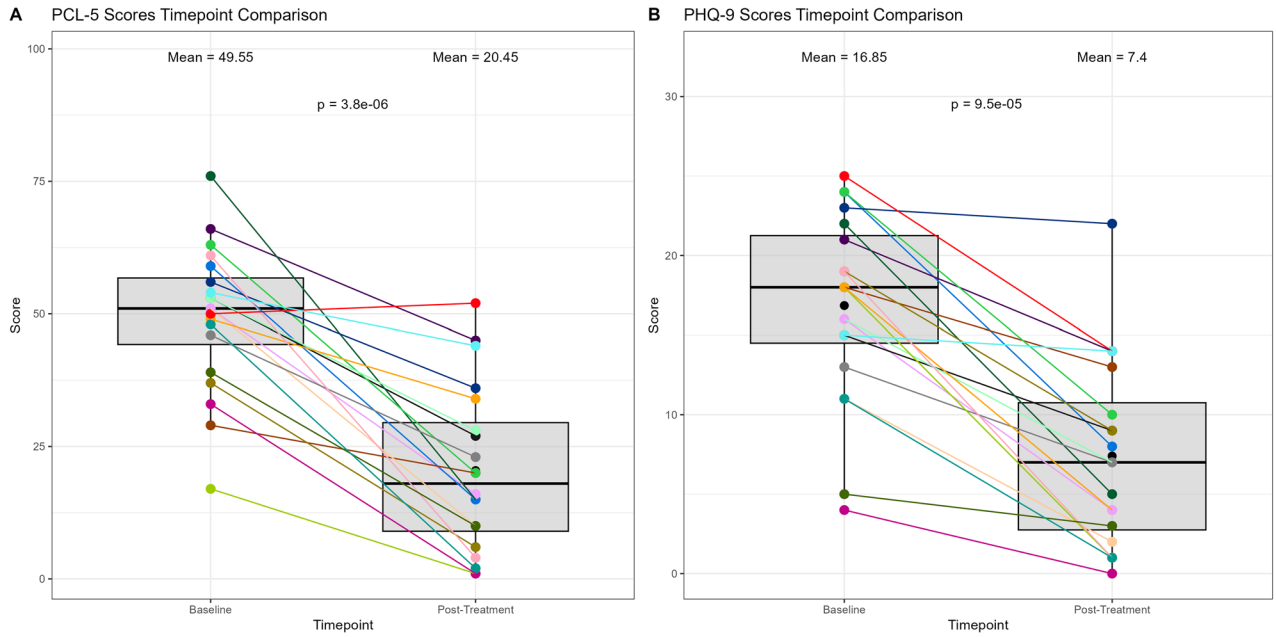

Fig. 1 Changes in PCL-5 and PHQ-9 Scores Before and After Ketamine Treatment Course. A PCL-5. **B** PHQ-9. The y-axis shows the reported score for each evaluation, while the x-axis represents the timepoints for treatment with ketamine. Each color corresponds to one patient, with a line connecting their evaluation score from baseline to post-treatment. Colors across panels also correspond to the same individual. P-values reported are unadjusted.

investigated DNAmTL, a telomere length proxy, as telomere length has been reported to be reduced in MDD and PTSD [57]. Where applicable, we utilized the principal component versions of the biomarkers, which have high test-retest reliability, which is especially important for longitudinal studies [58].

Results revealed a significant reduction in epigenetic age between timepoints, PhenoAge ($p = 0.024$), GrimAge V2 ($p = 0.021$), and OMICmAge ($p = 0.0094$) (Fig. 1). However, after adjusting by principal component variance explaining clocks, only OMICmAge retained significance (PC-adjusted Bonferroni p-value = 0.047). Other epigenetic markers did not demonstrate any significant changes; however, most epigenetic biomarkers exhibited a downward trend in epigenetic age after Ketamine treatment (Supplementary Table 2). Interestingly, Systems Age Inflammation approached significance, with an unadjusted p-value of 0.076.

