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Peripheral blood microRNA levels in females with cocaine use disorder

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ABSTRACT

Background: There is growing emphasis in the field of psychiatry on the need to identify candidate biomarkers to aid in diagnosis and clinical management of addictive disorders. MicroRNAs (miRNAs) are small nucleotide sequences with the ability to regulate gene expression at the transcriptomic level. However, the role of miRNAs as potential biomarkers for addiction is still underexplored. Based on translational and clinical findings, we compared the expression levels of microRNA-124 (miR-124), microRNA-181 (miR-181), and microRNA-212 (miR-212) between a group of females with cocaine use disorder (CUD; n = 30) and a group of healthy female controls (HC; n = 20).

Methods: Blood expression levels of miR-124, miR-181, and miR-212 in the HC and CUD group were determined by qPCR, using two miRNAs as endogenous controls (miR-24 and miR-126). Substance use behavior was assessed by self-report using the Addiction Severity Index (ASI-6) and depressive symptoms severity was measured using the Beck Depressive Inventory (BDI-II). Urine screen test was performed to detect cocaine metabolites.

Results: Mir-124 and miR-181 were upregulated in the CUD group ($p > 0.01$). Furthermore, increased cognitive/affective depression symptoms were identified among a CUD subgroup with the higher miR-181 expression levels ($p > 0.05$). No significant difference in expression levels was found for miR-212.

Conclusions: MiR-124 and miR-181 show promise as biomarkers for CUD when assessed in the peripheral blood. Further investigation is needed to elucidate the molecular mechanisms underlying these associations and to validate target genes regulated by these miRNAs.

1. Introduction

The hallmarks of the systemic pathophysiology of cocaine use disorder (CUD) include metabolic syndrome, as well as altered levels of neurotrophins, oxidative stress parameters, neuroendocrine hormones, and inflammatory cytokines (Grassi-Oliveira et al., 2012; Levandowski et al., 2016; Viola et al., 2015; Viola et al., 2014a,b; Virmani et al., 2007; Zaparte et al., 2015). Recent findings suggest that cocaine-induced regulation of these signaling pathways in peripheral blood cells are mediated through persistent changes in gene expression (Fries et al., 2018). Therefore, epigenetic mechanisms, including DNA modifications, chromatin remodeling factors, histone modifications, and various classes of non-coding RNAs, may be fundamental processes implicated in the molecular signature of CUD in both the brain and systemically (Doura and Unterwald, 2016; Leighton et al., 2018; Nestler, 2014; Viola et al., 2016).

MicroRNAs (miRNAs) are small non-coding RNAs composed of 18–25 nucleotides. These molecules act by repressing messenger RNA translation, playing a critical role as a further layer of gene regulation between DNA transcription and protein translation (Shi and Jin, 2009; Stefani and Slack, 2008). Moreover, miRNAs are known not only to target several genes, but also to target significant parts of molecular and biological pathways (Kehl et al., 2017). Many miRNAs were reported to be up- or downregulated by cocaine exposure in rodent studies that explored their role in many regions of the brain (Nestler, 2014). Some examples are miR-9, miR-19, miR-26, miR-34, miR-124, miR-132, miR-134, miR-135, miR-181, miR-183, miR-212, miR-375, miR-449 and Let-7d (Doura and Unterwald, 2016). Overall, these miRNAs may function as putative biomarkers for CUD.

Based on convincing pre-clinical work suggesting a promising role

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for these epigenetic regulators in the pathophysiology of mental disorders (Ding et al., 2016; He et al., 2016; Maffioletti et al., 2016), recent studies have started to explore peripheral miRNAs levels in humans. Findings include elevated blood expression of miR-124 in patients with major depression disorder (He et al., 2016), a significant association of a single nucleotide polymorphism (SNP) within the miR-137 gene with schizophrenia, and reduced blood expression levels of miR-137 in patients with schizophrenia (Consortium, 2011; Guella et al., 2013), among other findings (Liu et al., 2017; Sánchez-Mora et al., 2019). Specifically regarding drug addiction, decreased expression of miR-181, miR-15b, and Let-7d have been found in the blood of individuals with methamphetamine use disorder compared to healthy controls (Zhao et al., 2016).

