### Title

Increased cell-free mitochondrial DNA is a marker of ongoing inflammation and better neurocognitive function in virologically suppressed HIV-infected individuals




### Journal

Journal of NeuroVirology, 23(2)

### ISSN

1355-0284



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### Publication Date

2017-04-01

### DOI

10.1007/s13365-016-0497-5

Peer reviewed

J. Neurovirol. (2017) 23:283–289
DOI 10.1007/s13365-016-0497-5

# Increased cell-free mitochondrial DNA is a marker of ongoing inflammation and better neurocognitive function in virologically suppressed HIV-infected individuals

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Received: 20 June 2016 / Revised: 11 October 2016 / Accepted: 1 November 2016 / Published online: 5 December 2016


**Abstract** Cell-free mitochondrial DNA (mtDNA) is a highly immunogenic molecule that is associated with several inflammatory conditions and with neurocognitive impairment during untreated HIV infection. Here, we investigate how cell-free mtDNA in cerebrospinal fluid (CSF) is associated with inflammation, neuronal damage, and neurocognitive functioning in the context of long-term suppressive antiretroviral therapy (ART). We quantified the levels of cell-free mtDNA in the CSF from 41 HIV-infected individuals with completely suppressed HIV RNA levels in blood plasma (<50 copies/mL) by droplet digital PCR. We measured soluble CD14, soluble CD163, interferon γ-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor-α (TNF-α), neopterin, and neurofilament light chain (NFL) by immunoassays in CSF supernatant or blood plasma. Higher levels of mtDNA in CSF were associated with higher levels of MCP-1 ($r = 0.56$, $p < 0.01$) in CSF and TNF-α ($r = 0.43$, $p < 0.01$) and IL-8 ($r = 0.44$, $p < 0.01$) in blood plasma. Subjects with a previous diagnosis of AIDS showed significantly higher levels of mtDNA ($p < 0.01$) than subjects without AIDS. The associations between mtDNA and MCP-1 in CSF and TNF-α in blood remained significant after adjusting for previous diagnosis of AIDS ($p < 0.01$). Additionally, higher levels of mtDNA were associated with a lower CD4 nadir ($r = -0.41$, $p < 0.01$) and lower current CD4% ($r = -0.34$, $p = 0.03$). Paradoxically, higher levels of mtDNA in CSF were significantly associated with better neurocognitive performance ($r = 0.43$, $p = 0.02$) and with less neuronal damage (i.e. lower NFL). Higher cell-free mtDNA is associated with inflammation during treated HIV infection, but the impact on neurocognitive functioning and neuronal damage remains unclear and may differ in the setting of suppressive ART.

**Keywords** mtDNA · ddPCR · Inflammation · Neurocognitive performance · Neurodegeneration

Josué Pérez-Santiago, Michelli F. De Oliveira, Sara Gianella, and Sanjay R. Mehta contributed equally to this work.

Some of these data were presented at the Conference of Retroviruses and Opportunistic Infections in Seattle, Washington, from February 23–26, 2015.

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## Introduction

Persistent immune activation is the hallmark of human immunodeficiency virus (HIV) infection, and it is both associated with disease progression and can persist despite long-term suppressive antiretroviral therapy (ART) (Hunt et al. 2003). Immune activation is detectable shortly after infection in the brain and cerebrospinal fluid (CSF) of HIV-infected individuals (Hunt et al. 2003). During the course of infection, HIV enters the central nervous system (CNS) and replicates in permissive cells (Zayyad and Spudich 2015), resulting in an immune activation cascade that may contribute to neurodegeneration (Erlandson et al. 2013; Gonzalez-Scarano and Martin-Garcia 2005), and neurocognitive impairment (Joseph et al.

2016) Apart from active replication, HIV viral proteins released from infected cells can be proinflammatory, resulting in further toxicity to bystander cells (Gonzalez-Scarano and Martin-Garcia 2005). As a consequence of neurodegenerative processes, cellular components are also released into the CSF. Some of these components, such as mitochondria, contain damage-associated molecular patterns (DAMPs), which can stimulate the innate immune system and initiate a non-infectious inflammatory response (Iwasaki and Medzhitov 2010). These mitochondrial DAMPs include cell-free mitochondrial DNA (mtDNA), formyl peptides, and others (Zhang et al. 2010). Higher levels of cell-free mtDNA in CSF are associated with poor outcome in the setting of traumatic brain injuries (Walko et al. 2014; Wang et al. 2013; Zhang et al. 2010). In HIV infection, the release of mitochondrial DAMPs from dying cells may further contribute to inflammation and immune activation in the CNS.

