AIDS, Publish Ahead of Print

DOI: 10.1097/QAD.0000000000002966

Plasma microRNAs are associated with domain-specific cognitive function in people with HIV

RUNNING HEAD: Plasma microRNA, cognition, HIV

Julissa MASSANETT APARICIO, B.S.1, Yanxun XU, PhD2,3, Yuliang LI, PhD2, Carlo

COLANTUONI, PhD4,5, Raha DASTGHEYB, PhD4, Dionna W. WILLIAMS, PhD6,7, Eugene L.

ASAHCHOP10 Jacqueline M. MCMILLIAN, MD8,9, Christopher POWER, MD9, 10, 11 Esther

FUJIWARA, PhD11,12, M. John GILL8.9, Leah H. RUBIN, PhD, MPH4, 13,14

1. Midwestern University, Glendale, AZ

2. Department of Applied Mathematics and Statistics, Johns Hopkins University, Baltimore, MD

3. Division of Biostatistics and Bioinformatics at The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD

4. Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD

5. Institute for Genome Sciences, University of Maryland, Baltimore MD

6. Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD

7. Division of Clinical Pharmacology, Johns Hopkins University School of Medicine, Baltimore, MD

8. Cumming School of Medicine, University of Calgary, Calgary, Canada

9. Southern Alberta Clinic, Calgary, AB, Canada

10. Department of Medicine, University of Alberta, AB, Canada

11. Neuroscience and Mental Health Institute, University of Alberta, Edmonton, Canada

12. Department of Psychiatry, University of Alberta, Edmonton, Canada

13. Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

14. Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Requests for reprints and correspondences should be addressed to: Leah H. Rubin, Ph.D., MPH,

Department of Neurology, Johns Hopkins University School of Medicine, 600 N. Wolfe Street/

Meyer 6-113, Baltimore, MD. 21287-7613, Phone: 443-287-0571, Fax: 410-955-0672, e-mail:

lrubin@jhmi.edu

Conflicts of Interest: M.J.G. has served as Ad hoc member on National HIV advisory boards to

ViiVhealth, Gilead, and Merck. All other authors declare that they have no conflicts of interest.

Study Funding: Research reported in this publication was supported by P30MH075773

(Haughey, Rubin, Sacktor), NIH T35 OD024982 (Aparicio), and CIHR FRN:201803 (Fujiwara,

Gill, Rubin, McMillan, Power).

Abstract

Objective: Cognitive impairment remains common in people with HIV(PWH) on antiretroviral therapy (ART). The clinical presentation and severity are highly variable in PWH suggesting that the pathophysiological mechanisms of cognitive complications are likely complex and multifactorial. MicroRNA(miRNA) expression changes may be linked to cognition as they are gene regulators involved in immune and stress responses as well as the development, plasticity, and differentiation of neurons. We examined plasma miRNA expression changes in relation to domain-specific and global cognitive function in PWH.

Design: Cross-sectional observational study

Methods: Thirty-three PWH receiving care at the Southern Alberta Clinic, Canada completed neuropsychological (NP) testing and blood draw. Plasma miRNA extraction was

followed by array hybridization. Random forest analysis was used to identify the top 10 miRNAs upregulated and downregulated in relation to cognition.

Results: Few miRNAs were identified across cognitive domains; however, when evident a miRNA was only associated with two or three domains. Notably, miR-127-3p was related to learning/memory and miR-485-5p to motor function, miRNAs previously identified in CSF or plasma in Alzheimer's and Parkinson's, respectively. Using miRNET 2.0, a software-platform for understanding the biological relevance of the miRNA-targets (genes) relating to cognition through a network-based approach, we identified genes involved in signaling, cell cycle, and transcription relating to executive function, learning/memory, and language.

Conclusion: Findings support the idea that evaluating miRNA expression (or any molecular measure) in the context of global NP function might exclude miRNAs that could be important contributors to the domain-specific mechanisms leading to the variable neuropsychiatric outcomes seen in PWH.