To the best of our knowledge, no study has been conducted to investigate the impact of CUD in the levels of peripheral miRNAs in humans. Therefore, we set out to compare the blood expression levels of candidate miRNAs between patients with CUD and healthy controls. We specifically focused on miR-124, miR-181 and miR-212, given that among many potentially relevant miRNAs, these have been the most explored by pre-clinical studies, with evidence indicating that: (1) these miRNAs are dynamically affected by cocaine acute exposure in both *in vivo* (e.g. rodent brain) and *in vitro* studies with neuronal and dopaminergic cell populations (Cabana-Domínguez et al., 2018; Chandrasekar and Dreyer, 2009; Xu et al., 2013); (2) they are molecular markers of the effects of chronic cocaine exposure in the brain reward system (Chandrasekar and Dreyer, 2011; Eipper-Mains et al., 2011; Quinn et al., 2018); (3) they are suitable for the identification of vulnerable/resilient subjects to cocaine dependence in behavioral animal models of drug addiction (Chandrasekar and Dreyer, 2011; Im et al., 2010; Quinn et al., 2015; Viola et al., 2016); and (4) the manipulation of these miRNAs in the brain resulted in altered cocaine-induced neuroplasticity and behavioral phenotypes (Doura and Unterwald, 2016).

The second aim of this study was to investigate whether differently expressed miRNAs could also be associated with the history of cocaine consumption or with the severity of depressive symptoms among participants with CUD. Finally, in order to identify relevant biological pathways regulated by differently expressed miRNAs, the third aim was to perform bioinformatics prediction of the miRNAs target genes.

The present study focused on female patients with CUD based on clinical studies showing that women: (1) start using cocaine earlier than do men (Dluzen and McDermott, 2008); (2) report higher amounts of cocaine consumption when compared to men seeking treatment (Elman et al., 2001); (3) report higher craving and withdrawal symptoms to cocaine than do men (Fattore et al., 2008); and (4) are more vulnerable to develop CUD, showing greater drug use escalation, than men (Abdalla et al., 2014).

2. Methods

2.1. Participants

This was a cross-sectional study performed with a convenience sample of females. Participants with CUD ($n = 33$) were undergoing treatment in an inpatient detoxification unit for females of a public hospital in Southern Brazil. Included participants met the following criteria: (1) age of 18–45 years old; and (2) diagnosis of CUD according to Structured Clinical Interview for DSM-5 (SCID) - physiological dependence of snorted or smoked cocaine (crack), (Shankman et al., 2018). Participants were excluded if they did not report or did not provide reliable information regarding their patterns of drug use behavior ($n = 3$); resulting in a final clinical sample size of 30. Females with CUD were invited to take part in the study during the first 3 days of treatment and provided written informed consent to participate. The detoxification treatment program consisted of 3 weeks of drug rehabilitation, including psychoeducation and support groups, moderate physical activity, a balanced diet (2200 Kcal/day), nursing care, and psychological and medical treatment. During detoxification, patients had no access to alcohol, cigarettes or drugs.

Healthy and unmedicated female control participants (HC, $n = 21$) were selected by convenience sampling (advertising) from the same age and socio-economic background of the CUD group. Any individuals with past or current Axis I disorders ($n = 1$), severe or unstable clinical illness, neurologic disorder, or any substance use in the 30 days preceding the study (self-report), or with a positive urine drug test by the time of assessment, were excluded; resulting in a final HC sample size of 20.

The research protocol was approved by the Ethical Committee of the detoxification unit. Before data collection, all participants were informed of the procedures and objectives of the study and provided written informed consent for participation. This study was conducted from December 2016, to December 2017.

2.2. Clinical assessment

Substance use history was obtained using the Addiction Severity Index (ASI-6) (Kessler et al., 2012; McLellan et al., 1980), which is a semi-structured interview and clinical/research instrument to assess several dimensions of patterns of substance use behavior. Data on the years of substance abuse (at least 3 days per week of high levels of substance consumption) and days of recent substance use (within the last 30 days before treatment enrollment) were inquired about cannabis, tobacco, alcohol, and cocaine. Moreover, sociodemographic data regarding age, income, and ethnicity were inquired during this interview.