Recently, we described associations between higher levels of mtDNA in CSF and increased markers of inflammation and neurocognitive deficits in a cohort of 31 HIV-infected individuals (Perez-Santiago et al. 2016). This previous cohort was limited by variable uptake of ART, and most individuals were not virally suppressed in their blood plasma and CSF at the time of sampling (Perez-Santiago et al. 2016). The ongoing viral replication may have confounded our ability to use mtDNA as a marker of neurodegeneration. To better understand the role of cell-free mtDNA in CSF as a biomarker of CNS inflammation and neurocognitive impairment, we evaluated the relationship between cell-free mtDNA in CSF, inflammation and neurocognitive function in a well-characterized cohort of individuals on long-term ART with sustained viral suppression in blood plasma.

## Methods

### Study population and sample

This was a retrospective study of 41 HIV-infected individuals from the HIV Neurobehavioral Research Center (HNRC) prospective memory cohort (Oliveira et al. 2015). At the time of sampling, all participants were on ART with undetectable levels of HIV RNA in blood (<50 copies/μL of plasma; Amplicor HIV Monitor Test, Roche Molecular Systems Inc.). Blood lymphocyte profiles were obtained by flow cytometry (CLIA-certified local laboratories). Epidemiological, behavioral risk and clinical data were also collected from participants (Sheppard et al. 2015). Individuals were deemed to have AIDS if they met the Centers for Disease Control criteria (http://www.aidsmap.com/CDC-case-definitions/page/1391604/). The estimated duration of infection (EDI) was determined using results of serologic and virologic tests as described previously (Little et al. 2008). Paired stored CSF and blood samples were retrospectively selected and used for measurements of CSF and blood plasma markers of inflammation and cell-free mitochondrial DNA.

### CSF DNA extraction and quantification

CSF was collected by lumbar puncture, centrifuged at 250×*g* for 15 min to separate the supernatant from cells, and the supernatant was stored at −80 °C. DNA was extracted from CSF supernatant samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) per manufacturer's protocol. Levels of mtDNA were measured by droplet digital PCR (ddPCR, Bio-Rad, Hercules, CA) using primer-probe combinations targeting the mitochondrial NADH dehydrogenase 2 gene (MT-ND2, Integrated DNA Technologies, IA) and the human genomic DNA (gDNA) by targeting the ribonuclease P protein subunit p30 gene (RPP30, Integrated DNA Technologies, IA), as previously described (Perez-Santiago et al. 2016) but using ZEN quenched probes (Var et al. 2016). Each sample was run in triplicate in a 20 μL of reaction, which consisted of 10 μL of 2× Bio-Rad supermix for probes, 1 μL of either 20× Primer/FAM-Zen ND2 mix or 20× Primer/HEX-Zen RPP30 mix, 4 μL of molecular grade water and 5 μL of total DNA, using the following conditions: (1) an initial activation of 95 °C for 10 min, (2) 55 cycles of 94 °C for 30 s and 60 °C for 1 min, (3) enzyme inactivation at 98 °C for 10 min, and 4 °C hold. The endpoint PCR reactions were read and analyzed using the Bio-Rad droplet reader and the QuantaSoft (Bio-Rad, CA) analysis software (Hindson et al. 2011). Levels of mtDNA were expressed in $\log_{10}$ copies/mL of CSF.