Key Words: microRNA, HIV, cognition, cognitive function

INTRODUCTION

HIV-1 enters the central nervous system (CNS) shortly after primary infection [1], resulting in changes of both viral and host gene expression profiles that eventually lead to neurodegeneration and reduced brain function [2-5]. Despite the use of modern antiretroviral therapy (ART) suppressing viral replication, mild forms of cognitive impairment persist in people with HIV (PWH). However, to date, the pathophysiological mechanisms contributing to these mild forms of cognitive impairment remain largely unknown. One possibility is that the mechanisms previously investigated were primarily examined in relation to a global measure of cognitive function, such as HIV-associated neurocognitive disorder (HAND), global deficit score (GDS), or global z-score. The clinical presentation of neurocognitive deficits in PWH is highly variable suggesting that the mechanisms contributing to cognitive complications are heterogeneous and may uniquely affect different cognitive domains. Molecular measures in peripheral blood could monitor cellular processes linked to such varied cognitive presentations.

Altered expression of microRNAs (miRNAs) is one mechanism previously studied in relation to global measures of cognitive function in PWH. miRNAs are non-coding RNA molecules that regulate a vast majority of human genes [6] by post-transcriptionally inhibiting their expression when binding to a complimentary site in a target mRNA. As a result, they are fundamental in maintaining normal biological functions, including in the brain, where approximately 70% of all identified miRNAs are expressed [7]. Although the specific targets of most miRNAs are not yet understood, it has been established that an individual miRNA can target hundreds of different mRNAs. These interactions allow a wide array of miRNAs to act in concert and create gene regulation networks capable of controlling normal cellular functions throughout the body. Hence, since miRNAs are involved in many biological processes, changes in their expression could potentially be associated to pathology, making them candidate biomarkers and potentially therapeutic targets [8] for various diseases.

To date, gene and miRNA expression profiles measured in brain, blood (via plasma or serum), and cerebral spinal fluid (CSF) have been linked to HAND [9-11], HIV encephalitis [3, 9-12], global cognitive impairment (e.g., GDS) in PWH [13-15], aging [16-18], and other neurodegenerative diseases such as Parkinson's [19, 20], Alzheimer's [19, 21], and Huntington's disease [22]. However, little is known about the relationships between miRNA and certain specific cognitive functions such as memory and motor function in PWH. Thus, we aimed to examine miRNA expression changes in relation to domain-specific and global measures of cognitive function in PWH. Given the variability in patterns of cognitive function in PWH, we hypothesized that there would be little overlap in the miRNAs associating with each cognitive domain.

METHODS

Participants

Participants in the present analysis included 33 PWH who were receiving active care at the Southern Alberta Clinic, Alberta, Canada from a previously published study [9]. Inclusionary criteria included confirmed HIV seropositivity, English speaking, normal or corrected-to-normal vision and hearing, and willingness to provide informed consent. Exclusionary criteria included: less than 9 years of education, head injury with loss of consciousness (>5 minutes), acute intoxication during testing, severe neurologic or psychiatric disorders based on medical chart review (e.g., psychosis, opportunistic infections, stroke). To be included in the present analysis, participants had to have complete neuropsychological (NP) test data available as the focus of the present study was on domain-specific cognitive performance. NP data was available on 33 of the 36 PWH from the validation cohort of the previously published study focused on HAND [9]. Written consent was obtained from each participant and the study was approved by the University of Calgary Human Ethics Committee (E-17256).

microRNAs

Details of microRNA extraction from plasma samples and analysis have been previously published [9]. In brief, blood was collected in EDTA tubes from all participants on the same day as neuropsychological test assessment. miRNeasy Serum/Plasma kit (Qiagen) was used for extraction of total RNA including microRNA from plasma samples and Bioanalyzer 2100 was used to analyze the samples for the quality of RNA. miRCURY locked nucleic acid (LNA) universal RT microRNA PCR (Exiqon) protocol was used involving the first-strand cDNA synthesis and real time PCR amplification. For first-strand cDNA synthesis, 4 µl of total RNA was used for reverse transcription in a total reaction volume of 10µl. Exiqon miRCURY LNA PCR primer assay (product number: 206999 and 2100158 for miR-4516 and miR-3665 respectively) and diluted cDNA (1:20) was used for qRT-PCR amplification. Exiqon LNA PCR primer assay miR-4707-5p (product number 2116591 and 206999) was also used for qRT-PCR. MicroRNA expression data was normalized using Exiqon LNA primer assay miR-16-5p (product number 205702). There were a total of 3391 miRNAs.