Depressive symptoms were measured using the Beck Depression Inventory II (BDI-II) (Beck et al., 1996) at the first week of detoxification treatment for patients. The BDI-II is composed of 21 items, and it is a self-rated scale that evaluates behavioral, affective, cognitive, and somatic manifestations of depression. The 21 depressive symptoms are often grouped into the two interrelated symptom categories, i.e., “somatic” and “cognitive-affective” (Storch et al., 2004). These different symptoms were summarized in the following study by summing the subset of the 21 symptom scores that reflect somatic symptoms (tiredness or fatigue, loss of energy, concentration difficulty, changes in appetite, and changes in sleeping pattern), and the subset of the symptom scores which reflect cognitive-affective symptoms (sadness, crying, past failure, guilty feelings, punishment feelings, self-dislike, self-criticism, pessimism, loss of pleasure, loss of interest, worthlessness, suicidal thoughts, indecisiveness, irritability, agitation, loss of interest in sex).

2.3. Study procedures

At the first week of detoxification treatment, patients were assessed regarding psychiatric diagnosis, history of substance use, and depression symptoms. Body mass index (BMI) data for each participant was also assessed (kg/m^2). Two 5 ml samples of peripheral blood were collected by venipuncture and urine samples were collected for drug testing (marijuana, opiate, cocaine, amphetamine, and benzodiazepines) using the Easy@Home® 5 Panel Instant Urine Drug Test (EDOAP-754, Easy Healthcare Corporation, USA). Individuals recruited for the control group were interviewed and had their BMI assessed. Additionally, the control group blood was drawn and urine samples drug tested at the day of interview. All blood samples were stored in PaxGene® Blood RNA tubes (BD Biosciences, USA) at −20 °C prior to analyses. Urine samples were used to assess cocaine and/or cannabis use within the previous 15 days.

2.4. Analysis of blood miRNA levels

Total RNA was extracted using PaxGene Blood RNA Kit (Qiagen) according to the reagent manufacturer's protocol, and reconstituted in 40 µl of RNase-free water. The concentration of RNA was measured

using Qubit® RNA Broad Range Assay in a Qubit® Fluorometer 2.0 (Life Technologies). Two hundred nanograms of RNA from each sample were reverse transcribed using the miScript II RT Kit (Qiagen) and cDNA generated was used for miRNA gene expression measurements. Specifically, template RNA was combined with 4 µl 5 × miScript HiFlex Buffer, 2 µl 10 × miScript Nucleics Mix, 2 µl miScript Reverse Transcriptase Mix, and RNase-free water to a final reaction volume of 20 µl. Reactions were incubated for 60 min at 37 °C and for 5 min at 95 °C. The resulting 20 µl of reverse transcription products were diluted with 10 µl of RNase-free water to a total volume of 30 µl, and then samples were stored at −20 °C.

Two microliters of cDNA samples were used in each RT-qPCR reaction, performed in a RotorGene PCR machine (Qiagen), using the miScript SYBR Green PCR kit (Qiagen). MicroRNA assays were performed using 10 µl of SYBR Green, 2 µl of miScript miRNA primer (Qiagen), 2 µl of miRNA-specific universal primer (Qiagen), 4 µl of RNase-free water. The following Qiagen primers were used: Hs miR-124a_1 (MS00006622), Hs miR-212_1 (MS00003815), Hs miR-181a-2*_1 (MS00008834), Hs miR-24_1 (MS00006552), and Hs miR-126_1 (MS00003430). To verify primer specificities, melting curve analyses were performed. MiR-24 (Morata-Tarifa et al., 2017) and miR-126 (Marabita et al., 2016) Ct values (geometric mean) were used as endogenous controls for miR analyses. Fold change analyses were performed following the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.5. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 20 software. The Shapiro-Wilk test was used for the analysis of normality of data distributions for each variable. Sociodemographic and clinical data presented normal distribution and group differences were investigated using chi-square tests or *t*-tests. All miRNA variables presented non-normal distribution and Mann-Whitney U tests were applied to analyze group differences. Given that BMI levels were significantly different between groups, we also investigated the correlation between BMI and miRNA levels using the Spearman correlation coefficient.

