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Plasma microRNA expression levels and their targeted pathways in patients with major depressive disorder who are responsive to duloxetine treatment

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ABSTRACT

Major depressive disorder (MDD) is a complex disorder with many pathways known to contribute to its pathogenesis, such as apoptotic signaling, with antidepressants having been shown to target these pathways. In this study, we explored microRNAs as predictive markers of drug response to duloxetine, a serotonin-norepinephrine reuptake inhibiter, using peripheral blood samples from 3 independent clinical trials (NCT00635219; NCT0059991; NCT01140906) comparing 6–8 weeks of treatment with duloxetine to placebo treatment in patients with MDD. Plasma microRNA was extracted and sequenced using the Ion Proton Sequencer. Rank feature selection analysis was used to identify microRNAs in the top 10th percentile for their differentiating ability between patients who remitted and did not remit with duloxetine treatment. The results were then compared between the 3 trials to see their replicability. To further validate our findings, we reasoned that the pathways targeted by these microRNAs would be those shown to be altered in MDD in pathway enrichment analysis. Hsa-miR-23a-3p, hsa-miR-16-5p, hsa-miR-146a-5p and hsa-miR-21-5p were identified in 2 or more trials as being able to differentiate patients who would remit with duloxetine treatment using samples collected before treatment initiation, suggesting that they may be good candidates for identification of predictive biomarkers of duloxetine response. Pathway enrichment analysis further showed that microRNAs identified as differentiating for duloxetine response target the apoptosis signaling pathway. Future studies examining these microRNAs outside of a clinical trial setting and exploring their role in MDD may further our understanding of MDD and antidepressant response.

1. Introduction

Major depressive disorder (MDD) is a debilitating condition with a complex etiology. At the cellular level, studies show alterations in neurotrophic factors, inflammation, metabolism, and apoptosis (Ernst et al., 2017; Kim et al., 2016; Sibille and French, 2013). Antidepressants, in addition to targeting serotonergic, noradrenergic, and/or dopaminergic pathways, are now well-known to have neuroprotective effects, which are potentially mediated by modulating apoptotic signaling (Miguel-Hidalgo et al., 2014a), decreasing pro-inflammatory cytokines (Wiedlocha et al., 2018) and increasing neurotrophic signaling (Rantamaki and Yalcin, 2016). MDD can be a challenging condition to treat, at times requiring multiple trials with different antidepressants, perhaps owing to its complex etiology. Therefore, identification of biomarkers that allow for prediction of treatment response to specific classes of antidepressants have recently garnered much attention. Ideally, these would be stable peripheral markers amenable to accurate measurement after sample collection, storage and processing within a tertiary care center setting.

MicroRNAs, which are post-transcriptional regulators of gene expression, have been explored as potential biomarkers of disease due to their stability (Koberle et al., 2013). Interestingly, there is accumulating evidence that patients with MDD have different expression levels of certain microRNAs compared to unaffected controls in both central and

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peripheral samples (Camkurt et al., 2015; Maffioletti et al., 2016; Wan et al., 2015), and some studies suggest that commonly used antidepressants, such as escitalopram, can affect changes in microRNA levels in patients with MDD (Bocchio-Chiavetto et al., 2013). Duloxetine, as well, may alter specific microRNAs that target genes in the MAPK/Wnt pathway, although these microRNAs are also altered in patients treated with escitalopram and placebo (Lopez et al., 2017). These studies suggest that microRNAs may be potential candidates for biomarkers of antidepressant response.

We explored plasma microRNA expression levels in samples from three independent clinical trials comparing duloxetine, a serotonin-norepinephrine reuptake inhibitor (SNRI), to placebo control in patients with MDD. Our aim was to identify levels of cell free microRNAs that could be explored as candidates for the prediction of remittance in patients with MDD treated with duloxetine. We also examined the replicability of these findings by comparing the results between the three clinical trials, and identified common pathways targeted by these microRNAs.

