American Pain Society
RESEARCH
EDUCATION
TREATMENT
ADVOCACY
PUBLISHED BY ELSEVIER

The Journal of Pain, Vol 16, No 9 (September), 2015: pp 814-824
Available online at www.jpain.org and www.sciencedirect.com

Original Reports

Analgesic Response to Intravenous Ketamine Is Linked to a Circulating microRNA Signature in Female Patients With Complex Regional Pain Syndrome

Sabrina R. Douglas,* Botros B. Shenoda,* Rehman A. Qureshi,† Ahmet Sacan,† Guillermo M. Alexander,‡ Marielle Perreault,‡ James E. Barrett,* Enrique Aradillas-Lopez,‡ Robert J. Schwartzman,‡ and Seena K. Ajit*

*Departments of *Pharmacology and Physiology and ‡Neurology, Drexel University College of Medicine, Philadelphia, Pennsylvania.*
†School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, Pennsylvania.

Abstract: Although ketamine is beneficial in treating complex regional pain syndrome (CRPS), a subset of patients respond poorly to therapy. We investigated treatment-induced microRNA (miRNA) changes and their predictive validity in determining treatment outcome by assessing miRNA changes in whole blood from patients with CRPS. Blood samples from female patients were collected before and after 5 days of intravenous ketamine administration. Seven patients were responders and 6 were poor responders. Differential miRNA expression was observed in whole blood before and after treatment. In addition, 33 miRNAs differed between responders and poor responders before therapy, suggesting the predictive utility of miRNAs as biomarkers. Investigation of the mechanistic significance of hsa-miR-548d-5p downregulation in poor responders showed that this miRNA can downregulate UDP-glucuronosyltransferase UGT1A1 mRNA. Poor responders had a higher conjugated/unconjugated bilirubin ratio, indicating increased UGT1A1 activity. We propose that lower pretreatment levels of miR-548d-5p may result in higher UDP-GT activity, leading to higher levels of inactive glucuronide conjugates, thereby minimizing the therapeutic efficacy of ketamine in poor responders. Differences in miRNA signatures can provide molecular insights distinguishing responders from poor responders. Extending this approach to other treatment and outcome assessments might permit stratification of patients for maximal therapeutic outcome.

***Perspective:** This study suggests the usefulness of circulating miRNAs as potential biomarkers. Assessing miRNA signatures before and after treatment demonstrated miRNA alterations from therapy; differences in miRNA signature in responders and poor responders before therapy indicate prognostic value. Mechanistic studies on altered miRNAs can provide new insights into disease.*

***Keywords:** MicroRNA, biomarker, complex regional pain syndrome, ketamine.*

Received March 18, 2015; Revised May 4, 2015; Accepted May 28, 2015.
S.R.D. and B.B.S. contributed equally to this work.
This study was supported by funds from a Rita Allen Foundation grant to S.K.A. B.B.S. is a recipient of the Fulbright Foreign Student Program Fellowship funded by the U.S. Department of State Bureau of Educational and Cultural Affairs. The authors report no disclosures. This study was not industry sponsored.
Supplementary data accompanying this article are available online at www.jpain.org and www.sciencedirect.com.
Address reprint requests to Dr. Seena K. Ajit, Assistant Professor, Pharmacology & Physiology, Drexel University College of Medicine, 245 North 15th Street, Mail Stop 488, Philadelphia, PA 19102. E-mail: seena.ajit@drexelmed.edu
1526-5900/$36.00

http://dx.doi.org/10.1016/j.jpain.2015.05.008

Complex regional pain syndrome (CRPS) is a chronic neuropathic pain condition with a broad array of symptoms, including pain, inflammation, sensory dysfunction, impaired motor function, and trophic disturbances.[6,8,12,17,31,49] Ketamine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, is used to treat patients with CRPS for whom all other treatments have failed.[47] Although the treatment is generally effective, approximately 30% of patients have an inadequate response to ketamine.[10] It has been proposed that using biomarkers in blood and cerebrospinal fluid, in conjunction with the symptom-

defining subtypes of patients with CRPS, may be beneficial in predicting disease severity and progression.[47]

Small noncoding microRNAs (miRNAs) negatively regulate gene expression by binding to the 3′ untranslated region (3′UTR) of target mRNAs to induce translational repression or mRNA degradation.[4] miRNAs regulate a wide range of biological processes, and their dysregulation is observed in a number of diseases.[34] The presence of stable miRNAs in bodily fluids and the noninvasive nature of assessing them has resulted in many studies exploring their usefulness as sensitive and specific biomarkers.[11] Our previous study investigating miRNAs in whole blood from patients with CRPS identified differential expression of 18 miRNAs between patients and control individuals.[39] In addition, clustering of 60% of the patients using miRNA profiles enabled clinically relevant stratification of heterogeneous patient populations.[39] Our objective here was to perform a proof-of-concept study to assess miRNA changes in response to therapy and explore the feasibility of using circulating miRNA signatures in predicting treatment response. We investigated whether intravenous (i.v.) ketamine treatment induced miRNA alterations in patients with CRPS and whether ketamine-induced miRNA changes, if any, would differ between responders and nonresponders. The present pilot study, including 7 responders and 6 poor responders, demonstrates the potential utility of miRNAs as biomarkers for both evaluating efficacy of ketamine therapy and predicting treatment response.

We investigated the mechanistic significance of differentially expressed miRNAs by studying the role of hsa-miR-548d-5p, a miRNA that showed significant downregulation in poor responders before treatment. hsa-miR-548d-5p is predicted to target the mRNA of cytochrome P450 3A4 (CYP3A4) and UDP-glucuronyl transferase (UGT1A family), 2 enzymes important for biotransformation of ketamine.[24,56,57] The role of miR-548d-5p in inducing different ketamine pharmacokinetic profiles between responders and poor responders, potentially minimizing the therapeutic efficacy of ketamine and pain relief, was also explored.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All participants were enrolled after giving informed consent as approved by the Drexel University College of Medicine institutional review board.

Inclusion and Exclusion Criteria

All patients meeting the clinical Budapest criteria for CRPS[22] undergoing i.v. ketamine therapy as treatment were asked to participate in this study. The patients were asked to provide blood samples before and after treatment. Patients with autoimmune or immunodeficiency conditions were excluded. Blood samples were collected between July 2012 and March 2013.