### Markers of innate immune activation, inflammation, and neurofilament light chain

The levels of selected markers of monocyte activation (neopterin, soluble CD163 (sCD163), and soluble CD14 (sCD14)), inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8)), and chemokine (monocyte chemoattractant protein-1 (MCP-1) and interferon γ-induced protein 10 (IP-10)) and axonal damages (neurofilament light chain (NFL)) were measured in all participants. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the levels of sCD163 (Trillium Diagnostics, Brewer, ME, USA), sCD14 (Quantikine ELISA Human sCD14 R&D Systems, MN, USA), and neopterin

(BRAHMS Neopterin EIA GmbH, Hennigsdorf, Germany) from blood plasma and CSF and NFL in CSF (Uman Diagnostics, Sweden). An electrochemiluminescence multiplex assay (Meso Scale Diagnostics, Rockville, MD, USA) was used to quantify the levels of TNF-α, IL-6, IL-8 and MCP-1, and IP-10 in CSF and blood plasma. All assessments were performed according to the manufacturer's procedures.

### Neuropsychological performance assessments

Individuals underwent neurocognitive testing using a standardized clinical battery testing seven ability areas consistent with Frascati recommendations for NeuroAIDS research (Antinori et al. 2007). The details of this battery can be found in de Oliveira et al. (2015). All raw neurocognitive test scores were converted to demographically adjusted T-scores and averaged to create a summary T-score, which was used as our primary measure of neurocognitive performance.

### Statistical analyses

Normality of the variables was assessed using a Shapiro test with a significance threshold of $p \leq 0.05$. A two-tailed $t$ test was used to detect differences in the levels of cell-free mtDNA between AIDS vs. non-AIDS study groups. Univariate and multivariate associations between cell-free mtDNA in CSF, supernatant, and clinical and immunological variables were assessed using the Pearson correlation test and fixed effects regression analysis, respectively. All statistical analyses were performed using the R statistical software (R Core Team 2016). Transformations and non-parametric analyses were performed if variables did not follow a normal distribution.

## Results

### Study participants characteristics

Our study cohort consisted of 41 HIV-infected subjects (31 males and 10 females), of which 66% were Caucasian (27/41) and with a median age of 51 years and a median CD4+ T cell count of 669 cells/μL at the time of sampling. Most subjects (40/41) were on ART, but all had suppressed HIV RNA levels in blood plasma. A summary of the clinical characteristics of the subjects is provided in Table 1.

**Table 1** Characteristics of our study participants

| | Study participants ($n = 41$) |
|---|---|
| Sex (M:F) | 31:10 |
| Race (C:NC) | 27:14 |
| Age (years) | 51 (33–58) |
| EDI (months) | 170.9 (93.2–210.3) |
| CD4 absolute (cells/μL) | 669 (482–893) |
| CD4 percent (%) | 33 (24.4–39.7) |
| CD8 absolute (cells/μL) | 918 (718–1195) |
| CD8 percent (%) | 43.9 (35.1–52.5) |
| CD4 Nadir (cells/μL) | 237 (100–330) |
| CD4/CD8 | 0.83 (0.51–0.99) |
| mtDNA levels ($\log_{10}$ copies/mL) | 3.99 (3.77–5.67) |

Median with and interquartile range values are shown

*M* male, *F* female, *C* Caucasian, *NC* non-Caucasian

### Cell-free mtDNA in CSF, inflammation, and immune dysfunction

We first evaluated whether higher levels of cell-free mtDNA in CSF supernatant were associated with increased inflammation and with evidence of greater immune dysfunction (as defined by CD4 nadir or previous diagnoses of AIDS) in HIV-infected subjects on suppressive ART. All samples showed detectable levels of mtDNA in CSF (median 3.99 $\log_{10}$ copies/mL, Table 1). Univariate correlations demonstrated that higher levels of free mtDNA were associated with increased levels of MCP-1 in CSF ($r = 0.56$, $p < 0.01$, Fig. 1a) and TNF-α ($r = 0.43$, $p < 0.01$, Fig. 1b) and IL-8 ($r = 0.44$, $p < 0.01$, Fig. 1c) in peripheral blood plasma. Additionally, higher levels of mtDNA were associated with lower CD4+ T cell nadir ($r = -0.41$, $p < 0.01$, Fig. 1d), and lower current CD4% ($r = -0.34$, $p = 0.03$, Fig. 1e). Individuals with a previous diagnosis of AIDS had significantly higher levels of mtDNA (mean ± SD 5.83 ± 1.56 $\log_{10}$ copies/mL) when compared to those without AIDS (mean ± SD 3.93 ± 0.35 $\log_{10}$ copies/mL, $p < 0.01$, Fig. 1f)(Perez-Santiago et al. 2015).