2. Materials and methods

2.1. Sample characteristics

Peripheral blood samples were received from 3 independent randomized, double-blind, placebo-controlled clinical trials, addressed A, B and C from here on (NCT00635219 (Lundbeck, 2008b); NCT00599911 (Lundbeck, 2008a); NCT01140906 (Lundbeck, 2008c), respectively), examining the effect of 6–8 weeks of 60 mg duloxetine treatment compared to placebo in patients with moderate to severe MDD in collaboration with the Canadian Biomarker Network for Depression (CAN-BIND). Study A (NCT00635219) was conducted between February 2008 to April 2009, with sample collection at baseline and at the end of treatment being 8 weeks apart. Study B (NCT00599911) was conducted between October 2007 to March 2009, with 6 weeks between sample collection at baseline and at the end of the treatment. Study C (NCT01140906) was conducted between May 2010 to September 2011, with 8 weeks between sample collection at baseline and at the end of treatment. Detailed information regarding the clinical trials can be found in www.ClinicalTrials.gov using the NCT numbers provided above. Blood collection was performed at the beginning of the trial (baseline) and at the end of treatment (time 2). Therefore, our samples were divided into 4 groups within each of the 3 trials: placebo baseline, placebo time 2, duloxetine baseline, and duloxetine time 2. Description of participants after outlier removal can be found in Table 1. Patients who remitted with duloxetine or placebo treatment at the end of the study period, as defined by Montgomery-Asberg Depression Scale (MADRS) score ≤ 10, were considered treatment responsive. Each clinical trial was analyzed separately.

2.2. MicroRNA extraction and sequencing

Plasma was collected and stored according to a standardized protocol for all 3 clinical trials. Briefly, blood was collected in EDTA tubes and centrifuged at 2000g for 10–15 min at 4 °C. Separated plasma samples were then stored at −20 °C for a maximum of 5 days, then stored at below −70 °C. Samples were stored frozen for 7–8 years in Study A, 9-7 years in Study B, and 5–6 years in Study C at −70 °C before performing microRNA extraction and sequencing. Plasma total RNA was isolated using the mirVana miRNA isolation kit (AM1560, ThermoFisher Scientific). Ion Total RNA-Seq V2 kit (4475936, ThermoFisher Scientific) was used to prepare the cDNA library, and its quantity and quality were measured using the Agilent High Sensitivity DNA Kit (5067-4626, Agilent) with the Agilent 2100 Bioanalyzer. MicroRNA sequencing was performed using the Ion Proton Sequencer. All assays were performed using manufacturers' instructions. Expression levels were quantified using MirDeep2 (Friedlander et al., 2012). Preprocessing was performed, where expression levels were normalized using a sample-specific normalization factor generated by dividing the geometric mean of all microRNAs across all samples by the sample-specific geometric mean of the control microRNAs. Outliers were removed using the IQR (interquartile range) outlier labeling method (Hoaglin and Iglewicz, 1987) on Matlab, where samples were identified and removed from analysis if they had a geometric mean of 0 or if normalized expression levels were outside of the interquartile range. Using this method, 45 samples from study B, 13 samples from study A, and 23 samples from study C were removed from analysis.

2.3. Rank feature selection analysis

Rank feature selection analysis was performed to examine between-group differences using a previously published method (Ren et al., 2017). Because our aim was to identify microRNAs predictive of response to drug treatment, we made the following comparisons: a) samples collected at baseline from individuals treated with duloxetine who remitted vs. samples collected at baseline from individuals treated with duloxetine who did not remit, and b) samples collected at baseline from individuals treated with placebo who remitted vs. samples collected at baseline from individuals treated with placebo who did not remit. For examining treatment effect, we also examined: a) samples collected at time 2 from individuals treated with duloxetine who remitted vs. time 2 samples from individuals treated with duloxetine who did not remit, and b) samples collected at time 2 from individuals treated with placebo who remitted vs. time 2 samples from individuals treated with placebo who did not remit. Rank feature selection with 10-fold validation was performed using the Statistics and Machine Learning Toolbox™ in Matlab 2016b. MicroRNAs were ranked for their ability to differentiate between the 2 groups within a comparison by performing feature selection with 10-fold validation, where each run randomly chose 90% of the subjects to be included in the analysis. The resulting 10 rankings were then averaged to produce the final ranked list of microRNAs. MicroRNAs ranked in the top 10th percentile were considered to have differentiating ability between the two groups.

2.4. Pathway enrichment analysis

If these microRNAs are indeed specific for duloxetine effect in MDD, we reasoned that there should be common pathways targeted by the identified microRNAs, and that these pathways should include those found to be altered in MDD. As some pathways are targeted by hundreds of microRNAs, to ensure the validity of our findings, we only

Table 1
Participant and clinical trial characteristics (Dul = duloxetine, P = placebo).