Ketamine treatment decreases CD4T memory cells in the blood

We quantified 12 different immune cell subsets between timepoints using EpiDISH (2023), and found a significant reduction in CD4T memory cells (unadjusted $p = 0.038$) after ketamine treatment (Fig. 2D). There was also a trend towards increased neutrophils (unadjusted $p = 0.053$). Meanwhile, CD4T Naïve, CD8T Naïve, CD8T Memory, B Naïve, B Memory, Basophils, regulatory T-cells, Eosinophils, Monocytes, and Natural Killer cells did not demonstrate any notable difference after ketamine treatment (Supplementary Table 3). Because the immune cell subset analysis was limited to 12 comparisons and guided by prior biological hypotheses, we report unadjusted p-values but provide adjusted p-values in Supplementary Table S3 for reference.

To verify that our clock findings were not confounded by shifts in leukocyte composition, we fit linear mixed-effects models with a random intercept for participants and two levels of immune-cell adjustment (Supplementary Table 4). In Model 1 (age, sex, PCs 1–3, CD4 T-memory, neutrophils), OMICmAge EAA remained significantly reduced after treatment ($\beta = -1.81 \pm 0.65$ yrs, $p = 0.017$), whereas changes in GrimAge v2 and PhenoAge were not significant ($p > 0.15$). Model 2, which added all 12 imputed blood-cell fractions, yielded an almost identical point estimate for OMICmAge ($\beta = -1.77 \pm 0.86$ yrs) but a marginal p-value ($p = 0.053$) owing to the loss of residual degrees of freedom in this 20-participant cohort. Direction and magnitude of the β-coefficients for all clocks were stable across both models, indicating that leukocyte composition explains little, if any, of the ketamine-associated age reversal. Together with the primary Wilcoxon results, these sensitivity analyses confirm that the observed reduction in OMICmAge is robust to model specification and blood-cell adjustment.

Changes in clinical variables did not correlate strongly with ketamine-induced differences in epigenetic age, but were consistent among samples

To investigate whether there is a relationship between the changes in clinical variables and biological age, we conducted a correlation analysis between all these markers (Supplementary Fig. 2). OMICmAge showed a moderate negative correlation with HbA1c ($r^2 = -0.58$, $p < 0.001$) and red blood cell distribution width ($r^2 = -0.52$, $p < 0.001$), and a positive correlation with serum folate levels ($r^2 = 0.62$, $p < 0.001$), which negatively correlated with PhenoAge ($r^2 = -0.72$, $p < 0.001$) and GrimAge V2 ($r^2 = -0.17$, $p < 0.05$). Both OMICmAge ($r^2 = -0.48$, $p < 0.001$) and GrimAge V2 ($r^2 = -0.49$, $p < 0.001$) showed moderate negative correlations with absolute lymphocyte count, while PhenoAge did not ($r^2 = 0.13$, $p > 0.05$). GrimAge V2 also had a negative correlation with lymphocyte count ($r^2 = -0.61$, $p < 0.001$) and a positive correlation with neutrophils ($r^2 = 0.64$, $p < 0.001$). PhenoAge differed by showing positive correlations with creatinine ($r^2 = 0.52$, $p < 0.001$), total protein ($r^2 = 0.65$, $p < 0.001$), WBC ($r^2 = 0.5$, $p < 0.001$), globulin ($r^2 = 0.59$, $p < 0.001$), and albumin ($r^2 = 0.5$, $p < 0.001$), which were weakly or negatively correlated with the other biomarkers. PhenoAge also had negative correlations with eGFR ($r^2 = -0.58$, $p < 0.001$) and BUN/Creatinine Ratio ($r^2 = -0.5$, $p < 0.001$), whereas these were positively correlated with GrimAge V2 (eGFR: $r^2 = 0.43$, $p < 0.001$; BUN/Creatinine Ratio: $r^2 = 0.17$, $p > 0.05$) and OMICmAge (eGFR: $r^2 = 0.41$, $p < 0.001$; BUN/Creatinine Ratio: $r^2 = 0.33$, $p < 0.001$). Notably, changes in