To investigate whether miRNAs levels could also be associated with the history of cocaine consumption or with the severity of depressive symptoms among participants with CUD, we split the clinical sample into groups of individuals showing expression levels below or above the highest expression value of the control group, particularly for differently expressed miRNAs. The significance level for all analysis was set at $\alpha = 0.05$ (two tailed).

2.6. Bioinformatics analysis

The miRTarget R package (https://github.com/kassambara/miRTarget) was used to identify the predicted targets of miRNAs differently expressed between groups. This package summarizes data from 11 online miRNA prediction databases (diana, microinspector, miranda, mirtarget2, mitarget, nbmirtar, pictar, pita, rna22, rnahybrid, and targetscan). Target genes were ranked according to the number of databases that scored positive for miRNA prediction. A list of the predicted targets was exported to the online database PANTHER 14.0 in order to perform a gene ontology (GO) analysis, in which the list of genes was categorized according with gene function. Data was presented as the percentage of genes for each biological process.

3. Results

Sociodemographic and clinical characteristics of the sample are depicted in Table 1. All participants of the CUD group had a positive score for cocaine metabolites in urine samples, and 23% of them ($n = 7$) had also a positive score for cannabis. There were significant differences between HC and CUD groups regarding BMI, while no significant group effect was observed for age, income, and ethnicity (Table 1). Therefore, associations between BMI and the levels of miR-124 (Spearman's rho = −0.16, $p = 0.244$), miR-181 (Spearman's rho = −0.18, $p = 0.195$), and miR-212 (Spearman's rho = 0.45, $p = 0.755$) were tested and no significant effect was observed.

There was a significant group effect on miRNA levels, specifically showing higher levels of miR-124 (U = 449, $p = 0.003$, Fig. 1A) and miR-181 (U = 459, $p = 0.002$, Fig. 1B) in the peripheral blood of the CUD group compared with HC group. No significant group differences were observed regarding miR-212 levels (U = 279, p = 0.678, Fig. 1C). The highest expression value for the HC group was 0.329 for miR-124 and 0.027 for miR-181. Based on these values the CUD group was split into two groups for each miRNA, i.e., those with expression above or below the highest levels in HC. Individuals with CUD and that had a higher expression than the reference value of the HC group for miR-181 ($n = 12$) reported significantly higher cognitive-affective depressive symptoms (group mean = 33.16, SD = 5.62, t = 2.34, $p = 0.027$) relative to individuals with CUD that had a lower expression of miR-181 regarding the highest HC value ($n = 18$, group mean = 25.27, SD = 12.53). No significant differences were observed between these groups regarding the levels of somatic depressive symptoms, as well as regarding their self-report of the years of substance abuse and days of recent substance use (all *p*-values > 0.05). Moreover, there were no differences between groups established based on miR-124 levels, considering the levels of cognitive-affective and somatic depressive symptoms, and for the years of substance abuse and days of recent substance use (all *p*-values > 0.05).

The GO analysis for miR-124 (Fig. 1D) revealed 401 predicted gene targets (full list of genes in supplementary material 1). Eleven different biological pathways regulated by this miRNA were exhibited, showing higher percentage of genes involved with biological regulation (26%) and metabolic process (22%), and a fewer genes implicated with pathways of localization (13%), cellular process (9%), biogenesis (8%), developmental process (6%), response to stimulus (3%), multicellular process (3%), adhesion (2%), signaling (2%) and immune system (1%). Similarly, these 11 biological pathways were found to be regulated by 445 gene targets (full list of genes in supplementary material 2) of miR-181(Fig. 1E), and the highest percentage of gene regulation was observed for metabolic process (25%) as well as for biological regulation (25%). In addition, this miRNA affects localization (10%), biogenesis (9%), cellular process (8%), developmental process (5%), multicellular process (5%), adhesion (4%), response to stimulus (3%), signaling (2%) and immune system (1%).