	N (placebo: duloxetine)	Gender	(M:F)	Age (placebo: duloxetine)	Study length
A (NCT00635219)	238:212	Dul	60:152	43.0:45.7	8 weeks
		P	73:165		
B (NCT00599911)	180:181	Dul	66:115	45.3:47.7	6 weeks
		P	66:113		
C (NCT01140906)	245:230	Dul	68:162	48.1:45.7	8 weeks
		P	75:170		

considered pathways that were enriched in all three trials to be differentiating. For this, we used Mirpath v.3 GO analysis (Ashburner et al., 2000; The Gene Ontology, 2017; Vlachos et al., 2015) to identify pathways that were significantly targeted by the top 10th percentile of ranked microRNAs within each comparison. False detection rate correction was applied, and pathways with $q < 0.05$ were considered to be significantly enriched. Only pathways enriched in all 3 trials for the same comparison were identified to be significantly targeted. To examine pathways specific for duloxetine effect, we took pathways enriched for duloxetine comparisons and subtracted those pathways also enriched for placebo comparisons for the same timeframe (baseline or time 2).

3. Results

3.1. MicroRNAs in the top 10th percentile in rank feature selection analysis for remission in baseline samples

To identify microRNAs that had potential for predicting response to duloxetine treatment, we examined between-group differences in microRNA expression levels from samples collected at baseline in patients who remitted with duloxetine treatment and patients who did not. MicroRNAs ranked in the top 10th percentile for comparisons in samples collected at baseline for both the placebo and duloxetine comparisons are listed in Table 2. Hsa-miR-16-5p, hsa-miR-146a-5p, hsa-miR-21-5p, and hsa-miR-30b-5p were identified in 2 of the 3 trials for the comparison between patients who remitted with duloxetine treatment and those who did not when examining baseline samples. Hsa-miR-23a-3p was identified in all 3 trials. Hsa-miR-30b-5p and hsa-miR-23a-3p were also ranked in 1 trial in the baseline placebo comparison. The other 3 microRNAs did not rank in the top 10th percentile for any of the 3 trials in the comparison between placebo treated patients who remitted and those who did not remit in baseline samples. In patients treated with placebo, hsa-miR-151a-5p, hsa-miR-185-5p and hsa-miR-183-5p were identified in 2 of the 3 trials in the comparison between patients who remitted and those who did not.

3.2. MicroRNAs in the top 10th percentile in rank feature selection analysis for remission in samples collected at time 2

While it was not the main aim of this study, we examined microRNA expression level differences between patients treated with duloxetine who remitted and who did not remit in samples collected at the end of the trials (time 2) as well. MicroRNAs ranked in the top 10th percentile for time 2 comparisons for both placebo and duloxetine treatment are listed in Table 3. Hsa-miR-21-5p, hsa-miR-144-3p, hsa-let-7c-5p, and hsa-miR-30c-5p were identified in 2 of the 3 trials as differentiating for remission with duloxetine treatment. Hsa-miR-30c-5p was also ranked in one of the 3 studies in the time 2 placebo comparison. In the placebo treatment group, hsa-miR-9-50, hsa-miR-29c-3p, hsa-miR-30d-5p, and hsa-miR-183-5p differentiated between patients who remitted and those who did not in 2 of the 3 trials. Hsa-miR-29c-3p was identified in all 3 trials for this comparison.

3.3. Pathway enrichment analysis for microRNAs ranked in the top 10th percentile for remission

Pathway enrichment analysis was performed with microRNAs ranked in the top 10th percentile for the differentiation of patients who remitted with duloxetine treatment and who did not in samples collected at baseline. Each trial was analyzed separately to generate a list of pathways targeted by the top 10th percentile of the microRNAs identified in rank feature selection analysis for baseline samples. Then, the pathways were compared between the 3 trials and only pathways enriched in all 3 trials for the same comparison (e.g. duloxetine remitters vs. non-remitters in baseline) were considered as significant. In

Table 2
MicroRNAs that are in the top 10th percentile in rank feature selection analysis from samples collected at baseline (microRNAs that are identified in 2 trials within the same comparison are bolded and those identified in 3 trials are in italics).