Ketamine Infusion and Patient Evaluation

Ketamine was infused to patients with refractory CRPS over 5.5 days in the intensive care unit of Hahnemann University Hospital in Philadelphia, PA. All patients had full cardiac and neuropsychological clearance before the ketamine infusion. The infusion rate began at 10 mg/h and was increased by 10 mg/h every 2 hours until a maximum rate of 40 mg/h was reached. This rate was continued for 120 hours and was then tapered off by 10 mg/h every 2 hours (Fig 1A). Clonidine (.1 mg tablet twice daily) was added if the patient developed an increase of 10 mm Hg or more from baseline systolic or diastolic blood pressure; midazolam i.v. (2 mg every 4 hours) was added as needed for anxiety. Patients were asked to rate their overall pain before, during, and after the infusion using a 10-point numerical rating scale (NRS) from 0 (no pain) to 10 (the worst pain). Patients were also asked to rate their pain before and after the infusion using the short-form McGill Pain Questionnaire (Fig 1A).

miRNA Profiling

Blood samples were collected before and after treatment in BD Vacutainer plastic blood collection tubes from molecular diagnostics (BD Biosciences, San Jose, CA) and stored at −20°C. RNA was isolated from whole blood using a PAXgene blood miRNA kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Taqman low-density array microfluidic cards version A and B (Applied Biosystems, Foster City, CA) were used to profile miRNAs in 100 ng of total RNA as previously described.[39] Mean normalization was applied to the cycle threshold (CT) values. miRNA species with CT values 35 and higher were treated as undetected. Fold change was calculated from raw CT values using the $2^{-\Delta\Delta CT}$ method.[45] The mean CT values of the 10 miRNAs with the lowest standard deviations were used as the endogenous control in the calculation of ΔCT. Statistical significance of differences in ΔCT values was calculated by a 2-tailed paired t-test for comparison of pretreatment and posttreatment samples and by a 2-tailed independent t-test for comparison of other experimental groups. The Benjamini-Hochberg false discovery rate correction was applied to the *P* values. Hierarchical clustering of miRNAs and samples was performed along with the generation of a heatmap of miRNA expression. Pairwise Spearman correlation was calculated between various clinical parameters and miRNAs.

Luciferase Reporter Assay

The 3′UTR clones for human UGT1A1 (HmiT013615-MT01), CYP3A4 (HmiT055240-MT01), and the precursor miRNA clone hsa-miR-548d-5p (HmiR0132) were purchased from GeneCopoeia (Rockville, MD). Reporter assays were performed 24 hours after transfection of HEK293 cells. Statistically significant differences from control were calculated using a Student t-test.

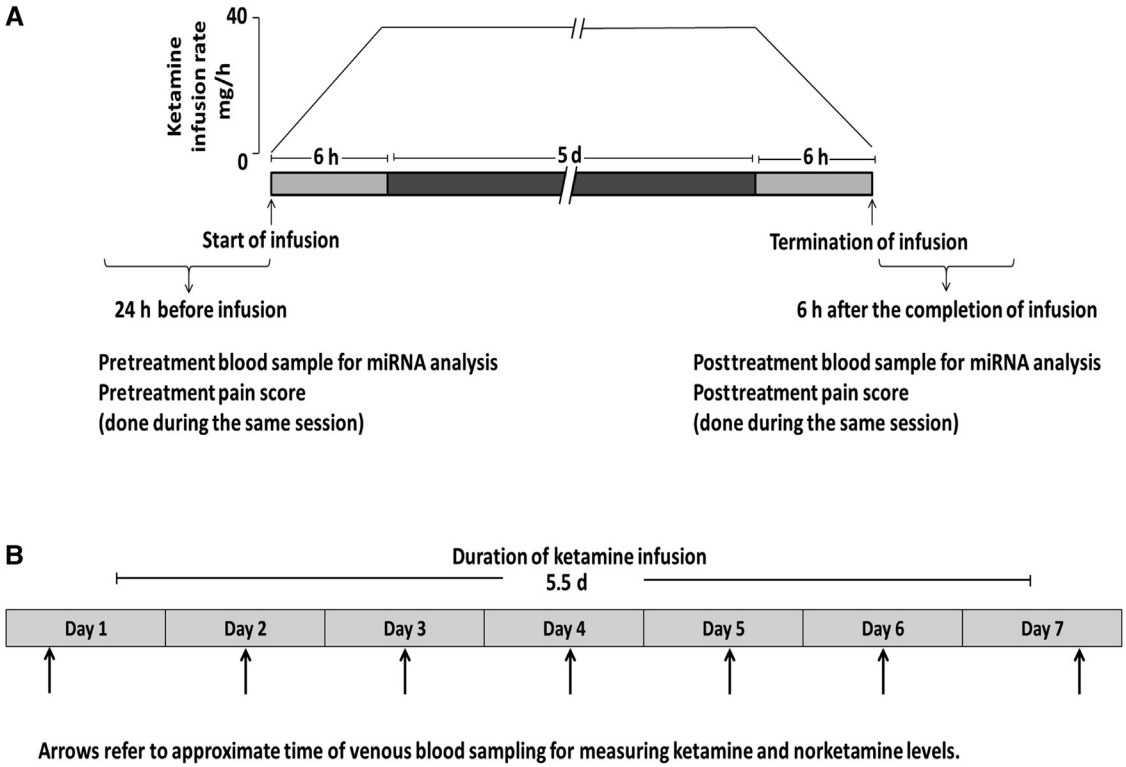

Figure 1. Schematic representation of study design. **(A)** Ketamine infusion schedule, time of blood collection, and pain scoring. **(B)** Time points blood samples were obtained for determining ketamine and NK levels.

RNA Isolation and Quantitative Polymerase Chain Reaction

HepG2 cells were transfected with miR-548d-5p plasmid using Xtreme gene HP DNA reagent (Roche, Indianapolis, IN). RNA was isolated 24 hours after transfection using a mirVana RNA isolation kit (Ambion, Austin, TX). cDNA was synthesized using a Maxima first strand cDNA synthesis kit (Thermo Fischer, Waltham, MA). The relative expression of UDP-GT1A1 was determined using quantitative polymerase chain reaction (UGT1A1 Assay ID: Hs02511055_s1; Applied Biosystems) with 18S as the normalizer. Statistical analysis was performed using a Student t-test.

UDP-UGT-1A1 Assay

Microsomes from the HepG2 cells transfected with empty vector or miR-548d-5p were isolated as described.[53] UDP-GT activity assay was performed using the UGT-Glo UGT1A1 screening system (Promega, Madison, WI). UDP-GT1A activity was measured as a function of relative substrate consumption.

Bilirubin Ratio and Norketamine Clearance

Routine daily laboratory workup included a complete chemistry test, liver function tests (total and direct bilirubin, serum alanine transaminase and aspartate transaminase levels), ketamine and norketamine (NK) levels, differential blood count, and troponin I level (Fig 1B). These values were used to determine direct/indirect bilirubin ratio, ketamine, and NK clearance. Serum levels of ketamine and NK were determined by liquid chromatography coupled with tandem mass spectrometry (Atlantic Diagnostic Laboratories, Bensalem, PA). Pharmacokinetic analysis was performed as previously described.[20,23] Ketamine clearance = C_{ss} × rate of infusion/kg (body weight). NK clearance = ketamine clearance × area under the curve $(AUC)_{0-\infty\,ketamine}$/$AUC_{0-\infty\,norketamine}$. $AUC_{0-\infty}$ was determined using the trapezoidal method, and the slope was determined according to the half-life of ketamine and NK, as previously reported for patients with CRPS.[20]

Results

miRNA Changes in Response to Ketamine Treatment in Responders and Poor Responders

We evaluated 13 female patients with CRPS. The incidence of CRPS is greater in females than males by approximately 4:1,[49] and given our small sample of males, only the 13 female patients were included in this study, removing gender as a confounding factor in our data analysis. The patients demonstrated disease duration of at least 2 years and an average NRS pain score ≥5. The pretreatment and posttreatment pain scores are tabulated in Table 1. Patients were grouped as responders if after treatment their average pain score decreased by at least 50% and as poor responders if their average pain score either increased or decreased less than 50%. Based on this criterion, the patients were grouped as 7 responders and 6 poor responders.