4. Discussion

To the best of our knowledge, this is the first study investigating the impact of CUD on miRNA levels in the peripheral blood among human subjects. We found significant group differences between women with and without CUD in the levels of miR-124 and miR-181, showing higher expression values associated with the disorder during the period of early abstinence (e.g. first week of detoxification treatment). In addition, we observed that those individuals with CUD that had a higher expression of miR-181 relative to controls also presented significantly higher cognitive-affective symptoms of depression. Regarding GO analysis, both miR-124 and miR-181 are predicted to regulate genes with biological functions that include biogenesis, biological regulation, cellular and developmental process, localization and metabolic process, although miR-124 was also associated with the functions of cell proliferation, immune system, and response to stimulus. Our preliminary findings are consistent with the growing body of evidence showing that miRNAs levels in the whole blood, as well as in plasma/serum samples, may be altered and are related to mental disorders, including schizophrenia, mood, and addiction disorders.

In rodents, chronic cocaine treatment increases the expression of miR-181 while it suppresses the expression of miR-124 in the brain mesolimbic pathway (Chandrasekar and Dreyer, 2009; Giannotti et al.,

2014). These miRNAs together with let-7d and miR-212 are highly conserved over different species and have been found to target a large number of classical genes involved with cocaine-induced neuroplasticity. For instance, miR-124 has predicted target sites of high binding energy for *BDNF*, *CREB*, *SEMA6A*, *NAC1*, and *NRP2*, while miR-181 targets *GRM5*, *GRIA2* and *RGS4*, among others. The manipulation of these miRNAs specifically in the nucleus accumbens has also been found to regulate the behavior of animals during the consolidation, extinction, and reinstatement of cocaine-induced conditioned place preference (Chandrasekar and Dreyer, 2011). Therefore, both miR-124 and miR-181 are critical for the maintenance and establishment of cocaine addiction phenotypes, since when they pair to their target mRNAs in the brain reward system, post-transcriptional silencing mechanisms are initiated to regulate gene expression and neuroplasticity.

In the peripheral blood, we found increased expression of both miR-124 and miR-181 in women with CUD. In addition to their role in cocaine-induced neuroplasticity, both miRNAs were predicted to target genes implicated with metabolic processes, with a more pronounced list of hits associated with miR-181 functioning (25%). Previous studies have suggested a role for miR-124 and miR-181 on the regulation of metabolic traits in fat tissues and in the liver (Pan et al., 2018; Ponsuksili et al., 2017). Interestingly, the systemic pathophysiology of CUD includes several metabolic alterations. For instance, chronic cocaine use is associated with increased prevalence of hypertension (Brecklin et al., 1998) and myocardial infarction (Behar et al., 2009), which are hallmark features of metabolic syndrome (Virmani et al., 2007). In addition, we and others have found elevated blood levels of adiponectin, a protein hormone that modulates a number of metabolic processes such as glucose uptake and fatty acid oxidation, among individuals with CUD during early abstinence (Levandowski et al., 2013; You et al., 2019). Moreover, evidence suggests that adiponectin regulates glutaminase activity (Ampuero et al., 2012). The *GLS* gene, which encodes the protein glutaminase, was one of the predicted targets found for miR-181 regarding its role on the regulation of metabolic processes in our GO analysis. Furthermore, the *PDK4* gene, which is another regulator of glucose metabolism, was also a predicted target of miR-181. Evidence suggests that individuals with CUD present altered mRNA levels of *PDK4* in their peripheral blood cells, particularly in those patients with high levels of depression (Fries et al., 2018). Therefore, it is possible that alterations in miR-181 and miR-124 expression in the blood are relevant to metabolic changes that occur in individuals with CUD, particularly during periods of drug withdrawal.