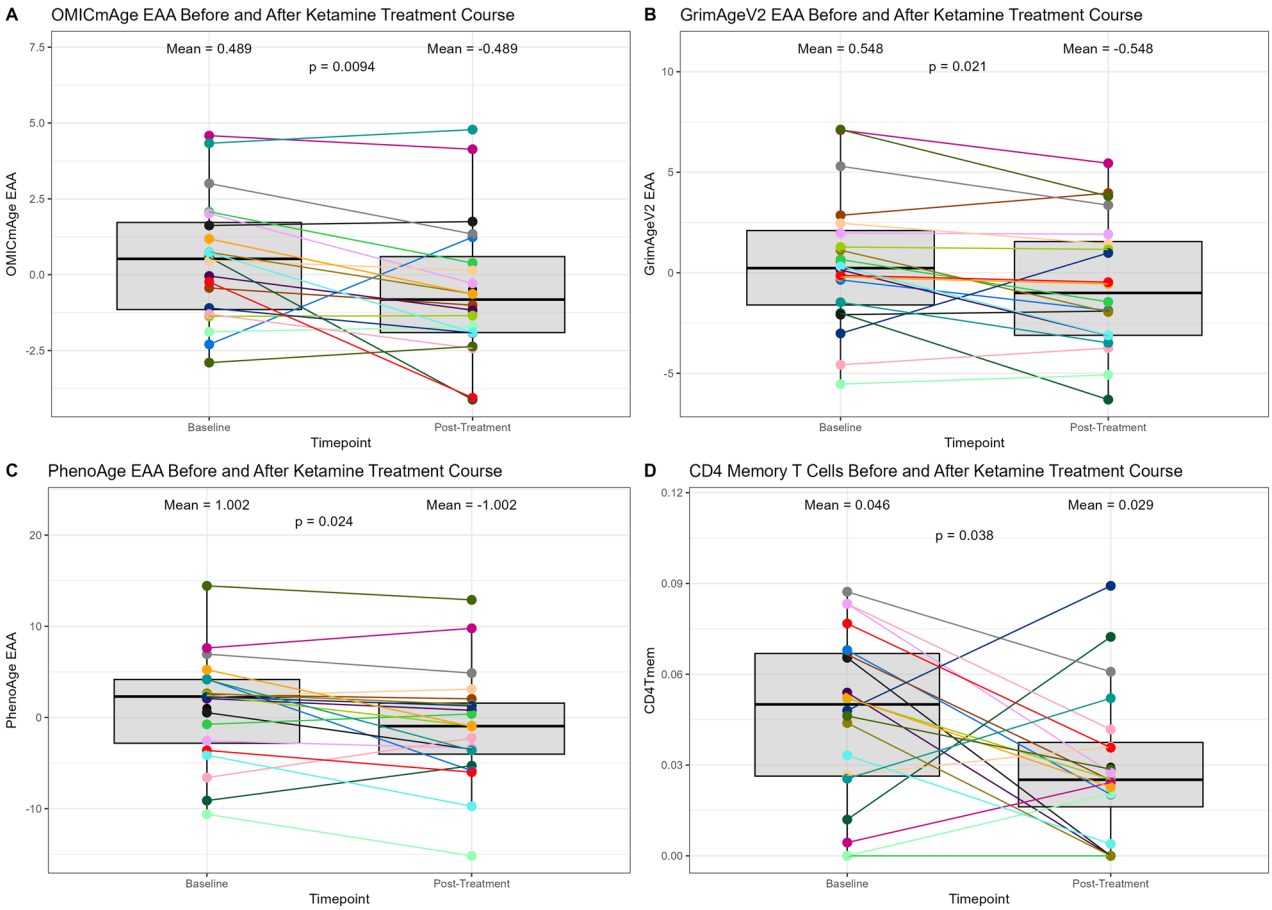

Fig. 2 Changes in Epigenetic Biomarkers of Aging Before and After Ketamine Treatment Course. A OMICmAge. **B** GrimAge V2. **C** PhenoAge. **D** CD4 Memory T-cells. The y-axis shows the epigenetic age acceleration (EAA) for each biomarker of aging and the immune cell proportion of CD4 Memory T-cells. The x-axis represents the timepoints for treatment with ketamine. Each color corresponds to one patient, with a line connecting their predicted marker from baseline to post-treatment. Colors across panels also correspond to the same individual. P-values reported are unadjusted.

PCL-5 and PHQ-9 scores did not correlate with OMICmAge, despite significant decreases in all three measures after ketamine treatment. GrimAge V2 did not significantly correlate with PCL-5 but had a weak correlation with PHQ-9 ($r^2 = 0.04$, $p < 0.01$). PhenoAge exhibited a negative correlation with both PCL-5 ($r^2 = -0.39$, $p < 0.001$) and PHQ-9 ($r^2 = -0.16$, $p < 0.05$).