Table 1. McGill and NRS Scores of Patients with CRPS and Their Average Percent Change After Receiving Ketamine Therapy

Patients (n)	Gender	Age (y)	Duration of the Disease/Pain (y)	Body Weight (kg)	McGill Pain Score		NRS Pain Score		Pain Score Change: Ratio		Average Percent Change
					Pretreatment	Posttreatment	Pretreatment	Posttreatment	McGill	NRS	
Responders											
17	F	43	5.0	118.18	73	3	8	1	.96	.88	91.7
13	F	54	23.0	112.73	113	12	10	1	.89	.9	89.69
15	F	51	4.0	80.00	114	2	8	2	.98	.75	86.62
12	F	51	12.0	90.91	122	19	8	1	.84	.88	85.96
16	F	39	4.0	88.18	95	13	7	2	.86	.71	78.87
5	F	65	7.0	75.00	139	11	7	3	.92	.57	74.61
10	F	50	2.0	85.91	167	15	8	4	.91	.5	70.51
Poor responders											
3	F	45	8.0	45.45	180	99	8	5	.45	.38	41.25
8	F	69	24.0	69.09	110	108	10	9	.02	.1	5.91
9	F	35	6.0	68.18	118	145	8	8	−.23	0	−11.44
22	F	56	6.4	65.91	57	74	8	8	−.3	0	−14.91
24	F	33	3.6	75.45	118	143	7	8	−.21	−.14	−17.74
11	F	45	12.0	72.73	50	96	8	10	−.92	−.25	−58.5

Abbreviation: F, female.

Comparison of miRNA profiles before and after ketamine treatment showed differential expression of 14 miRNAs in both responders (Fig 2) and poor responders (Supplementary Fig 1, Supplementary Table 1). The 14 miRNAs were not identical between the 2 groups, indicating that ketamine induced molecular changes that differed between responders and poor responders.

Predictive Validity of miRNA Signature

Comparison of miRNAs in responders relative to poor responders before treatment showed differential expression of 33 miRNAs, indicating that miRNA signatures can be used as biomarkers for predicting treatment response (Table 2, Supplementary Fig 2). In the posttreatment samples, 43 miRNAs differed between responders and poor responders (Supplementary Fig 3, Supplementary Table 1).

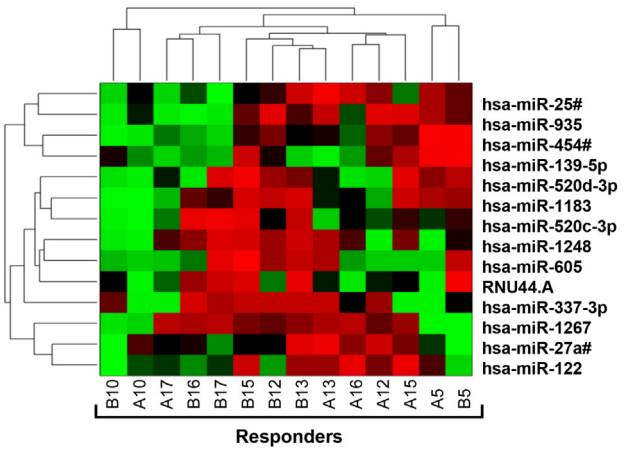

Figure 2. Expression patterns of miRNAs in responders before and after treatment. A clustergram of the significant differentially expressed miRNAs in patients with CRPS who responded to ketamine therapy before and after treatment. Each column represents an individual patient (B, before treatment; A, after treatment). The heat map represents normalized expression values for each miRNA. Red, high; black, average; green, low.

Comparison of miRNAs in Ketamine-Treated Patients With Control

We compared patient miRNAs with female control samples from our previous study.[39] Five miRNAs (hsa-miR-320, hsa-let-7c, hsa-miR-181a-2#, hsa-miR-320B, hsa-miR-720) that were identified in our previous study were also significant in this study, indicating reproducibility in independent patient cohorts. miR-320, miR-320B, let-7c, and miR-181a-2# were downregulated in patients with CRPS in both studies. However, miR-720 was upregulated in the first study but found to be decreasing in this study. Comparison of control individuals with responders showed differential expression of 33 miRNAs before treatment and 54 miRNAs after treatment; in poor responders, 48 and 29 miRNAs were altered before and after treatment, respectively (Supplementary Figs 4–9, Supplementary Table 1). Correlation analysis of selected clinical parameters and miRNAs is shown in Fig 3 and Table 3.

Regulation of UGT1A1 by hsa-miR-548d-5p

The expression of miRNA-548d-5p was significantly lower in poor responders relative to responders. Bioinformatics prediction using multiple algorithms[42] showed that mRNAs for UGT1A1 and CYP3A4 are predicted targets for miRNA-548d-5p. A luciferase reporter assay confirmed the binding of miR-548d-5p to the 3′UTR of UGT1A1, but not CYP3A4. In HepG2 cells transfected with miR-548d-5p, UGT1A1 transcripts and enzymatic activity were significantly reduced relative to empty vector transfected cells. Responders had a lower conjugated/unconjugated bilirubin ratio (Fig 4). There

Table 2. miRNAs Significantly Altered Between Responders and Poor Responders Before Ketamine Treatment

miRNA	P *Value*	*Fold Change*
hsa-miR-367	.0067	−1512.59
hsa-miR-15a	.0072	−432.79
hsa-miR-1276	.0103	−30.18
hsa-miR-337-3p	.0036	−29.76
hsa-miR-34a	.0483	−28.3
hsa-miR-26a-2#	.0233	−23.33
hsa-miR-605	.0242	−22.38
hsa-miR-450a	.0294	−21.4
hsa-miR-410	.0104	−21.27
hsa-miR-645	.0078	−20.98
hsa-miR-548d-5p	.0328	−18.08
hsa-miR-1303	.0324	−15.86
hsa-miR-376a	.0079	−15.18
hsa-miR-1225-3P	.0123	−13.99
hsa-miR-29c	.0454	−13.41
hsa-miR-337-5p	.0448	−12.67
hsa-let-7g#	.0077	−12.58
hsa-miR-149	.0186	−9.39
hsa-miR-212	.0054	−7.59
hsa-miR-21#	.0486	−6.77
hsa-let-let-7f	.0453	−6.44
hsa-miR-643	.0474	−5.44
hsa-miR-365	.0124	−5.32
hsa-miR-1260	.0476	−5.09
hsa-miR-504	.018	−5.072
hsa-miR-29a	.0445	−4.7
hsa-miR-576-3p	.036	−3.84
hsa-miR-186	.0273	−2.25
hsa-miR-146a	.0437	−2.08
hsa-miR-197	.0011	−1.9
hsa-miR-374b	.0227	−1.83
hsa-miR-16	.0331	−1.48
hsa-miR-150	.0159	1.27

NOTE. Poor responders had lower expression in almost all of the differentially expressed miRNAs compared with good responders. hsa-miR-150 was the only miRNA that was upregulated in poor responders. Data are sorted based on fold change.

was no difference in ketamine or NK clearance between responders and poor responders (Table 4).