In a multivariate model, only the relationships between mtDNA and CSF MCP-1 and TNF-α remained significant after adjusting for a previous diagnosis of AIDS ($p < 0.01$ for both models, Table 2).

Although age was not directly associated with the levels of cell-free mtDNA in CSF, age has been previously associated with inflammation (Nasi et al. 2014; Wallet et al. 2015). In a multivariate regression analysis, levels of mtDNA remained associated with inflammation markers MCP-1 in CSF and TNF-α and IL-8 in blood plasma ($p < 0.01$) after correcting for age.

### Cell-free mtDNA in CSF and neurocognitive performance

We next evaluated if a higher level of cell-free CSF mtDNA was associated with worse neurocognitive impairment, as previously described (Perez-Santiago et al. 2016). In contrast to our previous work, and similar to other studies conducted in subjects with Alzheimer's and Parkinson's diseases

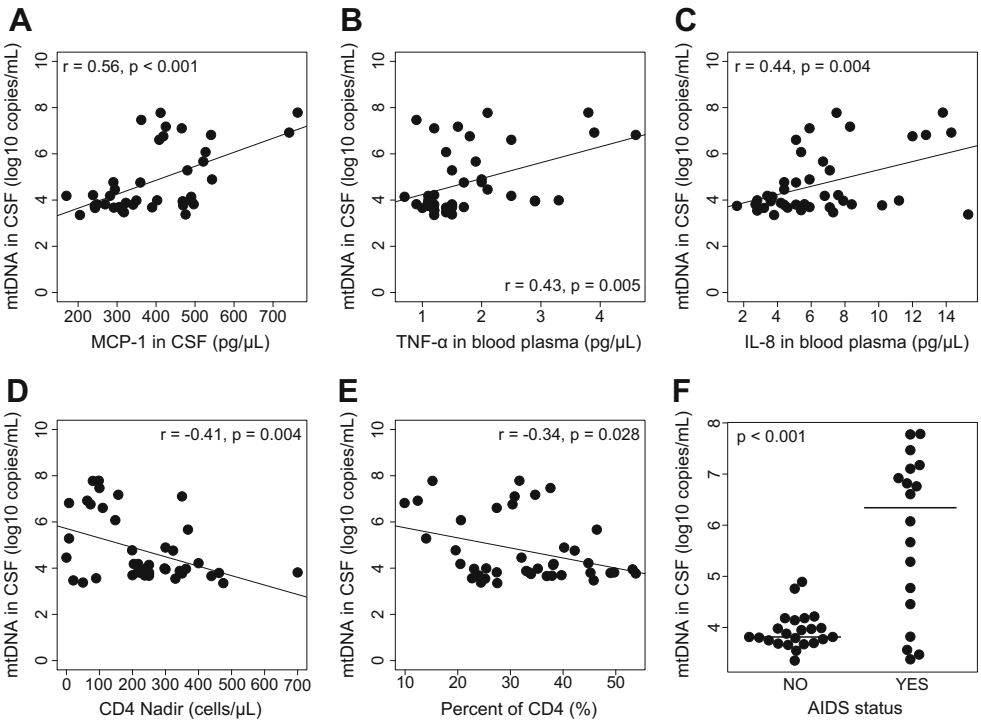


**Fig. 1** Mitochondrial DNA, inflammation and immunosuppression. Higher levels of mtDNA in CSF were associated with higher levels of MCP-1 in CSF (**a**), TNF-α (**b**) and IL-8 (**c**) in blood plasma. Additionally, higher levels of mtDNA in CSF were associated with a lower CD4 Nadir (**d**) and less CD4%. Individuals (**e**) with a previous AIDS diagnosis had higher levels of mtDNA in CSF than those without AIDS diagnosis