Duloxetine remission vs. no remission at baseline		
Study1	***Study2***	***Study 3***
hsa-miR-16-5p	*hsa-miR-23a-3p*	hsa-miR-22-5p
hsa-miR-23b-3p	**hsa-miR-21-5p**	hsa-let-7a-5p
hsa-miR-4433b-3p	**hsa-miR-30b-5p**	hsa-let-7g-5p
hsa-miR-144-5p	hsa-miR-24-3p	**hsa-miR-21-5p**
hsa-miR-92a-3p	hsa-let-7c-5p	**hsa-miR-146a-5p**
hsa-miR-486-5p	hsa-miR-26a-5p	hsa-let-7i-5p
hsa-miR-28-3p	hsa-miR-151a-3p	hsa-miR-4429
hsa-miR-361-5p	hsa-miR-210-3p	**hsa-miR-30b-5p**
hsa-miR-142-5p	hsa-let-7d-3p	hsa-miR-17-5p
hsa-miR-146a-5p	hsa-miR-335-5p	hsa-miR-320b
hsa-miR-451a	hsa-miR-139-5p	**hsa-miR-16-5p**
hsa-miR-23a-3p	hsa-miR-301a-3p	*hsa-miR-23a-3p*
hsa-miR-151a-5p	hsa-miR-144-3p	hsa-miR-193a-5p
hsa-miR-15b-3p	hsa-miR-625-5p	hsa-miR-98-5p
hsa-miR-652-3p	hsa-miR-505-3p	hsa-miR-378a-3p
Placebo remission vs. no remission at baseline		
Study1	***Study2***	***Study3***
hsa-miR-590-5p	hsa-miR-30b-5p	hsa-miR-4433b-3p
hsa-miR-140-3p	hsa-miR-3615	hsa-miR-139-5p
hsa-miR-151a-5p	hsa-miR-15b-5p	hsa-miR-30c-5p
hsa-miR-425-5p	hsa-miR-100-5p	hsa-miR-326
hsa-miR-221-3p	hsa-miR-489-3p	hsa-let-7f-5p
hsa-miR-107	hsa-miR-9-5p	hsa-miR-1260a
hsa-miR-23b-3p	hsa-miR-744-5p	hsa-miR-195-5p
hsa-miR-103a-3p	hsa-miR-23a-3p	hsa-miR-1260b
hsa-miR-185-5p	hsa-miR-181a-5p	hsa-miR-17-3p
hsa-miR-150-5p	**hsa-miR-183-5p**	hsa-miR-1307-3p
hsa-miR-320a	hsa-miR-192-5p	hsa-miR-766-3p
hsa-miR-130a-3p	**hsa-miR-151a-5p**	hsa-miR-197-3p
hsa-miR-101-3p	hsa-let-7e-5p	**hsa-miR-183-5p**
hsa-miR-20a-5p	hsa-miR-550a-3p	hsa-miR-148b-3p
hsa-miR-320b	hsa-miR-18a-5p	**hsa-miR-185-5p**

Table 3
MicroRNAs that are in the top 10^{th} percentile in rank feature selection analysis from samples collected at time 2 (microRNAs that are identified in 2 trials within the same comparison are bolded and those identified in 3 trials are in italics).

Duloxetine remission vs. no remission at time 2		
Study1	***Study2***	***Study3***
hsa-miR-21-5p	hsa-miR-142-3p	hsa-miR-424-5p
hsa-miR-144-3p	hsa-miR-15b-5p	hsa-miR-19a-3p
hsa-miR-194-5p	hsa-miR-27b-3p	hsa-miR-421
hsa-miR-221-3p	**hsa-let-7c-5p**	hsa-miR-409-3p
hsa-miR-320b	hsa-miR-139-5p	hsa-miR-485-3p
hsa-miR-106b-3p	hsa-miR-99b-5p	**hsa-miR-30c-5p**
hsa-let-7c-5p	hsa-miR-106a-5p	hsa-miR-4433b-3p
hsa-miR-574-3p	**hsa-miR-144-3p**	hsa-miR-4286
hsa-miR-30c-5p	hsa-miR-181b-5p	hsa-miR-4429
hsa-miR-425-5p	hsa-miR-451a	hsa-miR-148b-3p
hsa-miR-23b-3p	hsa-miR-23a-3p	hsa-miR-19b-3p
hsa-miR-150-5p	**hsa-miR-21-5p**	hsa-miR-22-5p
hsa-miR-378a-3p	hsa-miR-1260a	hsa-miR-151a-3p
hsa-miR-2110	hsa-miR-27a-3p	hsa-miR-101-3p
hsa-miR-30d-5p	hsa-let-7d-5p	hsa-miR-28-3p
Placebo remission vs. no remission at time 2		
Study1	***Study2***	***Study3***
hsa-miR-151a-3p	hsa-miR-146b-5p	hsa-miR-3591-3p
hsa-miR-7-5p	hsa-miR-19a-3p	hsa-miR-590-5p
hsa-miR-125b-5p	hsa-miR-10a-5p	hsa-miR-223-3p
hsa-miR-409-3p	*hsa-miR-29c-3p*	hsa-miR-195-5p
hsa-miR-103a-3p	hsa-miR-101-3p	hsa-miR-28-3p
hsa-miR-9-5p	**hsa-miR-9-5p**	hsa-miR-454-3p
hsa-miR-29c-3p	hsa-miR-181b-5p	**hsa-miR-30d-5p**
hsa-miR-376a-3p	**hsa-miR-30d-5p**	hsa-miR-125a-5p
hsa-miR-335-5p	**hsa-miR-183-5p**	hsa-miR-139-5p
hsa-miR-27b-3p	hsa-miR-30c-5p	hsa-miR-374b-5p
hsa-miR-340-5p	hsa-miR-489-3p	**hsa-miR-183-5p**
hsa-miR-134-5p	hsa-miR-19b-3p	hsa-miR-148b-3p
hsa-miR-148a-3p	hsa-miR-376c-3p	hsa-miR-192-5p
hsa-miR-16-2-3p	hsa-miR-155-5p	hsa-let-7i-5p
hsa-miR-99a-5p	hsa-miR-766-3p	*hsa-miR-29c-3p*