Discussion

Stability in circulation and dysregulation in disease state are 2 features making extracellular miRNAs useful candidates for biomarker discovery.[59] Alterations in miRNA profiles have been reported for rheumatoid arthritis[2] and systemic lupus erythematosus[50] as well as for painful conditions such as irritable bowel syndrome,[14] chronic bladder syndrome,[16] endometriosis,[38] and migraine.[3] Cerebrospinal fluid from patients with fibromyalgia showed differential expression of 9 miRNAs.[5] Our previous study in patients with CRPS identified differential expression of 18 miRNAs and demonstrated the usefulness of circulating miRNAs in patient stratification.[39] Here, we evaluated miRNA changes in response to therapy and assessed the validity of circulating miRNA signature in predicting treatment response.

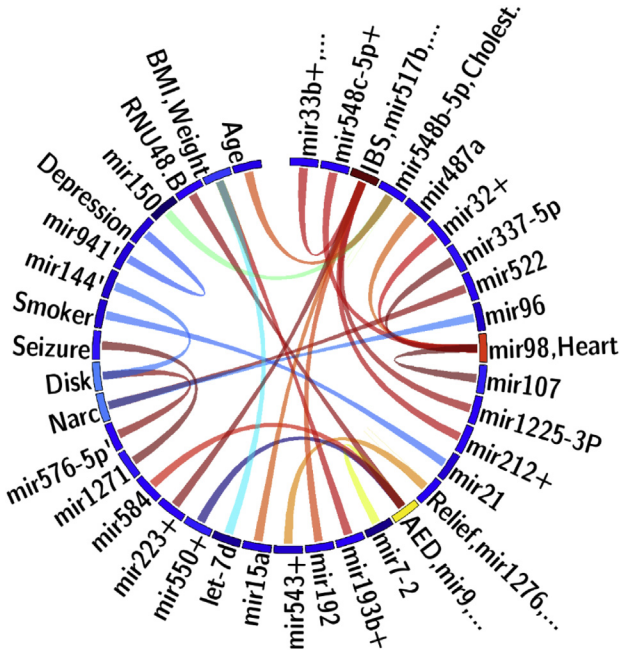

Figure 3. A Circos diagram showing the correlations between selected clinical parameters and miRNAs. The links between variables indicate pairwise Pearson correlation values, with positive correlations shown in red and negative correlations in blue. The variables along the circle are colored by their total strength of correlation. For clarity, only correlations of magnitude greater than .75 are shown; variables with correlation greater than .9 are merged into a single node, and groups of miRNAs are indicated with ellipsis. The list of all pairwise correlations is available in Table 3. miRNA levels after treatment are marked with a plus symbol and change in miRNA levels with treatment are marked with a single quote symbol. Abbreviations: BMI, body mass index; Disk, history of disk prolapse; Narc, history of narcotic therapy; AED, antiepileptic treatment; Relief, average pain relief from ketamine treatment; Heart, history of heart disease; Cholest, blood cholesterol level; IBS, irritable bowel syndrome.

Subanesthetic doses of systemic ketamine produce analgesia.[44] When administered i.v. over several days with antipsychotics and antihallucinogens, ketamine has been shown to provide relief to patients with CRPS[7] that often lasts for weeks or even months after treatment[9,10,18,25,26,48,51]; however, not all patients with CRPS respond to ketamine treatment.[37] Only moderate to severely affected patients with CRPS, for whom all other treatments have failed, are considered for ketamine treatment.[25] Ketamine is also considered to be the prototype for a new generation of glutamate-based antidepressants that can alleviate depression within hours of treatment.[1] Several biological measures have been explored to characterize treatment response and to gain insight into mechanisms underlying the rapid antidepressant effects of ketamine. A plasma metabolomics study in patients with bipolar depression suggested that the basal mitochondrial β-oxidation of fatty acids differed between responders and nonresponders to ketamine.[58] Other studies have shown differences in baseline plasma concentrations of *D*-serine,[35] serum levels of interleukin 6,[61] and plasma levels of Shank3, a postsynaptic density protein involved in NMDA receptor tethering and dendritic spine rearrangement.[40] Our analysis of circulating miRNAs in

Table 3. Correlations Between Selected Clinical Parameters and miRNAs

		Pearson		Spearman	
Variable 1	Variable 2	Correlation	FDR	Correlation	FDR
IBS	hsa-miR-367	.99	2.8E-13	.79	.016
IBS	hsa-miR-367+	.99	1.36E-11	.79	.016
IBS	hsa-miR-212	.95	4.08E-06	.79	.016
BMI	Weight	.95	5.08E-06	.87	.0014
Cholesterol	hsa-miR-548b-5p	.93	2.99E-05	.85	.0027
Relief	hsa-miR-337-3p	.90	.0023	.83	.031
IBS	hsa-miR-302c+	.89	.00024	.76	.027
Heart	hsa-miR-98	.89	.00031	.87	.0017
Relief	hsa-miR-365+	.88	.0044	.60	.3
IBS	hsa-miR-517b	.88	.00048	.74	.041
AED	hsa-miR-30d	.88	.00049	.74	.043
AED	hsa-miR-590-3p	.87	.00063	.74	.043
IBS	hsa-miR-346+	.85	.0012	.76	.027
AED	hsa-miR-130b+	.84	.0016	.71	.064
AED	hsa-miR-664	.84	.0021	.74	.043
Heart	hsa-miR-337-5p	.83	.0027	.79	.015
AED	hsa-miR-425	.82	.0032	.74	.043
IBS	hsa-miR-212+	.82	.0038	.76	.027
AED	RNU48.B+	.82	.0039	.74	.043
IBS	hsa-miR-33b+	.81	.0048	.61	.18
AED	hsa-miR-7-2	.81	.0049	.65	.12
Heart	hsa-miR-107	.81	.005	.87	.0017
IBS	hsa-miR-302c	.81	.0052	.79	.016
AED	hsa-miR-1255b	.80	.0056	.74	.043
IBS	hsa-miR-155+	.80	.0063	.74	.041
IBS	hsa-miR-628-5p	.79	.0071	.76	.027
AED	hsa-miR-1274A	.79	.0071	.71	.064
AED	hsa-miR-550+	−.79	.0071	−.62	.15
Heart	hsa-miR-548c-5p+	.79	.0073	.79	.015
IBS	hsa-miR-155	.79	.0077	.74	.041
AED	RNU48.B	.79	.0078	.62	.15
Weight	let-7d	−.79	.0083	−.68	.089
Seizure	hsa-miR-1271	.79	.0084	.74	.043
AED	hsa-miR-589.B	.78	.0086	.74	.043
AED	hsa-miR-584	.78	.0086	.74	.043
Depression	hsa-miR-941′	−.78	.0094	−.65	.12
AED	hsa-miR-1233	.78	.0097	.65	.12
Heart	hsa-miR-218	.71	.035	.82	.0082
Relief	hsa-miR-376a+	.71	.099	.88	.0096

NOTE. Correlations of magnitude at least .7 and false discovery rate (FDR) of at most .01 are shown. FDR is calculated using the Benjamini-Hochberg method. The raw *P* values and the list of comparison that do not meet the FDR criteria are shown in Supplementary Table 2.
Abbreviations: IBS, irritable bowel syndrome; +, miRNA after treatment, BMI, body mass index; Cholesterol, blood cholesterol level; Relief, average of the reduction in McGill and NRS pain scores as a result of ketamine treatment; Heart, history of heart disease; AED, antiepileptic treatment; ′, change in miRNA with treatment.

female patients with CRPS undergoing ketamine therapy demonstrated differential expression of miRNAs in responders and poor responders both before and after treatment. These differences indicate that ketamine elicited different responses in the 2 groups. It also suggests that the commonality in disease symptoms does not necessarily indicate dysregulation of the same molecular pathways. Investigating genes targeted by these differentially regulated miRNAs can be beneficial in obtaining insights into previously unexplored mediators in CRPS.