Ketamine exhibits significant effect on epigenetic biomarker proxies and Marioni protein markers

We further assessed the impact of ketamine usage upon epigenetic biomarker proxies (EBPs), which are DNA methylation surrogate markers for multiple plasma proteins, metabolites, and clinical variables [14]. We also examined Marioni Episcores [17], which are DNA methylation surrogates of additional plasma proteins trained in a different cohort than the EBPs. Results revealed 17 significantly different EBPs pre- and post-treatment (Table 2) out of 396 EBPs based on an PC-adjusted Bonferroni p-value < 0.05. Analysis of the Marioni Episcore protein estimates identified 13 Episcore protein markers out of 116 as exhibiting a significant (PC-adjusted Bonferroni p-value < 0.05) difference pre- and post-ketamine treatment after comparison with the Wilcox test (Table 2).

Ketamine treatment resulted in locus-specific epigenome wide changes

We conducted an epigenome-wide association study (EWAS) to examine DNA methylation changes after ketamine treatment, adjusting regression models for age, sex, 12 immune cell levels, and 3 principal components. To control for multiple comparisons, we used FDR and selected a model with a lambda value of 1.14, indicating no overfitting. We identified 1144 CpG sites ($p < 0.001$), with 764 hypermethylated and 380 hypomethylated after treatment (Fig. 3). Only one CpG site at Chromosome 12 (cg03703650) was significantly hypomethylated (FDR = 0.0025), located in the promoter of the advillin gene, involved in actin bundling and neurite outgrowth.

Using the 1144 CpG sites meeting the uncorrected p-value < 0.001, we performed two enrichment analyses, one for hypermethylated and another for hypomethylated sites. Among hypermethylated CpG sites, we identified an enrichment in processes regulating the nuclear cycle and DNA replication biological processes related to the immune system, such as regulation of T cell differentiation and mast cells, and molecular functions linked mainly to 1-phosphatidylinositol regulator activity. On the other hand, hypomethylated CpG sites were enriched in the regulation of circadian sleep, the growth plate cartilage morphogenesis, cervix development, and hindbrain tangential cell migration. We also found that these sites are mainly related to indoleamine 2,3-dioxygenase activity. The full list of associated gene ontology terms can be found in Supplementary Tables 5 and 6.

DISCUSSION

This study found that administering ketamine, a rapid-acting antidepressant and off-label drug for PTSD, led to significant

Table 2. Effect of Ketamine treatment on epigenetic biomarker proxies and Marioni EpiScore markers.

Epigenetic Biomarker Proxies			
	Glass Δ	**Wilcox p-value**	**PC-adjusted Bonferroni**
HAP28_HUMAN	0.01186	0.007296	0.007296
Eicosenoylcarnitine (C20:1)*	0.29262	0.010689	0.010689
Glucuronide of piperine metabolite C17H21NO3 (4)*	−0.18962	0.015312	0.015312
BUN_MRS	−0.02076	0.017181	0.017181
adenosine	0.21264	0.017181	0.017181
malonate	0.04982	0.017181	0.017181
Ethyl alpha-glucopyranoside	0.21809	0.019234	0.019234
cytosine	0.05052	0.019234	0.019234
3beta-hydroxy-5-cholenoate	0.12882	0.023951	0.023951
F10A1_HUMAN	−0.02942	0.029575	0.029575
Eicosenedioate (C20:1-DC)*	0.34356	0.029575	0.029575
3-methoxytyrosine	−0.0808	0.032768	0.032768
deoxycarnitine	0.21411	0.036234	0.036234
BMP1_HUMAN	0.01636	0.039989	0.039989
lactose	0.1088	0.044054	0.044054
Sebacate (C10-DC)	9.06761	0.048441	0.048441
proline	−0.30485	0.048441	0.048441
Marioni EpiScores			
	Glass Δ	**Wilcox p-value**	**PC-adjusted Bonferroni**
NRTK3	0.02811	0.008308	0.008308
RARRES2	−0.05664	0.010689	0.010689
MMP.1	−0.03449	0.013617	0.013617
Coagulation.factor.VII	0.01541	0.019234	0.019234
Galectin.4	−0.02226	0.021484	0.021484
Contactin.4	0.02742	0.026642	0.026642
MMP.2	0.00186	0.029575	0.029575
TNFRSF17	0.03661	0.029575	0.029575
CCL10	0.24618	0.032768	0.032768
TPO	−0.01753	0.032768	0.032768
SHBG	−0.02132	0.036234	0.036234
CD48.antigen	0.00873	0.044054	0.044054
Granzyme.A	0.0149	0.044054	0.044054