addition, only pathways that were not also enriched for the microRNAs identified in time 2 duloxetine comparison and the baseline placebo comparison were considered specific for the prediction of duloxetine treatment response. Pathway enrichment analysis results can be found in Table 4. Ten pathways were identified as being targeted by microRNAs differentiating those who will remit with duloxetine treatment and those who will not, including the apoptotic signaling pathway (GO:0097190) and the glucose metabolic process pathway (GO:0006006).

Pathway enrichment analysis was also performed for microRNAs ranked in the top 10th percentile for the duloxetine comparison in samples collected at time 2. After removing pathways also enriched in the corresponding placebo comparison and baseline duloxetine comparison, 17 pathways were found to be significantly enriched, including the cytokine-mediated signaling pathway (GO:0019221).

To examine pathways that may differ in individuals who remit without pharmacological treatment, pathways targeted by microRNAs ranked in the top 10th percentile for the differentiation between patients who did and did not remit with placebo treatment at baseline were examined. From this set, pathways that were also enriched for the time 2 placebo comparison and baseline duloxetine comparison were not considered specific and removed. This resulted in 33 pathways (Table 4), including negative regulation of apoptotic process pathway (GO:0043066) and the positive regulation of apoptotic process pathway (GO:0043065).

This analysis was repeated for microRNAs ranked in the top 10th percentile for the differentiation between remitters and non-remitters who received placebo treatment in the analysis of time 2 samples. Fifteen pathways were identified after excluding pathways also identified in the baseline placebo comparison and duloxetine comparison at time 2 (Table 4), which included the apoptotic signaling pathway (GO:0097190).

Exploratory analyses were performed to examine pathways identified in both baseline and time 2 data. Both the intracellular transport of virus pathway and response to virus pathway were implicated in the analysis of both baseline data and post-treatment (time 2) data differentiating remitters and non-remitters with duloxetine treatment. In comparing baseline data and post-treatment data differentiating remitters and non-remitters with placebo treatment, the anatomical structure morphogenesis pathway, cellular metabolic process pathway, COPII vesicle coating pathway, and inositol phosphate metabolic process pathway were implicated for both time points.

Further analyses were done to explore whether common pathways were identified in the analysis of both duloxetine and placebo treatment groups, as these might reveal processes involved in a general response to treatment. Only the chondroitin sulfate metabolic process pathway was implicated in both treatment groups when examining data from baseline samples. When examining pathways specific to analysis of time 2 data implicated in both duloxetine and placebo comparisons, the apoptotic process pathway, cytoplasmic stress granule pathway, nitric oxide metabolic process pathway, regulation of nitric-oxide synthase activity pathway, and virion assembly pathway were found to be significant for both duloxetine and placebo groups.

4. Discussion

MDD is a complex disorder with multiple pathways potentially influencing its development and progression, including inflammation, apoptosis, and dysregulation of neurotrophin pathways (Czéh and Lucassen, 2007; Miguel-Hidalgo et al., 2014a; Shelton et al., 2011). Not surprisingly, antidepressants were shown to have neuroprotective effects likely through these pathways as well in addition to their ability to alter neurotransmitter signaling (Shimizu et al., 2003; Wiedlocha et al., 2018), although it remains to be known whether different classes of antidepressants differ in their ability to alter these pathways. If so, it may be beneficial to have biomarkers to aid in the prediction of

Table 4
List of pathways identified for duloxetine comparisons after the removal of pathways identified for placebo comparisons within the same time frame.