Comparison of miRNAs in ketamine-treated patients with female control samples from our previous study[39] showed that hsa-miR-320, hsa-let-7c, hsa-miR-181a-2#, and hsa-miR-320B were significantly downregulated in both our previous study and our current study. miR-720 was upregulated in the first study but found to be decreasing in this study. Further studies in a larger patient population of treatment-resistant patients with CRPS are needed to confirm if this difference in miR-720 expression is a result of the inclusion of female patients with treatment-resistant CRPS in the current study compared with all patients with CRPS included in the previous miRNA profiling study. Recently miRBase (http://www.mirbase.org/), the online searchable database for all published miRNA sequences and associated annotations,[21] removed miR-720 because the transfer RNA (tRNA) species $tRNA^{Thr}$ have an 18-nucleotide sequence at the 3′ end that matches miR-720. It has been proposed that further validation is

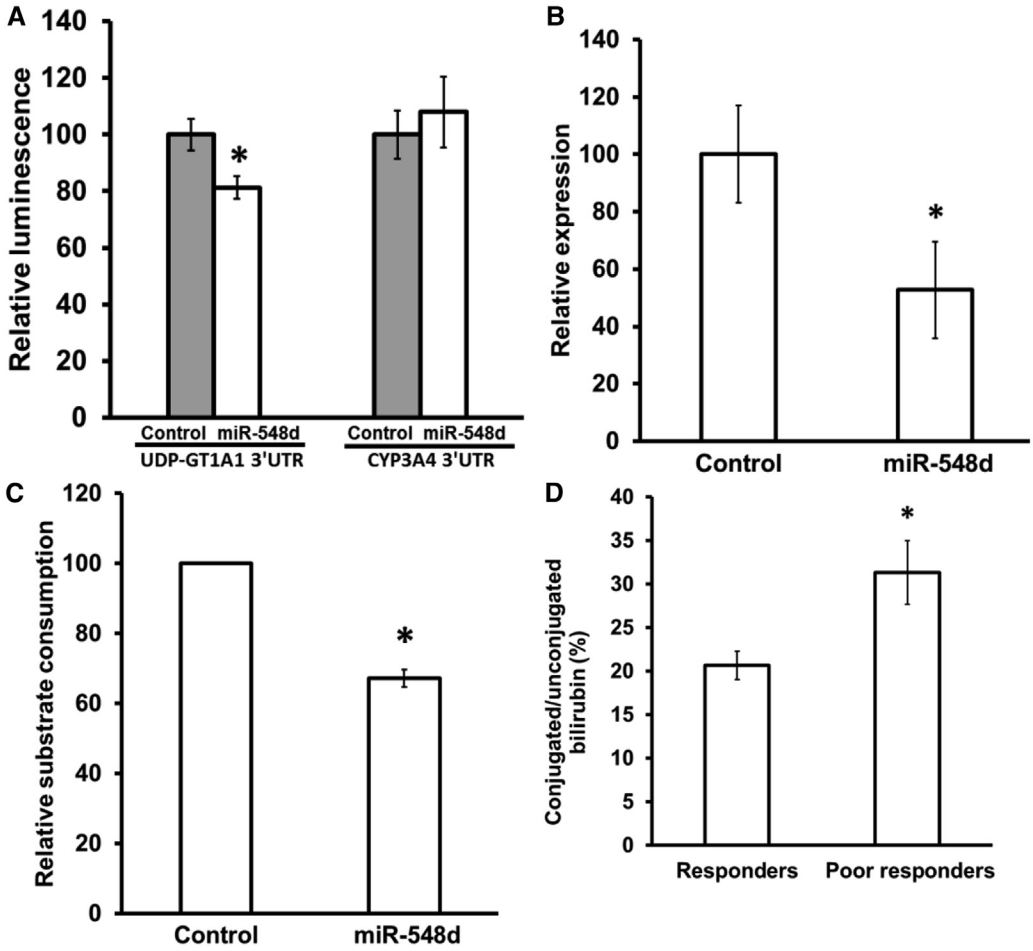

Figure 4. Regulation of UGT1A1 by hsa-miR-548d-5p. **(A)** Luciferase assay to determine miR-548d-5p binding to the 3′UTR of UGT1A1and CYP3A4. The 3′UTR was cloned downstream of the luciferase open-reading frame. The luciferase activity was measured 24 hours after transfection, and data are expressed as a percentage of the control. **(B)** Transcriptional regulation of UGT1A1 by hsa-miR-548d-5p. HepG2 cells were transfected with hsa-miR-548d-5p, and the relative expression of endogenous UGT1A1 mRNA was determined using quantitative polymerase chain reaction; 18S was used as the normalizer. **(C)** UDP-GT activity in hsa-miR-548d-5p transfected cells. Using microsomes isolated from the HepG2 cells transfected with empty vector or miR-548d, the enzymatic activity was measured as a function of relative substrate consumption. **(D)** Conjugated (direct) and unconjugated (indirect) serum bilirubin levels of patients with CRPS before treatment. Responders (n = 5) to ketamine therapy had lower conjugated/unconjugated bilirubin ratio relative to poor responders (n = 4). Patients with bilirubin reported as <1 were not included in the analysis. Statistical analysis was performed using a Student t-test. *$P < .05$.

required to confirm whether this small RNA fragment is derived from tRNA processing.[46] Nevertheless, from a biomarker perspective, miR-720 is still informative.[55] We also observed that miR-25#, an miRNA downregulated 3.9-fold in patients with CRPS,[39] was upregulated 2.1-fold (*P* value .04, Fig 2, Supplementary Table 1) in responders after treatment. This suggests that upregulation of miR-25# could be therapeutically beneficial in reversing pain, and we are validating potential target genes for miR-25# and their relevance in CRPS. The reproducibility in identifying similar miRNAs from independent patient cohorts warrants further investigation of all these miRNAs not only as a biomarker but also as signaling molecules. Confirmation of valid miRNA–mRNA interactions is challenging[32] and typically requires exploration of several bioinformatics prediction

Table 4. Pharmacokinetic Variables of Ketamine and NK in Patients With CRPS Grouped According to Their Response to Ketamine Therapy

	Ketamine					*NK*			
	t_{max} (h)	C_{max} (mg/L)	AUC	C_{ss} (mg/L)	cL (L/h/kg)	t_{max} (h)	C_{max} (mg/L)	AUC	cl (L/h/kg)
Responders	73.7 ± 9.9	.5 ± .07	60.6 ± 9.7	.43 ± .05	1.01 ± .12	53.7 ± .94	.27 ± .04	31.3 ± 4.2	2.45 ± .54
Poor responders	101.3 ± 11.1	.53 ± .05	66.9 ± 8.4	.44 ± .04	1.6 ± .11	58.6 ± 9.88	.39 ± .04	37.1 ± 3.39	2.67 ± .31

Abbreviations: t_{max}, time to reach C_{max}; C_{max}, maximum plasma concentration; AUC, area under the curve; C_{ss}, steady state concentration; cl, clearance.

algorithms, individual validation of binding, and translational suppression and forward genetic techniques to uncover new potential targets.[15] In addition, the differentially expressed miRNAs observed in this study are predicted to target genes with wide-ranging cellular functions. Target validation studies for individual miRNAs will be beneficial in determining the specific functional consequences of aberrant circulating miRNAs and their role in chronic pain.