Shown are the mean pre- and post-treatment values, mean differences, unadjusted p-values, and effect sizes (Glass's Δ). Glass's Δ is calculated as the mean change divided by the pre-treatment standard deviation and provides a robust effect size when pre- and post-treatment variances differ.

reductions in self-reported PTSD and MDD symptoms measured using PCL-5 and PHQ-9, as well as decreases in biological age according to the OMICmAge clock, but not among any other clock assessed. OMICmAge's significant decline highlights its unique biomarker proxies' ability to detect associations with depression, PTSD, and potentially other brain diseases. Created by integrating 16 protein, 14 metabolite, and 10 clinical epigenetic biomarkers across 8 biological systems, OMICmAge is particularly robust in identifying factors central to biological aging [14]. In fact, previous investigation showed OMICmAge having the highest odds and hazard ratios for depression compared to other aging biomarkers [14]. Ketamine administration has also been linked to changes in the metabolites and proteins used in OMICmAge, such as ribitol, uridine, triglycerides, and creatinine. Animal studies in rodents found a change in ribitol and other key elements in energy metabolism, such as phosphate and propanoic acid, post-ketamine treatment [59]. Creatine and phosphocreatine have also been observed to be downregulated in the hippocampus of both rats and mice after one dose of ketamine [60, 61]. There also seems to be a reduction in uridine and glutamine as opposed to an increase in urea concentrations in ketamine-treated rats [60, 62, 63]. Similarly, human studies have discovered an increase in triglycerides, cholesteryl esters, and several phosphatidylcholines in the human plasma of both healthy patients and those with treatment-resistant depression [64, 65]. This may explain OMICmAge's effectiveness in capturing epigenetic aging changes in patients with MDD and/or PTSD.

The reduction in epigenetic ages, particularly in OMICmAge, appears to reflect coordinated biological changes rather than a black-box artifact. Several methylation-based surrogate markers that contribute to OMICmAge, including adenosine, BUN, carnitines, and proline, significantly shifted following ketamine treatment, aligning with known ketamine-responsive pathways. Additionally, we observed a decline in CD4T memory T cells, a population positively associated with biological age and systemic inflammation. Epigenome-wide analyses further revealed changes in CpG sites enriched for genes involved in immune regulation and circadian rhythm, both mechanistically linked to mood and aging. Furthermore, while composite omic clocks like OMICmAge reduce Type I error by integrating across multiple biomarkers, our findings suggest that OMICmAge is not simply averaging noise. Rather, it appears uniquely sensitive to ketamine's biological effects. Unlike other clocks, OMICmAge integrates methylation surrogates for proteins, metabolites, and clinical markers across eight physiological systems - including several (e.g., adenosine, BUN, carnitines) that significantly changed post-treatment and are known to intersect with ketamine's pharmacodynamics. Taken together, these points support the idea that OMICmAge reflects coordinated neuroimmune and metabolic changes induced by ketamine, rather than capturing an arbitrary statistical signal.

Epigenetic biomarker proxies (EBPs) offer a rapid, scalable approach to survey molecular changes across metabolic, inflammatory, and signaling pathways implicated in depression and ketamine response [14]. Prior research has linked some of these EBPs, such as proline, blood urea nitrogen (BUN), carnitines, and adenosine, to depression. For instance, high proline levels may worsen depressive symptoms via the microbiota-gut-brain axis; BUN and carnitines in combination have shown protective effects against depression [66]; and BUN independently has shown protective effects against depression [35, 67, 68]. Carnitines were found to decrease in depressive patients and subjects with a history of trauma, and carnitine supplementation has been shown to improve symptoms [69–71]. Adenosine, associated with both depression and ketamine treatment, plays a role in ketamine's anti-inflammatory and antidepressant effects by regulating glutamate neurotransmission [72]. In mice models of depression, ketamine upregulated the adenosine 1 receptor, leading to antidepressant and anti-anxiety effects [73]. It also increased AMPAR activation, which inhibited glutamate release, suggesting that ketamine's antidepressant effects may involve the regulation of glutamate neurotransmission [74].