Cognitive Measures

The neuropsychological (NP) test battery included the Symbol Digit Modalities Test (SDMT), Number-Sequencing (TMT-2) and Number-Letter-Sequencing (TMT-4) from the Delis-Kaplan Executive Function System (D-KEFS), letter and category guided fluency, Hopkins Verbal Learning Test (HVLT), Grooved Pegboard (GPEG), and a short version of the Wisconsin Card Sorting Test (WCST). All NP test outcomes were normed to age, education, and if available, for sex [23]. NP outcomes were then combined into five cognitive domains: *Attention* (SDMT, TMT-2), *language/fluency* (letter and category fluency), *learning*

and memory (HVLT), *motor* (GPEG), and *executive functions* (WCST; TMT-4). A global NP score was also created by averaging the five domain scores.

Statistical Analysis

To identify important miRNA that could predict cognition, a random forest (RF) model (machine learning method) was fitted to each of the cognitive domain z-scores and to the global NP z-score, separately. Each model controlled for sex, years of education, CD4 nadir, and undetectable viral load (vs. detectable). We split the data into training (20 samples) and validation (14 samples) to search for the optimal hyperparameters in the RF model for better prediction power. Each RF predictive model consisted of n = 500 trees. Next, we computed the mean square error (MSE) based on all of the data. Variable Importance was defined by the mean decrease in MSE for each variable, and we focused on the top ten miRNAs (ranked by Variable Importance) that were positively associated with each cognitive domain z-score and with the global NP z-score; we also examined the top ten miRNAs that were negatively associated with these outcomes. All RF models were conducted in R, package "randomForest" version 4.6-14. Next, to better understand the functional mechanisms associated with the miRNA expression changes, we used mirNet 2.0 (https://www.mirnet.ca/) to link miRNAs to genes and associated pathways. Specifically, we input the top ten miRNAs that were positively (and negatively) associated with each cognitive outcome separately into mirNet (Organism=H. sapiens; ID type=miRBase ID; Targets=genes (miRTarBase v8.0)). Within mirNet we then selected "minimum network" which simplifies dense networks into more interpretable ones based on the shortest path network where pair-wise shortest paths are computed for all seed nodes. Nodes that are not on the shortest path are removed. We then used the REACTOME database (https://reactome.org) for functional enrichment and interpretation (open-source, open access pathway database). Only statistically significant genes are discussed where significance was set at $P<0.01$ after false discovery rate (FDR) corrections.

RESULTS

Table 1 provides sociodemographic, clinical, and behavioral characteristics of the sample. Participants were 81% male, 79% White with a mean age of 49.1 (SD=9.2), and an average of 13.9 years of education (SD=2.2). Seventy-eight percent had undetectable HIV RNA and the mean CD4 lymphocyte count at the time of assessment was 690.5 (SD=323). The mean log peak HIV RNA was 4.4 (SD=0.8), the mean CD4 nadir was 188.8 8 (SD=137.5), and the average duration of ART exposure was 11 years (SD=7.5). Fifteen percent were cigarette smokers, 51% were alcohol users, and none of the participants used illicit substances.

Expression of individual miRNAs is associated with domain-specific cognitive function

Cognitive dysfunction in PWH may be a result of multiple mechanisms including miRNA expression changes. To determine if there is variability in the miRNAs that are associated with each cognitive domain, RF machine learning analysis was used to identify from plasma

samples the top ten positively correlated and negatively correlated miRNAs for each domain. We found that there is significant variability across cognitive domains (**Figure 1a**). Distinct miRNAs were associated with individual cognitive domains, while global NP scores were associated with many of the same miRNAs seen in the individual domain associations which is to be expected given that the global NP score is computed by averaging the cognitive domain z-scores. These observations were consistent for both positively and negatively correlated miRNAs. Specifically, among the positively correlated miRNAs, six of the top ten miRNA (60%) were unique to motor (miR-337-5p, miR-664a-3p, miR-195-5p, miR-485-5p, miR-1202, miR-891b), 70% to attention (miR-369-3p, miR-25-3p, miR-600, miR-380-3p, miR-548-al, miR-378-g, miR-432-5p), 80% to executive function (miR-190b-5p, miR-4279, miR-31-5p, miR-3138, miR-2214, miR-374b-5p, miR-652-3p, miR-16-5p), 90% to language (miR-4533, miR-34b-5p, miR-4691-5p, miR-125a-5p, miR-598-3p, miR-4758-5p, miR-4505, miR-345-5p, miR-3166), and 100% to learning and memory (miR-4253, miR-1253, miR-154-5p, miR-937-3p, miR-127-3p, miR-1246, miR-301a-3p, miR-4663, miR-4507, miR-1237-3p). There were only four miRNA that overlapped across domains: miR-200c-3p (motor, executive function); miR-let-7i (motor, executive function, attention); miR-3120-3p (motor, attention); and miR-99b-5p (motor, attention, language). There were only five miRNA that were associated only with global NP scores: miR-2114-5p, 376a-3p, miR-185-5p, miR-431-5p, miR-4781-3p.