Bioinformatics prediction using multiple algorithms[29] showed that UGT1A1 and CYP3A4 harbor potential binding sites for hsa-miR-548d. Both genes are of particular importance in drug metabolism.[24,56,57] Thus, although there were other miRNAs that had higher fold change, we decided to focus on miR-548d for target validation studies because of its potential regulatory role in ketamine metabolism. Ketamine has a complex pharmacokinetic profile.[28] The principal enzyme responsible for ketamine *N*-demethylation in human liver is cytochrome P450, resulting in the production of a series of metabolites, including NK.[24] Ketamine and NK are extensively hydroxylated to a series of 6 hydroxynorketamine metabolites (HNK4a–4f) and 2 hydroxyketamine metabolites (HK5a and HK5b). NK is also transformed into a dehydronorketamine metabolite.[36,62] Most ketamine metabolites are rendered inactive and eliminated by conjugative enzymes, specifically UDP-GT, through glucoronidation.[56] The human UDP-glucuronosyltransferase (UGT) family is grouped into UGT1A and UGT2B subfamilies. The UGT1A family comprises 9 functional protein isoforms that share a common fifth exon and 3′UTR.[30] There was an 18-fold difference in expression of hsa-miR-548d-5p between responders and poor responders. The expression of this miRNA was higher in responders, indicating that the genes targeted by this miRNA are lower in responders because of the expected inverse correlation between an miRNA and its target mRNA. Our target validation studies showed that hsa-miR-548d-5p does not bind to the 3′UTR of CYP3A4. This suggests that hsa-miR-548d-5p does not play a role in regulating the expression of CYP3A4 and, thus, of the *N*-demethylation of ketamine into NK. However, hsa-miR-548d-5p did bind to the UGT1A1 3′UTR and reduced expression of UGT1A1 mRNA and protein in liver cells. These results suggest that the miR-548d-5p may mediate difference in UDP-GT activity between responders and poor responders. Poor responders had a higher percentage of direct/indirect bilirubin relative to responders, indicating that they may have increased UDP-GT enzyme activity and therefore increased removal of ketamine metabolites, potentially minimizing the therapeutic efficacy of ketamine.

Our pharmacokinetic analysis shows that there was no significant difference in ketamine and NK clearance between responders and poor responders. Previous pharmacokinetic study in patients with CRPS receiving 5 days of a continuous subanesthetic dose of ketamine also did not show significant differences in ketamine clearance and first-order elimination rate of NK between responders and poor responders.[19,20] Primary metabolites in the plasma were a series of hydroxynorketamine metabolites, and (R)- and (S)-dehydronorketamine were the major metabolites found in urine, indicating that NK is the initial but not the primary metabolite and that metabolites downstream of NK play a role in ketamine-related pain relief in patients with CRPS.[36] Thus, it was postulated that the therapeutic properties are not solely a result of the antagonism of ketamine and NK on the NMDA receptor but that other downstream ketamine metabolites may directly or indirectly contribute to the therapeutic efficacy.[36,43,47] Differences in the ability to metabolize ketamine because of interindividual differences and pharmacogenetic factors have been proposed to contribute to the varied responses to ketamine therapy and its clinical outcome.[20,43] Similar conclusions have been drawn for patients with depression; plasma from patients with treatment-resistant bipolar depression who had undergone a single 40-minute infusion of a subanesthetic dose of ketamine showed that although NK is an initial metabolite, it is not the major circulating metabolite. This again suggests that other downstream metabolites of ketamine may play a role in the pharmacological effects of the drug.[63] It is also known that (2S,6S)-hydroxynorketamine is an active and selective inhibitor of the α_7 subtype of the nicotinic acetylcholine receptor; this activity was shown to contribute to the pharmacological responses associated with the antidepressant activity of (R,S)-ketamine.[41,52] We postulate that in patients with CRPS, 1 factor contributing to resistance is an altered pharmacokinetic profile produced by enhanced elimination of active metabolites downstream of NK, which is mediated by hsa-miR-548d-5p. However, because we have relied on indirect evidence of a higher percentage of direct/indirect bilirubin in poor responders, indicating increased UDP-GT enzyme activity, additional studies investigating hydroxynorketamine and its downstream metabolites along with their glucuronide conjugates in plasma and urine will provide direct evidence for the role of miR-548d-5p in mediating response to ketamine therapy in responders and poor responders.

Our study focusing on 1 miRNA is an example of how circulating miRNAs can be used to explore disease mechanisms or response to therapeutic intervention. Future studies investigating the other 32 miRNAs that were differentially regulated in responders and poor responders before treatment could provide a glimpse of underlying aberrant molecular signaling in patients with CRPS. These studies together with the clinical symptoms can help us understand why only a subset of patients respond to treatment. Our analysis did not show any correlation between disease duration and analgesic response to ketamine, but we observed a significant difference in body weight between responders and poor responders (Table 1). We are investigating the link between miR-34a, which showed 28-fold reduction in poor responders relative to responders (Table 2), and the neuroendocrine system, and the findings will be

published as a separate study. Similarly, treatment-induced miRNA changes in responders can be explored to identify target genes that are being regulated by these miRNAs.

Our previous study showed that circulating miRNAs altered in patients with CRPS are trafficked by exosomes.[33] Exosomes are extracellular vesicles 30 to 100 nm in size and they carry mRNAs, proteins, lipid mediators, and miRNAs to recipient cells via systemic blood circulation.[13] Uptake of exosomes can result in modulation of gene expression in recipient cells and represents a novel mechanism of cellular communication.[54] Our previous findings suggest a role for exosomes in dysregulated inflammation and chronic pain states,[33] and further studies are needed to investigate the functional consequences of exosome-mediated delivery of miRNAs to recipient cells. Determining treatment-induced changes in exosomal miRNA composition will be beneficial both as a biomarker and as a novel therapeutic strategy.[13]

Conclusions

Although we used a limited number of samples, our data demonstrate the feasibility of using miRNA signatures in circulation as biomarkers to predict treatment response and the usefulness of miRNAs identified in understanding the molecular mechanisms underlying CRPS. Our studies showed that miR-548d-5p can regulate UDP-GT but not CYP3A4, suggesting that UDP-GT activity in responders and poor responders may be mediated by differences in the level of circulating miR-548d-5p. Lower levels of miR-548d-5p in poor responders before treatment could result in higher UDP-GT activity, leading to the production of more inactive glucuronide conjugates and faster elimination of active ketamine metabolites downstream of NK. Thus, the levels of hsa-miR-548d-5p could minimize the therapeutic efficacy of ketamine and pain relief. Differences in miRNA signature can thus provide molecular insights distinguishing responders from poor responders. High failure rates of drugs targeted to treat neuropathic pain warrant changes in approaches.[27,60] Studies targeting well-defined patient populations for clinical trials will play a crucial in developing drugs that may be efficacious in a subset of patients. Extending this approach to other treatment and outcome assessments might permit stratification of patients for maximal therapeutic outcome.