Previous studies have linked significant Marioni protein markers, such as Matrix metalloproteinases (MMPs) 1 and 2, blood coagulation factor VII, sex hormone binding globulin (SHBG), and TNFRSF17, to depression risk and prognosis. MMPs regulate

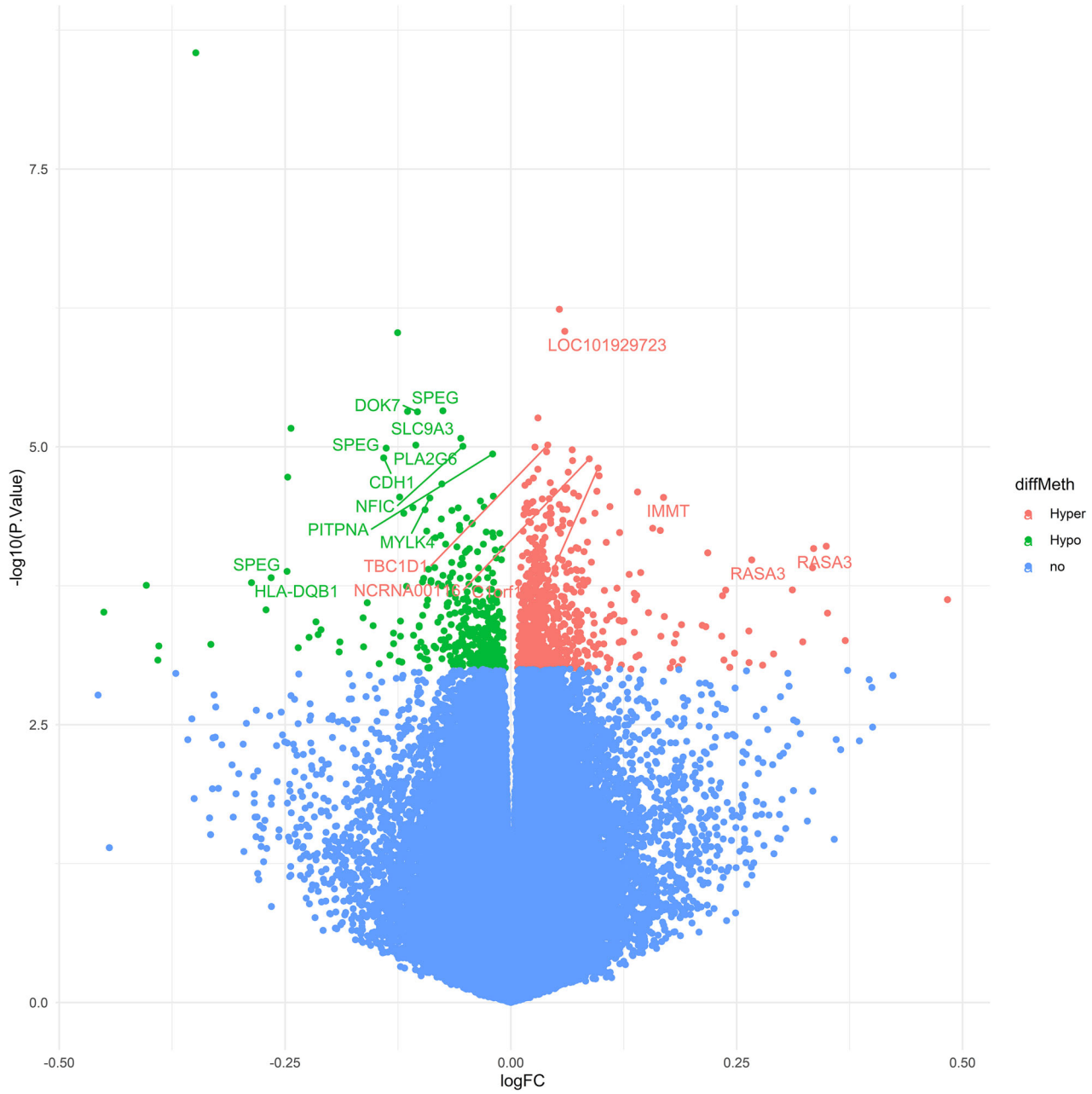

Fig. 3 Volcano plot showing hyper- and hypomethylation of CpG sites between timepoints. The x-axis represents the log 2 fold change in DNA methylation levels between cases and controls, while the y-axis displays the -log10 of the p-value for each CpG site. CpG sites with an FDR below 0.05 and exhibiting hypermethylation are shown in red, while those with an FDR below 0.05 and displaying hypomethylation are colored green. Moreover, we annotated the significant CpG sites with the genes where these probes are mapped. Dots without gene annotations are intergenic CpG sites.

inflammation and cytokine processing, with elevated MMP2 levels in cerebrospinal fluid associated with depression and schizophrenia, proportional to symptom severity. However, some studies report decreased MMP2 expression in depression [75]. Coagulation factor VII has also been linked to increased depression risk [76] and suicidal behavior [77], especially in aging populations. Meanwhile, SHBG has been linked to cognitive impairment in patients with comorbid schizophrenia and depression, with elevated SHBG levels associated with higher depression risk in women, particularly post-menopause [74, 78]. Finally, TNFRSF17 has been found to be upregulated [79] in women with postpartum depression [80], although no link has been established with MDD or PTSD. Together, these findings show a clear link between MDD and several EBPs and Marioni protein markers, highlighting their ability to capture disease-specific changes in MDD and PTSD.

Additionally, this study revealed pathways related to hypomethylated and hypermethylated CpG sites post-ketamine treatment, offering insights into ketamine's antidepressant and PTSD effects. Notably, regulation of T cell differentiation was enriched in hypermethylated CpG sites, aligning with studies demonstrating the involvement of ketamine in the inhibition of the differentiation and reactivation pathogenic Th17 cells, which is upregulated in patients with MDD [81, 82]. Ketamine's suppression of Th17-mediated neuroinflammation via IL-16/STAT3 inhibition supports its role in reducing CNS inflammation, a key mechanism behind its antidepressant and PTSD effects. Additionally, hypomethylation of