For the negatively correlated miRNAs, three of the top ten miRNA (30%) were unique to attention (miR-1299, miR-3620-3p, miR-3154), 50% to executive function (miR-3917, miR-518e-3p, miR-1282, miR-3201, miR-520a-5p), motor (miR-149-5p, miR-3621, miR-3940-5p, miR-4530, miR-4445-5p), and language (miR-1301-3p, miR-216-5p, miR-4792, miR-3910, miR-105-5p), and 70% to learning and memory (miR-3128, miR-130b-3p, miR-486-3p, miR-185-5p, miR-4419b, miR-19b-3p, miR-448ag)(**Figure 1b**). The miRNA that overlapped across domains including the following nine: miR-663a, miR-1910-5p, and miR-4734 (motor, executive function, attention); miR-4516 (motor, attention); miR-3163, miR-921, and miR-520h (attention, language); miR-1207-5p (motor, executive function); miR-4423-3p (learning and memory, executive function); and miR-542-3p and miR-3671 (learning and language, memory). There were three miRNA that were only negatively associated with global NP scores: miR-520g-3p, miR-4685-5p, and miR-4440.

In order to put the results into the context of traditional statistics, we also calculated Pearson's correlations for each of the top 10 miRNA identified from the RF models that were positively and negatively associated with domain-specific and global NP function. In general, correlations were significant for the miRNA positively associating, but not miRNA negatively associating, with domain-specific and global NP function (Supplemental Tables 1 and 2, http://links.lww.com/QAD/C162, respectively). It is important to point out that the results from univariate analysis are not necessarily expected to match those from the RF models, as RF is a nonlinear model. Therefore, it is possible to have a top predictor variable from RF that does not have $P<0.05$. RF models are also multivariate, and predictive capabilities of variables are always observed within the context of other variables. This is important considering that none of these factors exist in isolation in clinical studies.

Genes targeted by miRNAs correlated with specific cognitive domains

To understand the biological relevance of the top miRNAs associating with cognitive function, we used the bioinformatics tool mirNET (https://www.mirnet.ca/). Across every cognitive domain, we found that there were commonalities in the genes targeted by each list of miRNAs. Additionally, this network analysis showed that target genes were mostly involved in cellular functions related to signaling, cell cycle and transcription. Furthermore, significant target genes were found for three positively correlated (executive function, learning and memory, and global NP function; **Figure 2a-c**) and three negatively correlated (attention, language/fluency, and learning and memory; **Figure 3a-c**) miRNA lists (Supplemental Tables 3-8, http://links.lww.com/QAD/C166). Specifically, significant target genes positively related to executive function included ABL2, ACTB, ACTG1, AP2B1, ARHGDIA, CCND1, CCNT2, FLNA, SP1, FZD6, E2F2, AGO1, and RAC1. Positive correlates of learning and memory included CALM2, CSNK2A1, DLC1, ARF1, and CALM3 whereas for global NP correlates included AGO2, CCND1, and TFDP2. Significant target genes negatively relating to attention included ATP1B3, WEE1, RHOB, MAFK, SLC7A5, GATA6, CALR, and CANX. Negative correlates of language/fluency include AKT1, CDk6, H3F3B, PTEN, CDK4, CDKN1A, WEE1, and RAD51 whereas for learning and memory correlates included CALM1 and CAMK4. Of these target genes, several that have been associated to other neurodegenerative diseases in the literature were selected for further discussion.

DISCUSSION

We aimed to examine changes in peripherally expressed miRNAs in relation to domain-specific and global cognitive function in PWH. Our machine learning analyses indicated significant variability in the miRNAs relating to each cognitive domain. Few miRNAs were identified across cognitive domains; however, when evident a miRNA was only associated with two or three cognitive domains. In contrast, the lists for global NP function were mostly comprised of overlapping miRNAs which is expected given that the global NP score is computed based on the average of the domain scores. These findings support the idea that evaluating miRNA expression (or any molecular measure) in the context of global NP function might exclude miRNAs that could be important contributors to the domain-specific mechanisms leading to the variable neuropsychiatric outcomes seen in PWH. These results suggest that different miRNAs are likely linked to different cognitive functions.