**Table 2** Associations between mtDNA and clinical variables while adjusting for AIDS status

| | $r^2$ | *p* value |
|---|---|---|
| **MCP-1 CSF** | **0.52** | **0.016** |
| **TNF-α plasma** | **0.52** | **0.021** |
| **Mean T-score** | **0.54** | **0.037** |
| IP-10 CSF | 0.46 | 0.131 |
| CD8 percentage | 0.48 | 0.145 |
| IL-8 plasma | 0.46 | 0.164 |
| Neopterin plasma | 0.45 | 0.185 |
| IP-10 plasma | 0.47 | 0.239 |
| Neopterin CSF | 0.47 | 0.271 |
| CD4/CD8 | 0.46 | 0.335 |
| CD4 absolute count | 0.46 | 0.347 |
| IL-6 CSF | 0.39 | 0.376 |
| CD8 absolute count | 0.46 | 0.450 |
| sCD14 CSF | 0.46 | 0.450 |
| NFL | 0.41 | 0.515 |
| TNF-α CSF | 0.74 | 0.565 |
| MCP-1 plasma | 0.44 | 0.583 |
| IL-8 CSF | 0.45 | 0.633 |
| sCD163 CSF | 0.45 | 0.646 |
| sCD14 plasma | 0.45 | 0.708 |
| CD4 percentage | 0.45 | 0.717 |
| IL-6 plasma | 0.47 | 0.799 |
| CD4 Nadir | 0.45 | 0.802 |
| sCD163 plasma | 0.45 | 0.998 |

AIDS diagnosis was included in the multivariate model due to the significance in the univariate model

(Podlesniy et al. 2013; Pyle et al. 2015), in this cohort of virally suppressed individuals, better neurocognitive performance, as measured by the summary T-score, was correlated with higher cell-free mtDNA levels within the CSF supernatant ($r = 0.43$, $p = 0.02$; Fig. 2). In a multivariate analysis, higher levels of mtDNA remained associated with better neurocognitive performance after adjusting for AIDS ($p = 0.04$, Table 2).

## Cell-free mtDNA in CSF and markers of brain damage

Next, we investigated the association between mtDNA and NFL (a marker of axonal neurodegeneration). We found that

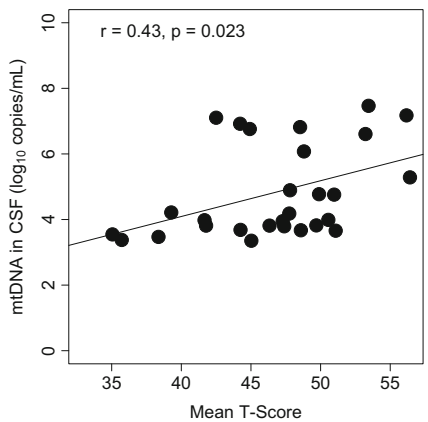


**Fig. 2** Associations of mtDNA and inflammation with neurocognitive function. Higher levels of mtDNA in CSF were associated with better neuropsychological performance

higher levels of NFL were associated with the lower levels of mtDNA in CSF even after adjusting for age ($p = 0.04$), but this association was no longer significant after adjusting for AIDS status.

## Discussion

In this cross-sectional study, we investigated the potential usefulness of cell-free mtDNA as a biomarker of HIV-associated CNS inflammation and neurocognitive impairment in the context of viral suppression with ART. We hypothesized that levels of free mtDNA in the CSF would reflect the levels of overall cell death in the CNS and potentially contribute to the ongoing inflammatory response during suppressive ART.

Consistent with this hypothesis, we found that cell-free mtDNA in CSF samples from our cohort was strongly associated with systemic and CNS inflammations (MCP-1 in CSF and TNF-$\alpha$ and IL-8 in blood plasma) during suppressive ART. These associations remained significant after adjusting for a previous diagnosis of AIDS. This was similar to our previous work (Perez-Santiago et al. 2016), where we observed associations between mtDNA and inflammation in a heterogeneous cohort of HIV-infected individuals both on and off therapy. Thus, mtDNA may be a potential marker of ongoing CSF inflammation in HIV-infected individuals regardless of detectable viral replication.