CpG sites linked to circadian regulation suggests ketamine may improve sleep duration and quality. Sleep disorders are a known symptom and risk factor for PTSD [83, 84], and research has shown an association between MDD and PTSD and poor sleep quality, resulting in abnormal mood [85–87], and aberrant expression of circadian clock genes in depressive patients [88]. Ketamine's rapid antidepressant effects may also involve resynchronizing the central clock to light cycles, causing decreased waking, and increased sleep, REM sleep, and slow-wave activity [89, 90]. Indeed, previous studies suggest ketamine has a chronotherapeutic effect, improving mood by shifting patients away from an "evening" body clock [91], further emphasizing the role of epigenetic changes in ketamine's antidepressant action and its impact on epigenetic age. These neuroimmune and circadian effects converge on key pathways regulating affective behavior, cognition, and stress reactivity, thus providing a plausible molecular basis for ketamine's rapid and sustained impact on depression and PTSD symptoms.

Emerging evidence suggests that T-cell–driven inflammation and circadian dysregulation can directly influence mood and stress-related behavior through several neuroimmune pathways. For example, proinflammatory $CD4^+$ T-cell subsets such as Th17 have been shown to promote neuroinflammation and synaptic dysfunction, both of which are implicated in MDD and PTSD pathophysiology [81, 82]. Ketamine's suppression of Th17 cell differentiation and IL-6/STAT3 signaling may help mitigate this inflammatory cascade. In parallel, disruptions in circadian clock genes can impair glucocorticoid regulation and monoamine neurotransmission, contributing to mood instability and sleep disturbances—core features of both depression and PTSD [86, 88]. By targeting both neuroimmune and circadian systems, ketamine may help restore homeostasis across brain regions involved in mood regulation, offering a mechanistic explanation for the observed improvements in symptoms and biological age markers.

Through our methylation analysis, we also discovered one CpG site with an adjusted p-value of 0.0025 associated with the advillin gene, which has been linked to axon regeneration and reduced neuropathic pain [76]. Advillin is expressed in the habenula [92], which plays a role in depression and the sustained antidepressant effects of ketamine [93]. However, it is not exclusive to that region. It is also broadly expressed in sensory neurons derived from the neural crest and has been implicated in neuronal outgrowth and cytoskeletal remodeling [92]. Further studies are needed to further establish a link between this gene and MDD or PTSD, and thus, further investigation is needed to better understand the role of the methylation of this specific location in the context of these diseases.