In addition to that, transcriptome-wide analysis of miRNA changes in the brain of animals exposed to chronic corticosterone treatment (an animal model of depressive-like phenotypes) revealed that both miR-181 and miR-124 were upregulated in the prefrontal cortex (Dwivedi et al., 2015). Interestingly, we observed that a subsample of the CUD group that had significantly higher miR-181 levels relative to reference values of the control group also presented more depressive symptoms, particularly the constellation of symptoms involved with affective and cognitive aspects of depression. This is relevant since previous evidence suggests that clinical outcomes related to CUD among depressed patients deteriorated to a greater degree during and after detoxification treatment compared to patients without depression (McKay et al., 2002; Wardle et al., 2017). Moreover, given that blood levels of miR-124 has been also pointed out as a putative epigenetic signature of major depressive disorder, and that CUD subjects also scored higher for depressive symptoms compared with controls, it is possible that the observation of higher levels of miR-124 and miR-181 in the CUD group might be associated with the cumulative effect of chronic cocaine consumption withdrawal, depression and stress. For instance, cocaine acutely strongly increases cortisol levels (Baumann et al., 1995) and inpatients with CUD display chronically elevated cortisol levels in blood, saliva and hair samples (Contoreggi et al., 2003; Fox et al., 2009; Grassi-Oliveira et al., 2012). Thus, changes in miR-124 and miR-181 expression might be directly linked to cortisol changes associated with cocaine intake and only indirectly with cocaine use itself.

These findings contributes to the current limited knowledge on the role of miRNAs in humans with CUD, and offer new molecular targets that in the future may help with the diagnosis and treatment of CUD. Nonetheless, the results of this study should be interpreted in light of important limitations. First, this study was performed with a small sample size, and we cannot rule out the possibility of type I error (false-positives) in our findings. Thus, our results should be seen as exploratory and require replication and validation. In addition, the small sample size might have led to an underpowered analysis of the association, for example, between miRNA and the past and current history of cocaine consumption. Second, we did not perform mRNA or protein analysis of the predicted targets of miRNAs. Therefore, whether changes in miRNA expression detected in our analyses would reflect alterations in the levels of their target genes remains unknown. Third, because of the exploratory nature of this report, including its cross-sectional design, it is not known if the altered miRNAs are persistent or state characteristics in CUD patients. The participants in the study were abstinent for some time (first week of detoxification). However, it is unclear if these expression changes for miR-124 and miR-181 would also exist when patients are actively drug users. Future longitudinal studies are needed to answer this question and explore the possibility of potential biomarkers for diagnoses and prognosis for CUD. Fourth, participants with CUD also presented a history of alcohol, tobacco and cannabis use, which can potentially confound the association between cocaine abuse and miRNAs upregulation. For example, chronic ethanol consumption has been demonstrated to up-regulate systemic expression of miR-181a and miR-221 in nonhuman primates (Asquith et al., 2014). However, people with drug addiction commonly report the consumption of multiple drugs (Aharonovich et al., 2006; Aharonovich et al., 2005; Martinotti et al., 2009; Viola et al., 2014a,b). Therefore, it is particularly difficult to recruit participants without a history of polysubstance use. Despite that, our inclusion criteria was stringent regarding the self-report of cocaine as the as the most harmful substance with regard to drug-related problems and treatment-seeking motive. There are also limitations inherent in the interpretation of the current findings with respect to the role of these miRNAs in the brain and how cocaine exposure seems to change their functionality in pre-clinical studies. Although some evidence suggest that blood and brain levels of miRNAs may correlate (Kanata et al., 2018; Liu et al., 2010), future studies might reveal whether such association would also be accurate for miR-124 and miR-181. Moreover, miRNA expression profiles can significantly vary between different cell types (Lai et al., 2018), and our analyses performed in whole blood samples might have covered important findings regarding the function of the investigated miRNAs in specific peripheral cell populations. In addition, we cannot be sure of where the miRNA signals are coming from, since it could be derived from the leukocytes, plasma/serum, or extracellular vesicles. Finally, this study focused on the investigation of only three potentially relevant miRNAs for CUD, although the literature suggests many others (e.g. miR-9, miR-19, miR-26, miR-34, miR-132, miR-134, miR-135, miR-183, miR-375, miR-449 and Let-7d) that could also be differentially expressed in blood samples of patients with CUD.