Acknowledgments

We thank Dr. Michael Bouchard for providing HepG2 cells.

Supplementary Data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jpain.2015.05.008.

References

1. Abdallah CG, Sanacora G, Duman RS, Krystal JH: Ketamine and rapid-acting antidepressants: a window into a new neurobiology for mood disorder therapeutics. Annu Rev Med 66:509-523, 2015

2. Ammari M, Jorgensen C, Apparailly F: Impact of microRNAs on the understanding and treatment of rheumatoid arthritis. Curr Opin Rheumatol 25: 225-233, 2013

3. Andersen H, Duroux M, Gazerani P: Serum microRNA signatures in migraineurs during attacks and in pain-free periods. Mol Neurobiol, 2015; http://dx.doi.org/10.1007/s12035-015-9106-5 [ePub ahead of print]

4. Bartel DP: MicroRNAs: target recognition and regulatory functions. Cell 136:215-233, 2009

5. Bjersing JL, Lundborg C, Bokarewa MI, Mannerkorpi K: Profile of cerebrospinal microRNAs in fibromyalgia. PLoS One 8:e78762, 2013

6. Borchers AT, Gershwin ME: Complex regional pain syndrome: a comprehensive and critical review. Autoimmun Rev 13:242-265, 2014

7. Borsook D: Ketamine and chronic pain–going the distance. Pain 145:271, 2009

8. Bruehl S: An update on the pathophysiology of complex regional pain syndrome. Anesthesiology 113:713-725, 2010

9. Correll GE, Maleki J, Gracely EJ, Muir JJ, Harbut RE: Subanesthetic ketamine infusion therapy: a retrospective analysis of a novel therapeutic approach to complex regional pain syndrome. Pain Med 5:263-275, 2004

10. Dahan A, Olofsen E, Sigtermans M, Noppers I, Niesters M, Aarts L, Bauer M, Sarton E: Population pharmacokinetic-pharmacodynamic modeling of ketamine-induced pain relief of chronic pain. Eur J Pain 15:258-267, 2011

11. De Guire V, Robitaille R, Tetreault N, Guerin R, Menard C, Bambace N, Sapieha P: Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: promises and challenges. Clin Biochem 46: 846-860, 2013

12. de Mos M, Sturkenboom MC, Huygen FJ: Current understandings on complex regional pain syndrome. Pain Pract 9:86-99, 2009

13. El Andaloussi S, Mager I, Breakefield XO, Wood MJ: Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 12:347-357, 2013

14. Fourie NH, Peace RM, Abey SK, Sherwin LB, Rahim-Williams B, Smyser PA, Wiley JW, Henderson WA: Elevated circulating miR-150 and miR-342-3p in patients with irritable bowel syndrome. Exp Mol Pathol 96:422-425, 2014

15. Friedman RC, Farh KK, Burge CB, Bartel DP: Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19:92-105, 2009

16. Gheinani A, Burkhard F, Monastyrskaya K: Deciphering microRNA code in pain and inflammation: lessons from bladder pain syndrome. Cell Mol Life Sci 70: 3773-3789, 2013

17. Gierthmuhlen J, Binder A, Baron R: Mechanism-based treatment in complex regional pain syndromes. Nat Rev Neurol 10:518-528, 2014

18. Goldberg ME, Domsky R, Scaringe D, Hirsh R, Dotson J, Sharaf I, Torjman MC, Schwartzman RJ: Multi-day low dose ketamine infusion for the treatment of complex regional pain syndrome. Pain Physician 8:175-179, 2005

19. Goldberg ME, Torjman MC, Schwartzman RJ, Mager DE, Wainer IW: Pharmacodynamic profiles of ketamine (R)- and (S)- with 5-day inpatient infusion for the treatment of complex regional pain syndrome. Pain Physician 13:379-387, 2010

20. Goldberg ME, Torjman MC, Schwartzman RJ, Mager DE, Wainer IW: Enantioselective pharmacokinetics of (R)- and (S)-ketamine after a 5-day infusion in patients with complex regional pain syndrome. Chirality 23:138-143, 2011

21. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ: miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34:D140-D144, 2006

22. Harden RN, Bruehl S, Perez RS, Birklein F, Marinus J, Maihofner C, Lubenow T, Buvanendran A, Mackey S, Graciosa J, Mogilevski M, Ramsden C, Chont M, Vatine JJ: Validation of proposed diagnostic criteria (the "Budapest Criteria") for complex regional pain syndrome. Pain 150: 268-274, 2010

23. Hijazi Y, Bodonian C, Bolon M, Salord F, Boulieu R: Pharmacokinetics and haemodynamics of ketamine in intensive care patients with brain or spinal cord injury. Br J Anaesth 90:155-160, 2003

24. Hijazi Y, Boulieu R: Contribution of CYP3A4, CYP2B6, and CYP2C9 isoforms to N-demethylation of ketamine in human liver microsomes. Drug Metab Dispos 30:853-858, 2002

25. Kiefer RT, Rohr P, Ploppa A, Dieterich HJ, Grothusen J, Koffler S, Altemeyer KH, Unertl K, Schwartzman RJ: Efficacy of ketamine in anesthetic dosage for the treatment of refractory complex regional pain syndrome: an open-label phase II study. Pain Med 9:1173-1201, 2008

26. Koffler SP, Hampstead BM, Irani F, Tinker J, Kiefer RT, Rohr P, Schwartzman RJ: The neurocognitive effects of 5 day anesthetic ketamine for the treatment of refractory complex regional pain syndrome. Arch Clin Neuropsychol 22:719-729, 2007

27. Kola I, Landis J: Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3:711-715, 2004

28. Leung LY, Baillie TA: Comparative pharmacology in the rat of ketamine and its two principal metabolites, norketamine and (Z)-6-hydroxynorketamine. J Med Chem 29: 2396-2399, 1986

29. Liu B, Li J, Cairns MJ: Identifying miRNAs, targets and functions. Brief Bioinform 15:1-19, 2014

30. Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, Miners JO, Owens IS, Nebert DW: Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. Pharmacogenet Genomics 15:677-685, 2005

31. Maihofner C, Seifert F, Markovic K: Complex regional pain syndromes: new pathophysiological concepts and therapies. Eur J Neurol 17:649-660, 2010

32. McDonald MK, Ajit SK: MicroRNA biology and pain. In: Theodore JP, Gregory D (eds): Progress in Molecular Biology and Translational Science. London, Academic Press, 2015, pp 215-249