Pathways targeted by microRNAs specific to duloxetine remission for samples collected at baseline	Pathways targeted by microRNAs specific to duloxetine remission for samples collected at time 2	Pathways targeted by microRNAs specific to placebo remission for samples collected at baseline	Pathways targeted by microRNAs specific to placebo remission for samples collected at time 2
1 activation of phospholipase C activity	1 antigen processing and presentation of exogenous peptide antigen via MHC class I	1 antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	1 activation of MAPKK activity
2 androgen receptor binding	2 cell-cell junction organization	2 antigen processing and presentation of exogenous peptide antigen via MHC class II	2 adherens junction organization
3 apoptotic signaling pathway	3 cytokine-mediated signaling pathway	3 carbohydrate metabolic process	3 apoptotic signaling pathway
4 cell proliferation	4 'de novo' posttranslational protein folding	4 cell cycle	4 CENP-A containing nucleosome assembly
5 glucose metabolic process	5 exonucleolytic nuclear-transcribed mRNA catabolic process involved in deadenylation-dependent decay	5 cellular response to hypoxia	5 extracellular matrix disassembly
6 homeostatic process	6 histone binding	6 'de novo' posttranslational protein folding	6 homeostatic process
7 JAK-STAT cascade involved in growth hormone signaling pathway	7 mRNA export from nucleus	7 endosome	7 JAK-STAT cascade involved in growth hormone signaling pathway
8 negative regulation of cell proliferation	8 negative regulation of transcription from RNA polymerase II promoter	8 glucose transport	8 protein maturation
9 nucleobase-containing small molecule interconversion	9 negative regulation of transforming growth factor beta receptor signaling pathway	9 Golgi membrane	9 regulation of defense response to virus by virus
10 protein binding, bridging	10 O-glycan processing	10 histone binding	10 sulfur compound metabolic process
	11 positive regulation of muscle cell differentiation	11 mRNA processing	11 transferrin transport
	12 proteasome-mediated ubiquitin-dependent protein catabolic process	12 mRNA splicing, via spliceosome	12 tRNA metabolic process
	13 protein ubiquitination	13 negative regulation of apoptotic process	13 virus receptor activity
	14 regulation of small GTPase mediated signal transduction	14 negative regulation of epidermal growth factor receptor signaling pathway	14 vitamin metabolic process
	15 translational initiation	15 negative regulation of transcription from RNA polymerase II promoter	15 water-soluble vitamin metabolic process
	16 ubiquitin protein ligase binding	16 Notch signaling pathway	
	17 vacuolar transport	17 nuclear-transcribed mRNA poly(A) tail shortening	
		18 nucleocytoplasmic transport	
		19 nucleotide-binding oligomerization domain containing signaling pathway	
		20 poly(A) RNA binding	
		21 positive regulation of apoptotic process	
		22 positive regulation of viral transcription	
		23 protein kinase binding	
		24 protein ubiquitination	
		25 protein ubiquitination involved in ubiquitin-dependent protein catabolic process	
		26 Ras protein signal transduction	
		27 regulation of cellular amino acid metabolic process	
		28 transcription elongation from RNA polymerase II promoter	
		29 transcription from RNA polymerase II promoter	
		30 translational initiation	
		31 transport	
		32 triglyceride biosynthetic process	
		33 ubiquitin protein ligase binding	

whether a patient would be responsive to a specific class of antidepressant. In this study, we aimed to identify microRNAs and their targeted pathways that could potentially predict whether a patient with MDD would remit with duloxetine, a SNRI. To the best of our knowledge, this is the first study to examine the potential of cell free circulating microRNAs to be used as biomarkers for the prediction of response to antidepressant treatment.

Our study examined plasma microRNAs from blood samples collected in 3 independent clinical trials. Lopez and colleagues (Lopez et al., 2017) examined microRNAs extracted from whole blood in a combined set of samples from the same 3 clinical trials and identified 4 microRNAs as being markers of response to both antidepressant and placebo treatment. In our study, we adopted a different approach, where we aimed to identify microRNAs that are specific to duloxetine treatment. By examining patients who were treated with duloxetine separately from patients who were treated with placebo, we were able to exclude microRNAs and pathways that are identified in both duloxetine and placebo comparisons, which are more likely to pertain to a general treatment effect not specific to drug response. We also examined the 3 clinical trials separately to allow for the comparison of the results between the trials to examine their replicability.