Changes in miRNA expression are also reported in other neurodegenerative diseases as well as in human aging. Some of the miRNAs highlighted in other diseases and with aging coincide with altered miRNAs seen in our analysis. For instance, we found lower miR-185-5p expression changes to relate to poorer memory in PWH, which is downregulated in plasma from Alzheimer's disease patients compared to age and sex-matched controls [21]. Higher miR-127-3p was also related to poorer memory in our sample of PWH, a marker that is altered in the CSF of Alzheimer's disease patients [19]. Additionally, we identified miR-485-5p to relate to motor function in PWH which has been found to be downregulated in

plasma in Parkinson's disease [24]. Moreover, microRNAs related to attention and motor domains in the present study, also show altered expression in serum in multiple sclerosis and schizophrenia [25, 26]. Higher miR-19b-3p was also related to poorer learning and memory which has been found to be downregulated with aging [18]. Additionally, lower let7i was associated with poorer performance in a number of domains (attention, executive function, motor), another marker of aging, specifically stem cell exhaustion [17]. These findings support specific alterations in circulating miRNA expression to relate to specific cognitive domains in PWH.

To have a better understanding of the functional mechanisms associated with miRNA expression changes obtained from our plasma samples, we investigated genes of significant mechanistic pathways. Among them was AKT1, which emerged as important in the negatively correlated miRNAs associated with language. This gene encodes for a protein involved in signaling and is known to play a critical role in neuronal survival and synaptic development as well an insulin signaling. Moreover, AKT1 is a contributing factor to schizophrenia [27]. Another notable gene was SP1, which was important to the positively correlated miRNAs that clustered for executive function. SP1 plays a role in preventing cell death of cortical neurons in response to oxidative stress, DNA damage or both [28]. SP1 is also a pro-inflammatory transcription factor for HIV and linked to Alzheimer's disease and its inhibition in mice models results in increased memory deficits [29]. Other significant genes in the minimum networks obtained were the cell cycle genes CCND1 and CDK4 which are linked to Alzheimer's disease and are thought to be responsible for region-specific neuronal death [30]. Three genes coding for the calcium binding protein calmodulin CALM1, CALM2, and CALM3 emerged as important for memory. Calmodulin is postulated to play a role in the calcium dysregulation that precedes the molecular events that result in the formation of amyloid beta plaques and phospho-tau neurofibrillary tangles characteristic of Alzheimer's disease [31]. Recently, the CALM gene family is also reported to have enhanced post-translational modification in Alzheimer's disease alone when compared to Huntington's disease and Alzheimer's disease [32]. These findings highlight the potential role of calcium regulation through calmodulin proteins in the neurodegenerative effects leading to Alzheimer's disease and perhaps other neurodegenerative diseases. Although these are only a few examples, we generally found genes involved in signaling, cell cycle, and transcription to predominate.

Several studies document the importance of signaling, cell cycle and transcription in the brain and how altered activity of these processes can potentially result poorer cognition. Cell cycle regulators have been implicated in apoptosis of adult neurons which results in neurodegeneration [33]. More specifically, cell cycle regulator Cyclin D1 which is encoded by one of the genes identified in our functional analysis using mirNet (CCND1) is essential in the regulation of neuronal death through activation of Cyclin D1 dependent kinases [34]. Signaling pathways are also known to play a role in the neuronal apoptotic events associated with neurodegeneration, as well as neural stem cell maintenance, learning, and memory [35, 36]. Of particular importance is the Notch signaling pathway which was identified in our functional analysis. Notch signaling is responsible for key mechanisms that occur in early

neural development and brain plasticity throughout mammalian life which is why it has been linked to various neurological diseases [35]. In close association with signaling, transcription also contributes to memory, particularly long-term memory [37]. The transcriptional changes that are involved include DNA methylation, histone methylation, histone acetylation, and chromatin remodeling [38]. These studies along with our findings, validate miRNA expression changes as a useful tool in unpacking and understanding the molecular mechanisms that may be linked to cognitive function in PWH.