This pilot study identified a significant decrease in CD4T memory cells, which typically increase with age as CD4T naïve cells decline [94]. CD4T memory cells are also positively associated with biological age and multimorbidity, implying that lower biological age correlates with reduced CD4 memory T-cells [95]. Reduction in CD4 memory T-cells after ketamine treatment may suggest its potential to lower biological age and improve outcomes in depression and PTSD. Notably, only SystemsAge Inflammation neared significance among epigenetic aging markers, highlighting the immune system's role in reducing epigenetic age post-treatment. The moderate negative correlation between lymphocyte count and OMICmAge and GrimAge V2 supports this link.

Further investigation is needed to clarify the relationship between OMICmAge, GrimAge V2, PhenoAge, and clinical variables. While ketamine treatment significantly reduced PCL-5 and PHQ-9 scores, these changes did not correlate with reductions in epigenetic age. Similarly, no significant changes were observed in standard laboratory tests from baseline to post-treatment. The weak correlations between epigenetic aging and clinical measures suggest that these domains may respond independently, or that larger samples are required to detect convergent effects. The small sample size ($n = 20$) likely limited statistical power to detect associations across measures. Additionally, the use of a fixed ketamine dose, rather than titration as used in clinical practice, may have constrained treatment response. The absence of a control group further limits causal interpretation, particularly for self-reported symptom scales. As such, this pilot study should be interpreted as preliminary, and future studies in larger, controlled cohorts will be needed to validate these findings.

In summary, this study discovered that a 2–3 week treatment course of six ketamine infusions reduced PTSD and MDD scores, evaluated using the PCL-5 and PHQ-9. Ketamine also reduced biological age in study participants, particularly as indicated by PhenoAge, GrimAge, and OMICmAge. Our findings on altered epigenetic biomarker proxies and Marioni protein markers support their association with depression and trauma disorders, offering insights into ketamine's clinical and epigenetic mechanisms. Additionally, we observed a decrease in CD4T memory cells, suggesting a link between ketamine and immune cell subsets, and how these may mediate a reduction in biological age. While our study supports ketamine's role in alleviating depressive and PTSD symptoms and its potential mechanisms involving the sleep/wake cycle and neuroinflammation, further research is needed to clarify these epigenetic alterations and their contribution to ketamine's antidepressant effects and its impact on biological age.

DATA AVAILABILITY

The data and code that support the findings of this study are available from the corresponding authors upon reasonable request. To request data presented here, please contact the corresponding author for a data use agreement (DUA) which will detail the terms and conditions for data usage.

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AUTHOR CONTRIBUTIONS

Design and conceptualization of the study: KLD, MSD, MPM, RMS, JK, BCA, VBD. Sample collection and recruitment: KLD, JK, SM, NP, BCA. Data generation: TLM. Processing and statistical analyses: AC, VBD. Scientific discussion and interpretation of results: AC, NCG, RS, ATH, VBD. Wrote the manuscript: KLD, AC, RS, ATH, RMS, VBD.

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COMPETING INTERESTS

KLD and SM are employed with Expedition Mental Health. AC, NCG, TLM, RMS, MSD, MPM, and VBD are employed with TruDiagnostic Inc. BCA is employed with Wild Health. ATH and RS have received consulting fees from TruDiagnostic Inc. for work unrelated to the present manuscript, and are named as inventors on a patent for the SystemsAge scores.

ETHICAL APPROVAL

All study procedures were performed in accordance with relevant guidelines and regulations (including the Declaration of Helsinki and ICH-GCP). The protocol was reviewed and approved by the The Institutional Review Board of INSTITUTE OF REGENERATIVE AND CELLULAR MEDICINE gave ethical approval for this work (Protocol: Tru-012-2022v2; Approval/Registration: IRCM-2022-369). Informed consent was obtained from all participants prior to any study procedures. No animal experiments were performed.

CONSENT FOR PUBLICATION

This article does not include identifiable images or other personal data from human participants; therefore, consent for publication is not applicable.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Varun B. Dwaraka.