33. McDonald MK, Tian Y, Qureshi RA, Gormley M, Ertel A, Gao R, Aradillas Lopez E, Alexander GM, Sacan A, Fortina P, Ajit SK: Functional significance of macrophage-derived exosomes in inflammation and pain. Pain 155: 1527-1539, 2014

34. Mendell JT, Olson EN: MicroRNAs in stress signaling and human disease. Cell 148:1172-1187, 2012

35. Moaddel R, Luckenbaugh D, Xie Y, Villaseñor A, Brutsche N, Machado-Vieira R, Ramamoorthy A, Lorenzo M, Garcia A, Bernier M, Torjman M, Barbas C, Zarate C Jr, Wainer I: D-serine plasma concentration is a potential biomarker of (R,S)-ketamine antidepressant response in subjects with treatment-resistant depression. Psychopharmacology 232:399-409, 2015

36. Moaddel R, Venkata SL, Tanga MJ, Bupp JE, Green CE, Iyer L, Furimsky A, Goldberg ME, Torjman MC, Wainer IW: A parallel chiral-achiral liquid chromatographic method for the determination of the stereoisomers of ketamine and ketamine metabolites in the plasma and urine of patients with complex regional pain syndrome. Talanta 82: 1892-1904, 2010

37. O'Connell NE, Wand BM, McAuley J, Marston L, Moseley GL: Interventions for treating pain and disability in adults with complex regional pain syndrome. Cochrane Database Syst Rev;CD009416, 2013

38. Ohlsson Teague EM, Print CG, Hull ML: The role of microRNAs in endometriosis and associated reproductive conditions. Hum Reprod Update 16:142-165, 2010

39. Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graziano A, Barrett JE, Schwartzman RJ, Ajit SK: MicroRNA modulation in complex regional pain syndrome. J Transl Med 9:195, 2011

40. Ortiz R, Niciu MJ, Lukkahati N, Saligan LN, Nugent AC, Luckenbaugh DA, Machado-Vieira R, Zarate CA Jr: Shank3 as a potential biomarker of antidepressant response to ketamine and its neural correlates in bipolar depression. J Affect Disord 172:307-311, 2015

41. Paul RK, Singh NS, Khadeer M, Moaddel R, Sanghvi M, Green CE, O'Loughlin K, Torjman MC, Bernier M, Wainer IW: (R,S)-Ketamine metabolites (R,S)-norketamine and (2S,6S)-hydroxynorketamine increase the mammalian target of rapamycin function. Anesthesiology 121:149-159, 2014

42. Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB: Common features of microRNA target prediction tools. Front Genet 5:23, 2014

43. Sabia M, Hirsh R, Torjman M, Wainer I, Cooper N, Domsky R, Goldberg M: Advances in translational neuropathic research: example of enantioselective pharmacokinetic–pharmacodynamic modeling of ketamine-induced pain relief in complex regional pain syndrome. Curr Pain Headache Rep 15:207-214, 2011

44. Sawynok J: Topical and peripheral ketamine as an analgesic. Anesth Analg 119:170-178, 2014

45. Schmittgen TD, Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108, 2008

46. Schopman NCT, Heynen S, Haasnoot J, Berkhout B: A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. RNA Biol 7:573-576, 2010

47. Schwartzman RJ, Alexander GM, Grothusen JR: The use of ketamine in complex regional pain syndrome: possible mechanisms. Expert Rev Neurother 11:719-734, 2011

48. Schwartzman RJ, Alexander GM, Grothusen JR, Paylor T, Reichenberger E, Perreault M: Outpatient intravenous ketamine for the treatment of complex regional pain syndrome: a double-blind placebo controlled study. Pain 147:107-115, 2009

49. Schwartzman RJ, Erwin KL, Alexander GM: The natural history of complex regional pain syndrome. Clin J Pain 25: 273-280, 2009

50. Shen N, Liang D, Tang Y, de Vries N, Tak PP: MicroRNAs–novel regulators of systemic lupus erythematosus pathogenesis. Nat Rev Rheumatol 8:701-709, 2012

51. Sigtermans MJ, van Hilten JJ, Bauer MC, Arbous MS, Marinus J, Sarton EY, Dahan A: Ketamine produces effective and long-term pain relief in patients with complex regional pain syndrome type 1. Pain 145:304-311, 2009

52. Singh NS, Zarate CA Jr, Moaddel R, Bernier M, Wainer IW: What is hydroxynorketamine and what can it bring to neurotherapeutics? Expert Rev Neurother 14: 1239-1242, 2014

53. Sivertsson L, Ek M, Darnell M, Edebert I, Ingelman-Sundberg M, Neve EPA: CYP3A4 catalytic activity is induced in confluent Huh7 hepatoma cells. Drug Metab Dispos 38: 995-1002, 2010

54. Stoorvogel W: Functional transfer of microRNA by exosomes. Blood 119:646-648, 2012

55. Strimbu K, Tavel JA: What are biomarkers? Curr Opin HIV AIDS 5:463-466, 2010

56. Turfus SC, Parkin MC, Cowan DA, Halket JM, Smith NW, Braithwaite RA, Elliot SP, Steventon GB, Kicman AT: Use of human microsomes and deuterated substrates: an alternative approach for the identification of novel metabolites of ketamine by mass spectrometry. Drug Metab Dispos 37:1769-1778, 2009

57. Uchaipichat V, Raungrut P, Chau N, Janchawee B, Evans AM, Miners JO: Effects of ketamine on human UDP-glucuronosyltransferases in vitro predict potential drug-drug interactions arising from ketamine inhibition of codeine and morphine glucuronidation. Drug Metab Dispos 39:1324-1328, 2011

58. Villaseñor A, Ramamoorthy A, Silva dos Santos M, Lorenzo MP, Laje G, Zarate C, Barbas C, Wainer IW: A pilot study of plasma metabolomic patterns from patients treated with ketamine for bipolar depression: evidence for a response-related difference in mitochondrial networks. Br J Pharmacol 171:2230-2242, 2014

59. Witwer KW: Circulating MicroRNA biomarker studies: pitfalls and potential solutions. Clin Chem 61:56-63, 2015

60. Woodcock J, Witter J, Dionne RA: Stimulating the development of mechanism-based, individualized pain therapies. Nat Rev Drug Discov 6:703-710, 2007

61. Yang J-j, Wang N, Yang C, Shi J-y, Yu H-y, Hashimoto K: Serum interleukin-6 is a predictive biomarker for ketamine's antidepressant effect in treatment-resistant patients with major depression. Biol Psychiatry 77:e19-e20, 2015

62. Zarate CA Jr, Brutsche N, Laje G, Luckenbaugh DA, Venkata SL, Ramamoorthy A, Moaddel R, Wainer IW: Relationship of ketamine's plasma metabolites with response, diagnosis, and side effects in major depression. Biol Psychiatry 72:331-338, 2012

63. Zhao X, Venkata SL, Moaddel R, Luckenbaugh DA, Brutsche NE, Ibrahim L, Zarate CA Jr, Mager DE, Wainer IW: Simultaneous population pharmacokinetic modelling of ketamine and three major metabolites in patients with treatment-resistant bipolar depression. Br J Clin Pharmacol 74:304-314, 2012