There were several study limitations including the small sample, cross-sectional study design, which precludes causality, and lack of a validation cohort. Additionally, we measured peripheral miRNA levels and peripheral levels are imperfects markers of central levels. However, our findings provide preliminary and hypothesis-generating data for future studies examining centrally expressed miRNAs in brain tissue. Finally, our study population was predominantly comprised of white male PWH which limits generalizability to other subgroups of PWH. The mechanisms may not be the same in women with HIV, particularly minority women who are more likely to demonstrate cognitive impairment compared to men with HIV [39].

Our findings emphasize the importance of studying potential pathophysiological mechanisms contributing to domain-specific cognitive function in PWH rather than only global cognitive measures. Given the clinical presentation of cognitive impairment in PWH is highly variable, the mechanisms contributing to cognitive function are likely multifactorial and this was evidenced by the lack of commonalities in miRNA expression across cognitive domains. Furthermore, being able to identify miRNAs linked to other neurodegenerative diseases strengthens their potential as a way of obtaining key insights into the functional mechanisms contributing to cognitive function in PWH and may serve as potential therapeutic targets.

ACKNOWLDEGEMENTS

Research reported in this publication was supported by P30MH075773 (Haughey, Rubin, Sacktor), NIH T35 OD024982 (Aparicio), and CIHR FRN:201803 (Fujiwara, Gill, Rubin, McMillan, Power). The authors thank the patients and staff at the SAC as without you this work would not have been possible.

CONTRIBUTORS

Dr. Rubin conceived the study idea. Drs. Xu and Yuliang Li conducted the statistical analyses. Drs. Power, Fujiwara, and Gill take responsibility for the ascertainment of the biospecimens and the integrity of the cognitive, behavioral, and clinical data. Drs. Asahchop and Power take responsibility for the miRNA analyses. Dr. Rubin and Julissa Massanett Aparicio wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript and approved the final version.

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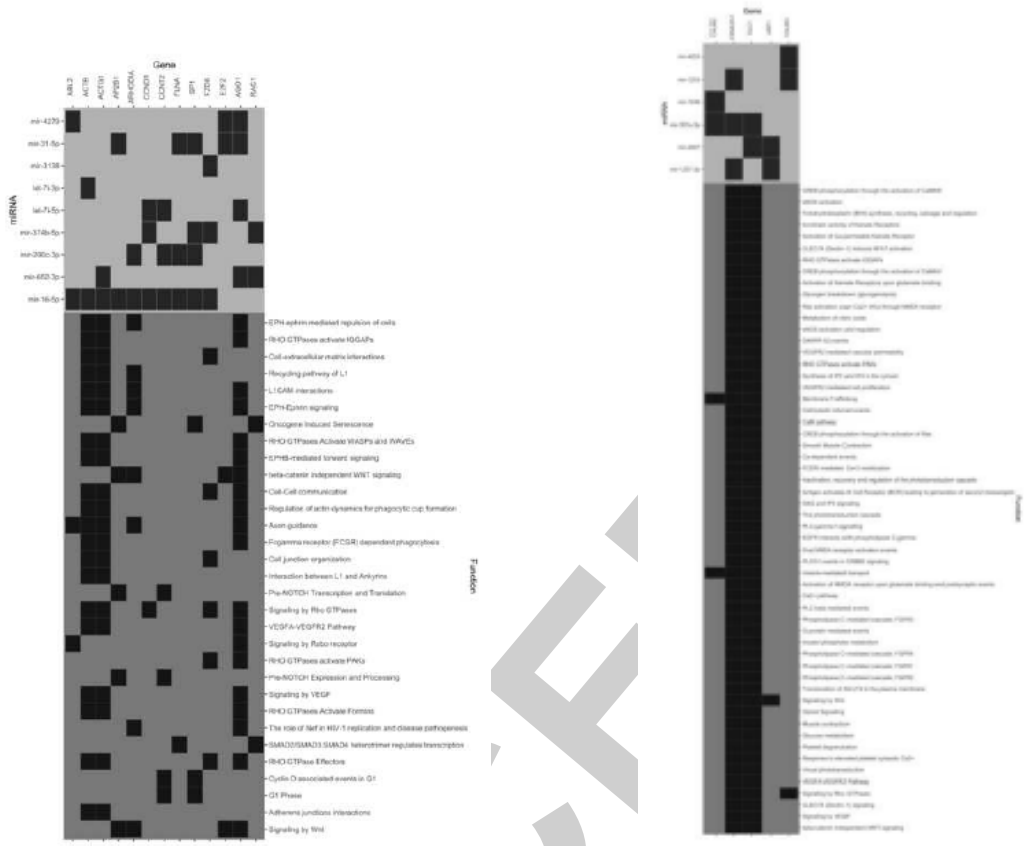
Figure Legends

Figure 1. Random forest model results for domain-specific and global neuropsychological (NP) function which include the top 10 miRNA that are positively associated with each cognitive outcome **(A)** and the top 10 miRNA that are negatively associated with each cognitive outcome **(B)**.