Examination of microRNA expression level differences between patients treated with duloxetine who remitted and did not remit in samples collected at baseline showed that hsa-miR-16-5p, hsa-miR-146a-5p, and hsa-miR-21-5p are ranked in the top 10th percentile in 2 of the 3 trials while not being ranked for the placebo comparison. Hsa-miR-23a-3p was ranked in all 3 studies for this comparison, although it was also ranked for 1 of the 3 studies in the baseline placebo comparison. These microRNAs may be good candidates for exploration of predictive markers for remission with duloxetine treatment. Hsa-miR-30b-5p and hsa-miR-30c-5p were ranked in both duloxetine and placebo comparisons when looking at baseline and time 2 comparisons, respectively, suggesting that these regulators may be involved in a general treatment response regardless of the type of treatment. From the full list of microRNAs that were in the top 10th percentile in any of the 3 trials for the baseline duloxetine comparison alone, we had overlapping results for duloxetine effect with Lopez and colleagues' findings (Lopez et al., 2017). These included hsa-miR-361-5p, hsa-miR-146a-5p and hsa-miR-24-3p. We identified only 2 microRNAs that were ranked in all 3 trials for the same comparison, although no single trial was always the discordant one, suggesting that this was not due to a particular trial characteristic. This was not unexpected, as the trials were conducted independently of each other and varied in study length (6 or 8 weeks), age range of participants (18–65 for Study B, and 18–75 for Studies A and C), and number of participants included (607–766), which could have contributed to the different results found in the studies. However, the identification of a subset of microRNAs in multiple studies suggests that despite these differences, we were able to identify important markers and pathways that may shed light on the difficult biological issue of treatment response in this common disorder.

If the microRNAs ranked in the top 10th percentile played a role in contributing to one's potential to respond to duloxetine, we reasoned that these microRNAs would target pathways that were previously shown to be altered in MDD. Therefore, we performed pathway enrichment analysis on microRNAs ranked in the top 10th percentile for baseline comparison of duloxetine remitters and non-remitters. This resulted in ten pathways, which included the apoptotic signaling pathway. Interestingly, the apoptotic signaling pathway was also found to be targeted by microRNAs that differ between patients who remit and do not remit after receiving only placebo in samples collected at the end of treatment. This suggests that microRNAs targeting the apoptotic process pathway may be involved in changes produced by placebo treatment in patients who remitted, and in one's likelihood to be successfully treated with duloxetine. Furthermore, positive and negative regulation of apoptotic process pathways were targeted by microRNAs that were ranked in the top 10th percentile for the discrimination between remitters and non-remitters in baseline samples from patients treated with placebo. Alterations in these two pathways, which are defined as any processes that increase or decrease cell death by apoptosis (Ashburner et al., 2000; The Gene Ontology, 2017), respectively, may change one's likelihood to recover from depression without pharmacological intervention. These findings are strongly supportive of the literature demonstrating the importance of apoptotic processes in the pathophysiology of MDD (Miguel-Hidalgo et al., 2014a; Shelton et al., 2011; Uranova et al., 2004). In fact, one of the most consistent postmortem brain findings in MDD is atrophy and decreased density and size of neurons and glia (Kim et al., 2016; O'Brien et al., 2004; Rajkowska, 2000; Sheline et al., 1996). Studies in the past decade have shown that increased apoptotic signaling may be a contributor to this phenomenon in MDD, with studies reporting altered expression of pro-apoptotic and anti-apoptotic genes in the post-mortem brain, with their expression differing between patients who did and did not receive antidepressant treatment (Miguel-Hidalgo et al., 2014a; Shelton et al., 2011).

Glucose metabolic process pathway was also implicated in the baseline comparison for duloxetine treatment. Disruption of glucose metabolism in the brain is well established in MDD (Baxter et al., 1989; Buchsbaum et al., 1986; Su et al., 2014), with a recent study demonstrating increased glucose and lactate levels in the pregenual anterior cingulate in MDD (Ernst et al., 2017). While there are no studies demonstrating the correlation between alterations in brain and peripheral microRNA levels, the findings of this study suggest that expression levels of plasma microRNAs in apoptosis and glucose metabolism pathways may offer insight into one's likelihood to respond to duloxetine treatment.