Despite these limitations, the results of this study suggest that the expression of miR-124 and miR-181 in the whole blood significantly differ between females with and without CUD. Our findings corroborate initial pre-clinical data on the role of these miRNAs in the brain and how they are implicated with cocaine-related phenotypes.

Contributors

Author Thiago Wendt Viola performed statistical analyses, wrote the manuscript and instructed author Bernardo Aguzzoli Heberle on PCR protocols. Author Bernardo Aguzzoli Heberle performed PCR, completed PCR data entry, and collaborated in the writing of the manuscript. Author Aline Zaparte collected blood samples and

collaborated in the writing of the manuscript. Authors Consuelo Walss-Bass and Gabriel Fries collaborated in the writing of the manuscript and with statistical analyses. Author Rodrigo Grassi-Oliveira designed the study protocol, managed literature searches and analyses, and reviewed the manuscript.

Conflicts of interest

All authors declare that they have no conflict of interest.

Role of funding source

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.03.028.

Appendix

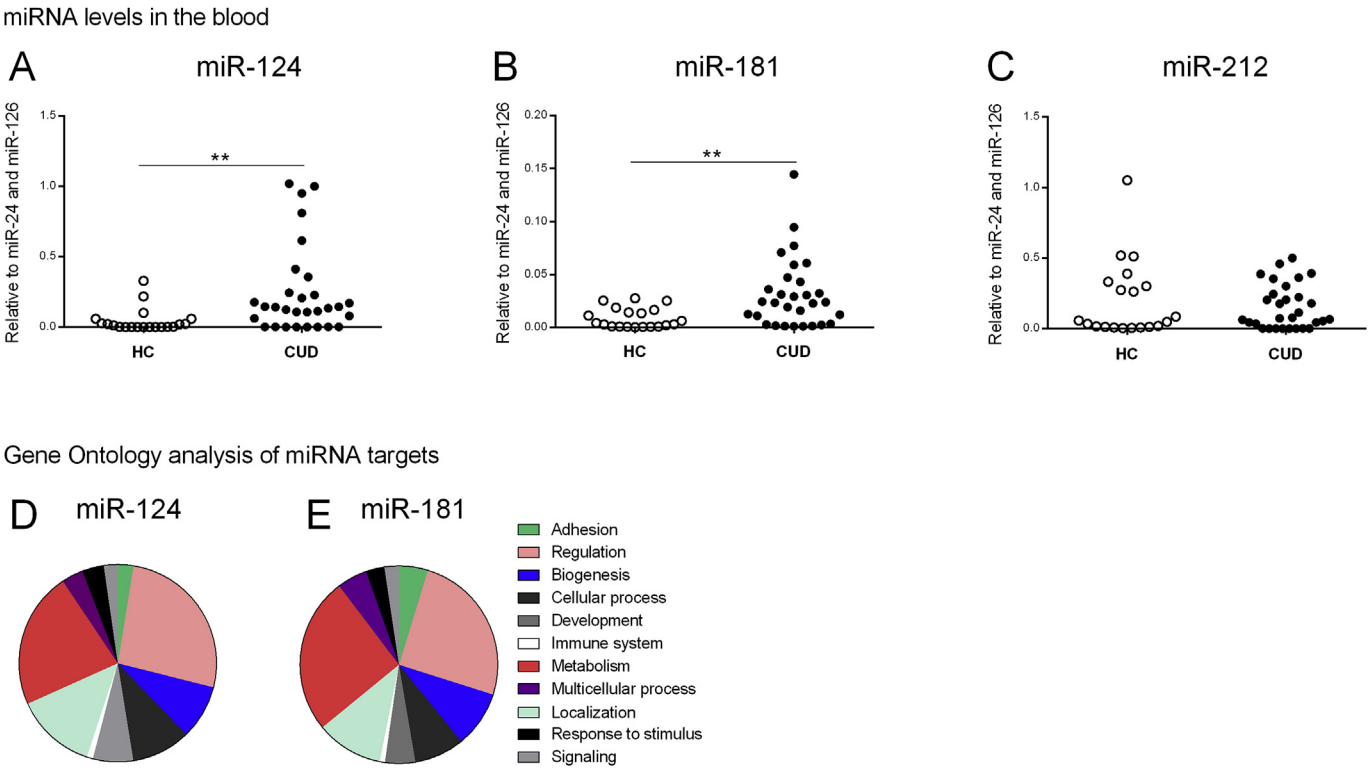

Fig. 1. miRNA levels and Gene Ontology analysis.
Legend: The miRNA levels in HC and CUD groups. $^{**}p < 0.01$ (Mann-Whitney *U* test). (A) miR-124 expression levels. (B) miR-181 expression levels. (C) miR-212 expression levels. Geno Ontology (GO) analyses were performed for the list of predicted targets of miR-124 (D) and miR-181 (E). GO analysis results for miR-124: biological regulation (26%, $n = 104$), metabolic process (22%, $n = 88$), localization (13%, $n = 52$), cellular process (9%, $n = 38$), biogenesis (8%, $n = 35$), developmental process (6%, $n = 26$), response to stimulus (3%, $n = 14$), multicellular process (3%, $n = 14$), adhesion (2%, $n = 10$), signaling (2%, $n = 9$) and