Figure 2. Tripartite adjacency matrix representation of miRNAs integrated to functional pathways through target genes. Only relationships with at least one interaction related to a significantly perturbed functional pathway (false discovery rate <= 0.01) are shown. The top rectangle represents the miRNAs and their gene targets that were positively related to **(A)** executive function, **(B)** learning and memory, **(C)** global neuropsychological function, while the bottom rectangle shows the relationship between the same gene targets and the significant functional pathways.



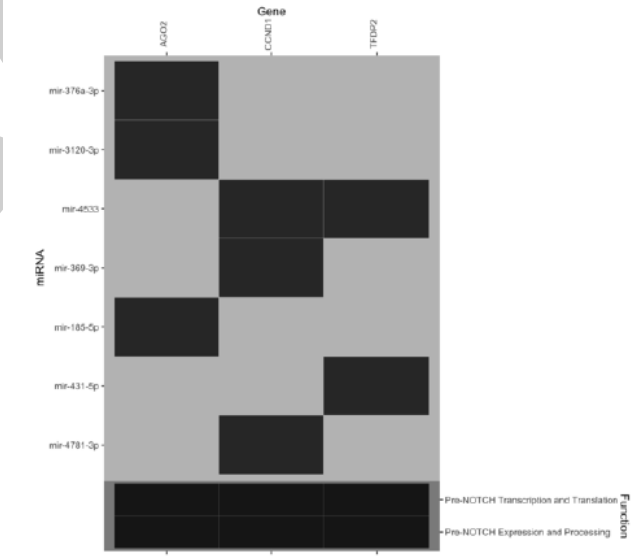
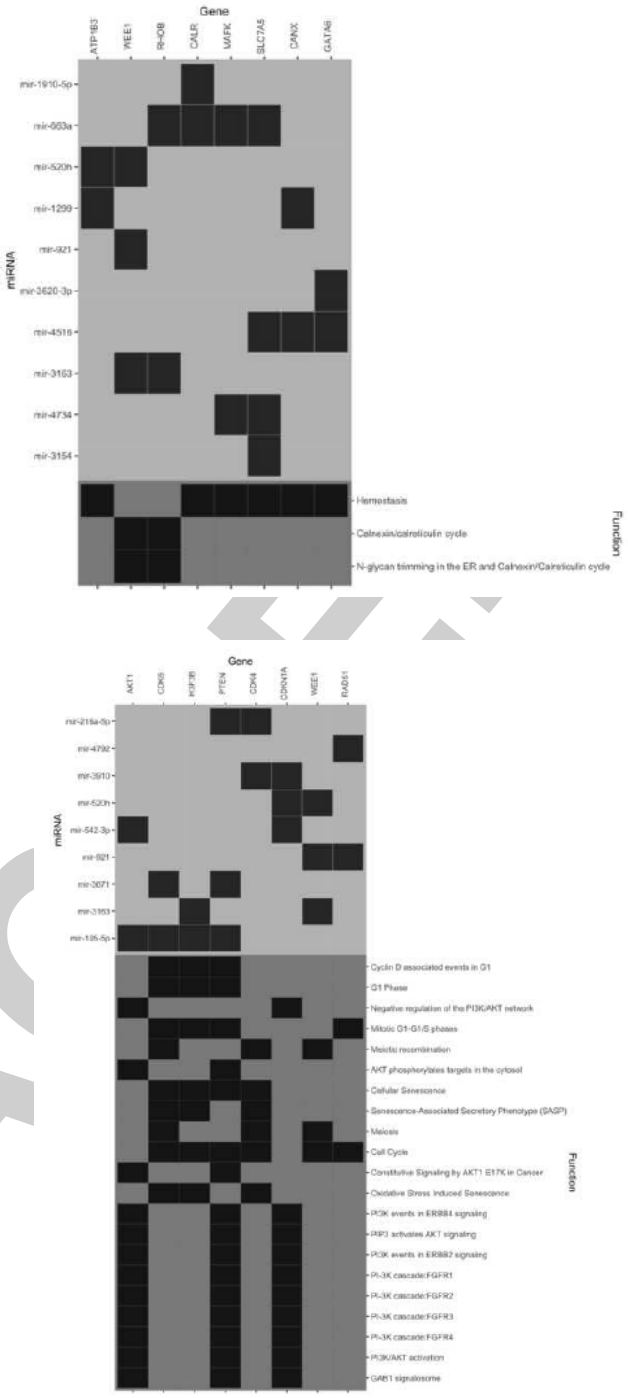