We also examined microRNA expression level changes produced by duloxetine treatment by performing rank feature selection on microRNA levels from samples collected in time 2 from patients who remitted and did not remit with duloxetine treatment. The same analysis was performed in the placebo comparison in time 2. Three microRNAs, hsa-miR-21-5p, hsa-miR-144-3p and hsa-let-7c-5p, were ranked in the top 10th percentile for duloxetine treatment for 2 of the 3 trials while not being ranked for the placebo comparison in any of the trials. These microRNAs may be good candidates for exploration as markers of duloxetine response. MiR-425-5p, which was in the top 10th percentile for one of the 3 trials for duloxetine treatment effect at time 2, was also identified by Lopez and colleagues (Lopez et al., 2017). When examining microRNAs ranked in 2 of 3 trials for differentiating duloxetine remission and non-remission at baseline and at time 2, hsa-miR-21-5p was ranked for both time points. For the placebo comparison, hsa-miR-183-5p was ranked for both time points. These microRNAs may be involved in both predisposing a person to remitting with duloxetine or placebo treatment, as well as the physiological changes produced as a result of these treatments in remitters.

Pathway enrichment analysis of microRNAs in the top 10th percentile for the comparison between duloxetine remitters and non-remitters in samples from time 2 was also performed. After only including pathways that were enriched in all 3 trials and removing those significant for the placebo comparison, 17 pathways were identified, which included the cytokine-mediated signaling pathway. Altered levels of pro and anti-inflammatory proteins and their genes are consistently found in the brain and peripheral samples from patients with MDD, with antidepressants having been shown to affect peripheral inflammatory markers (Alcocer-Gómez et al., 2014; Catena-Dell'Osso et al., 2011; Kim et al., 2016; O'Brien et al., 2006; Shelton et al., 2011; Wiedlocha et al., 2018). Our results are in support of these findings and suggest that duloxetine may be exerting its effects in part by modulating microRNAs in the cytokine pathway.

Furthermore, when comparing pathway analysis results from baseline data and post-treatment data, two pathways related to physiological response to viruses emerged from the duloxetine comparisons, and 4 pathways related to metabolism or synthesis and morphogenesis emerged from the placebo comparisons. Pathways implicated in the comparative analysis of data from both time points may be involved in one's likelihood to remit following duloxetine or placebo treatment, as well as the physiological changes experienced by those who do remit with these treatments. Identification of pathways that support metabolic, transporting, synthesizing, and immunological functions are in agreement with previous studies that demonstrate alterations in these processes in MDD (Adachi et al., 2014; Iwata et al., 2013; Miguel-Hidalgo et al., 2014b; O'Brien et al., 2004; Su et al., 2014). Further exploratory analysis was performed to identify pathways that may be involved in predisposing one to (baseline data) or change as a result of (time 2 data) remission with treatment in both duloxetine and placebo treatment groups, as it may identify processes that could be involved in treatment response regardless of the type of treatment used. Interestingly, the identified pathways included those involved in chondroitin sulfate synthesis, apoptosis, and nitric oxide synthesis and metabolism, which were suggested to be involved in the pathophysiology of MDD and antidepressant response in previous studies (Hunter et al., 2013; Oliveira et al., 2008; Shelton et al., 2011).

The findings of this study must be interpreted in light of its limitations. First, plasma samples were collected and stored outside of our laboratory, with the time of storage at $-70\,^{\circ}C$ ranging from 5 to 9 years. While we cannot ascertain the effect of storage time on the results, quality and quantity of cDNA were checked prior to sequencing to ensure that the samples were of adequate quality for analysis. Second, the microRNAs identified here were based on the analysis of patients who received 6–8 weeks of treatment. While the timeframe is sufficient to produce antidepressant response, whether these microRNAs can predict if patients will remain in remission for longer periods of time is unknown. Also, the generalizability of these findings is limited as the samples were obtained from clinical trials. Further exploration is needed to validate these findings in populations outside of a clinical trial setting. Also, as we focused on a limited number of implicated pathways, such as those involved in apoptosis, to allow us to check the congruency of our data with the existing knowledge on MDD, it should be noted that the full extent of the generated data was not discussed in this paper. Lastly, while the focus of this study was to identify potential predictive biomarkers by exploring microRNAs and pathways that may predict whether a patient would remit with duloxetine treatment, the same datamining method can be used to identify microRNAs that are discriminating between patients who received duloxetine and those who received placebo at the end of treatment. Future studies exploring this may further our understanding of duloxetine's pharmacodynamics.

The findings of this study suggest that microRNAs in the apoptosis signaling and glucose metabolism pathways may be good candidates for identification of peripheral biomarkers for predicting response to duloxetine. Hsa-miR-16-5p, hsa-miR-146a-5p and hsa-miR-21-5p, and hsa-miR-23a-3p may also yield interesting findings in future studies exploring predictive biomarkers of duloxetine response in MDD. Further validation of these findings in patients treated for longer periods of time with duloxetine and in different settings would be useful in improving the generalizability of these findings.

Conflict of interest

The authors declare no conflict of interest.

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