immune system (1%, $n = 4$). GO analysis results for miR-181: metabolism (25%, $n = 119$), biological regulation (25%, $n = 117$), localization (10%, $n = 51$), biogenesis (9%, $n = 43$), cellular process (8%, $n = 38$), developmental process (5%, $n = 23$), multicellular process (5%, $n = 23$), adhesion (4%, $n = 22$), response to stimulus (3%, $n = 14$), signaling (2%, $n = 11$) and immune system (1%, $n = 4$).

Table 1
Sociodemographic and clinical characteristics of the sample

	HC	CUD	Statistics	*p*-value
Sociodemographic				
Age (years) *(M/SD)*	29.10 (5.33)	33.00 (9.17)	−1.703	0.095[a]
Monthly Income (US$) *(M/SD)*	657.5 (340.25)	626.25 (996.25)	0.105	0.916[a]

(*continued on next page*)

Table 1 (*continued*)

	HC	CUD	Statistics	*p*-value
BMI (kg/m^2) *(M/SD)*	28.49 (4.43)	24.55 (4.36)	3.102	0.003[a]
Race (White) *(N/%)*	7 (35)	14 (46)	0.458	0.498[b]
Years of substance abuse (M/SD)				
Alcohol	0.40 (1.27)	4.32 (6.34)	–	–
Tobacco	4.15 (6.71)	15.61 (9.94)	–	–
Cannabis	0 (0)	7.69 (8.89)	–	–
Cocaine	0 (0)	9.04 (5.48)	–	–
Days of recent substance use (M/SD)				
Alcohol	0.89 (1.52)	7.33 (9.17)	–	–
Tobacco	2.30 (8.32)	16.76 (10.45)	–	–
Cannabis	0 (0)	6.38 (9.69)	–	–
Cocaine	0 (0)	16.29 (8.66)	–	–
Urine test for recent illegal substance use (N/%)				
Cannabis	0 (0)	7 (23)	–	–
Cocaine	0 (0)	30 (100)	–	–
Depressive symptoms (M/SD)				
BDI-II total score	6.20 (4.62)	39.23 (13.03)	−10.855	> 0.001[a]
BDI-II cognitive affective score	3.80 (2.85)	28.43 (10.93)	−11.755	> 0.001[a]
BDI-II somatic score	2.75 (2.61)	10.60 (3.52)	−9.025	> 0.001[a]

BMI – Body Mass Index; BDI-II – Beck Depression Inventory; Data presented in mean (M) and standard deviation (SD), or number of participants (N) and percentage (%); a *t*-Test; b chi-square. HC – Healthy and unmedicated control group (n = 20); CUD – Cocaine use disorder group (n = 30).

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