In HIV-infected individuals, persistent immune activation is common and associated with disease progression (Bofill et al. 1996; Giorgi et al. 1999; Mahalingam et al. 1993), even after a long-term suppressive ART (Hunt et al. 2003). Persistent T cell activation is associated with a blunted CD4+ T cell recovery during ART and is a marker of progression to AIDS (Bofill et al. 1996). The cell death associated with the inflammatory process may lead to an increased release of mtDNA. In fact, we observed that individuals with a previous diagnosis of AIDS had significantly higher levels of cell-free mtDNA in the CSF compared to people without any AIDS-defining illness. Consistently, acutely infected HIV individuals and late presenters taking ART both have higher plasma levels of cell-free mtDNA when compared to healthy individuals or long-term non-progressors (Cossarizza et al. 2011). Similarly to bacterial DNA, mitochondrial DNA includes repeated CpG motifs and can induce potent inflammatory reactions (Zhang et al. 2010). Cell-free mtDNA released during inflammatory cell death may induce further inflammation, leading to a persistent immune activation.

Next, we evaluated the associations between free mtDNA in CSF, neurodegeneration, and neurocognitive performance. Contrary to our previous study (Perez-Santiago et al. 2016), the current data show that higher levels of cell-free CSF mtDNA level were associated with less neurocognitive impairment in this cohort of virally suppressed subjects. This is despite the fact that mtDNA was associated with inflammation, and inflammation has been previously shown to be associated with NCI (Zayyad and Spudich 2015). Previous studies have examined the levels of cell-free mtDNA in the CSF of individuals with neurodegenerative diseases in non-acute settings, as in Parkinson's disease (PD) and Alzheimer's disease (AD) (Podlesniy et al. 2013; Podlesniy et al. 2016; Pyle et al. 2015). Interestingly, in the individuals with PD or AD, the levels of cell-free mtDNA were lower than those in healthy individuals (Podlesniy et al. 2013; Pyle et al. 2015), which is consistent with our findings. Additionally, we also found that decreased CSF mtDNA was associated with higher levels of NFL, another marker of neuronal degeneration. In the setting of ART-suppressed HIV infection, there might be alterations in cellular and mitochondrial physiology, similar to other neurodegenerative diseases such as AD and PD, which lead to mtDNA depletion, neuronal damage (as suggested by the association with NFL), and worse neurocognitive outcomes. We hypothesize that this decrease in the levels of mtDNA reflects decreased mitochondrial biogenesis in the brain tissue, which has been linked with AD and PD (Pyle et al. 2016; Rice et al. 2014). This supports the idea that in the context of suppressive ART, the lower levels of mtDNA maybe a marker of neurodegeneration. This association was not observable in non-suppressed individuals (Perez-Santiago et al. 2016) where active viral replication and associated inflammation likely resulted in higher levels of released mtDNA, masking the above-described relationship.

We recognize that this study has several limitations, including a small sample size and a lack of uninfected controls, which limit our conclusions. Further, future longitudinal studies are needed to better understand the interplay between free mtDNA and neurocognitive outcomes during HIV suppression. Finally, ex vivo and in vitro model approaches will be helpful to understand the mechanisms in which mitochondria and mtDNA may play a role on the development of neurocognitive dysfunction.

Despite these limitations, our provocative results suggest that despite being associated with increased inflammation, lower levels of cell-free mtDNA are associated with greater neurocognitive impairment and neurodegeneration during suppressive ART.

**Acknowledgments** We are grateful to all the participants in the HNRC Prospective Memory Cohort, the HNRC staff, and the CFAR Genomics Core. We would like to thank the CFAR Inflammation and Aging Scientific Working Group for the support. The work of MFO was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Brazil). This work was also supported by the NIH grants AI093163, MH073419, AI036214, and AG044325 and the Interdisciplinary Research Fellowship in NeuroAIDS (R25-MH081482).

**Author contributions** JPS designed and performed the ddPCR assays that quantified the mtDNA and RPP30 in CSF, performed the statistical analyses, and wrote the primary version of the manuscript. MFO

performed the soluble and inflammatory marker assays and wrote the primary version of the manuscript. SPW enrolled the participants, performed the neuropsychological testing of all participants, and participated in the data analyses. SRV and TRCD performed the DNA extractions and ddPCRs. SG and SRM designed the present study and participated in the data analysis. All authors read and approved the final manuscript.

**Compliance with ethical standards** All adult subjects provided their written informed consent. No children were included in this study. The Office of Human Research Protections Program of the University of California, San Diego, approved the study.

**Competing interests** The authors declare that they have no competing interests.

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