Figure 3. Tripartite adjacency matrix representation of miRNAs integrated to functional pathways through target genes. Only relationships with at least one interaction related to a significantly perturbed functional pathway (false discovery rate <= 0.01) are shown. The top rectangle represents the miRNAs and their gene targets that were negatively related to **(A)** attention, **(B)** language/fluency, and **(C)** learning and memory, while the bottom rectangle shows the relationship between the same gene targets and the significant functional pathways.



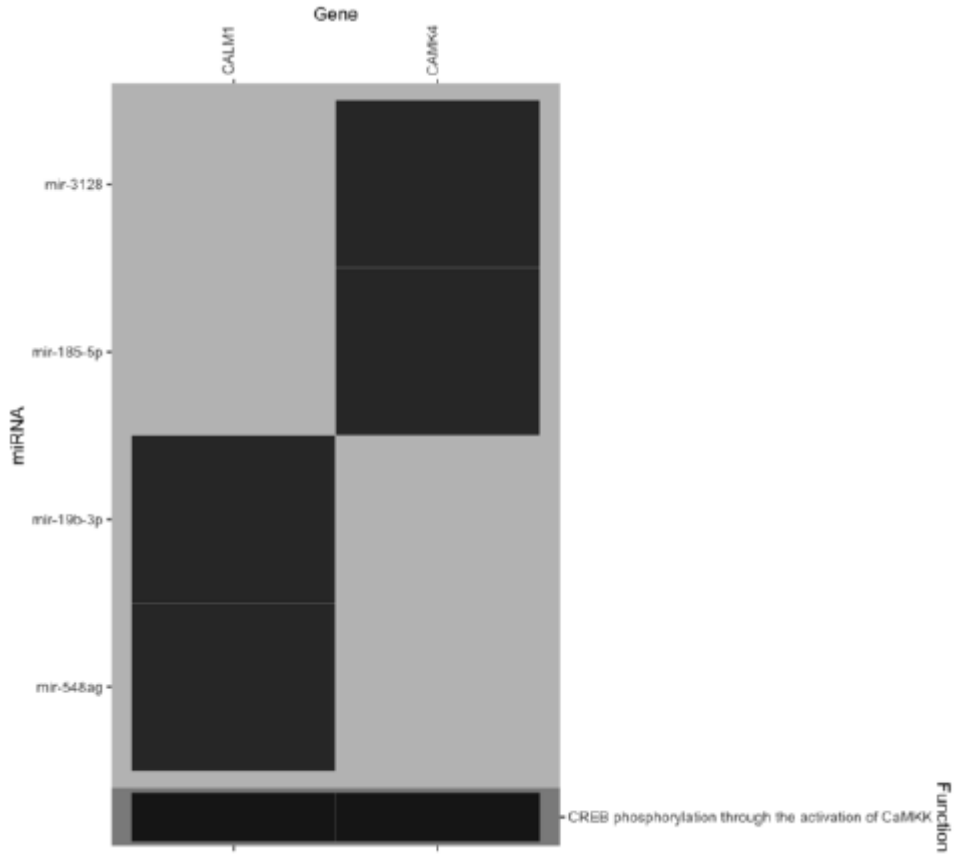


Table 1. Sociodemographic, behavioral, and clinical factors of the sample of people with HIV.

Variables	n (%)
Sociodemographic factors	
Age, M (SD)	49.6 (9.4)
Male	27 (81)
White	26 (79)
Years of Education, M (SD)	14.1 (2.3)
Behavioral factors	
Cigarette smokers	6 (18)
Alcohol users	17 (51)
HIV-related clinical factors	
HIV RNA undetectable	26 (79)
Log peak HIV RNA, M (SD)	4.4 (0.8)