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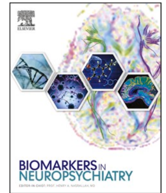

Differential expression of serum extracellular vesicle microRNAs and analysis of target-gene pathways in major depressive disorder

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A B S T R A C T

Background: Major depressive disorder (MDD) presents with both peripheral and central alterations, such that crosstalk between the periphery and the central nervous system could contribute to its aetio-pathophysiology. One putative mediating mechanism is circulating extracellular vesicles (EVs) and their microRNA (miRNA) cargo. In this study, we investigated differential expression of the serum EV miRNome in MDD patients versus controls with the aims of identifying potential EV miRNA biomarkers and downstream target gene pathways.
Methods: miRNA-Sequencing was performed on serum EVs isolated from MDD patients (n = 42) and matched healthy Controls (n = 18). Differential expression analysis was conducted, followed by diagnostic power analysis of dysregulated EV miRNAs, and pathway analysis of their target genes.
Results: Of 1800 serum EV miRNAs detected consistently, 33 were differentially expressed in MDD and Control subjects, 17 up-regulated and 16 down-regulated. Receiver-operating characteristic analysis identified an up-regulated and a down-regulated panel of EV miRNAs, each with additive diagnostic power as a differential biomarker for MDD. Predicted target gene-pathways were significantly enriched with respect to brain function, signal transduction and substance dependence ontology.
Conclusions: This study provides one of the first reports of dysregulation of the peripheral EV miRNome in MDD, including evidence for EV miRNAs as potential MDD biomarkers and identification of pathways via which they may contribute to MDD pathophysiology. Large-scale studies are required to confirm EV miRNome biomarker potential in MDD. Empirical evidence for involvement of the dysregulated EV miRNAs in the predicted target-gene pathways relevant to MDD pathophysiology is required.

1. Introduction

Major depressive disorder (MDD) is a heterogeneous disorder including symptoms of low mood, low interest in daily activities, and changes in homeostatic processes including appetite and sleep (DSM-V.; American Psychiatric Association, 2013). MDD is one of the leading causes of disability worldwide, currently affecting more than 260 million people (James et al., 2018). Psychosocial stress is a major risk factor for MDD (Fleshner et al., 2017). Stress, originating in the central nervous system (CNS), impacts on a number of systems in the periphery, including the sympathetic nervous system, the endocrine system, and the immune-inflammatory system (Wohleb et al., 2016). These peripheral changes provide information on the psychobiological state of the individual. Furthermore, they can feedback to and lead to alterations in

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the CNS, including: altered neurotransmitter signalling e.g. dopamine and glutamate (Duman et al., 2019; McEwen et al., 2015; Nestler & Carlezon, 2006), increased neuroinflammation (Hodes et al., 2015), and neurotoxicity (Felger & Treadway, 2017). These stress-related changes may well contribute to the aetio-pathophysiology of MDD. However, the physiological mechanisms underlying periphery-CNS ("body-brain") dysregulation in MDD are not well understood, and reliable peripheral biomarkers that would provide insights into these mechanisms and even serve diagnostic functions, remain to be identified.

Periphery-to-CNS communication can be mediated by cells e.g. immune cells, hormones e.g. cortisol, and soluble molecules e.g. cytokines (Miller & Raison, 2016). Recently, an additional communication pathway has gained attention, namely the transfer of signalling molecules via extracellular vesicles (EVs). EVs are membrane-enclosed structures that are 30–1000 nm in diameter and secreted by virtually all cell types, in the periphery and also in the CNS (Holm et al., 2018). In the cell of origin, a molecular cargo is encapsulated into nascent EVs, including cytosolic and membrane proteins, mRNAs, and high amounts of small non-coding RNAs, e.g. microRNAs (miRNAs). The loaded EVs are then released into the extracellular space and transported within body fluids (Holm et al., 2018). Recent *in vitro* and animal studies have demonstrated that, during inflammation or viral infection, EVs are able to cross the blood-brain barrier and enter the CNS, with the specific mediating mechanisms requiring further investigation (Ridder et al., 2014, Saint-Pol et al., 2020). Moreover, evidence from animal and human studies indicates that EVs released by CNS cells, including neurons and oligodendrocytes, can be transferred to the peripheral circulation (Galazka et al., 2018; García-Romero et al., 2017; Shi et al., 2014).

MicroRNAs are small non-coding RNAs, which play a role in the regulation of gene translation; they act via interaction with a complementary sequence on their respective target mRNAs, which in most cases leads to either degradation or translational repression of the latter (O'Brien et al., 2018). Individual miRNAs can regulate hundreds of mRNAs, and the same transcript can be bound by various miRNAs, rendering miRNA-mediated regulation complex and dynamic (Shu et al., 2017). Since the first study comparing the miRNA profile of peripheral blood mononuclear cells (PBMCs) between MDD and healthy subjects (Belzeaux et al., 2012), subsequent studies have investigated both intracellular and circulating extracellular miRNAs as biomarkers for MDD or antidepressant response (for reviews see Lopez et al., 2018; Yuan et al., 2018). Whilst miRNAs purportedly suitable for one or the other purpose have been proposed, there is relatively little between-study consistency in terms of the proposed MDD miRNA biomarkers. Such variability is most likely due to differences in sample characteristics, clinical characteristics of study subjects, and methods used to quantify miRNA expression levels.

Given that the miRNA cargo of EVs is affected by the physiological state of the releasing cell (Dalvi et al., 2017; Squadrito et al., 2014), the EV miRNome might provide a particularly sensitive indicator of pathological activity. In addition, since periphery-CNS EV passage is bidirectional (Saeedi et al., 2019), the peripheral EV miRNome might also include biomarkers of CNS cells status. Indeed, recent studies have provided evidence for the potential of specific peripheral EV miRNAs as biomarkers for bipolar disorder (Ceylan et al., 2020; Fries et al., 2019) and schizophrenia (Du et al., 2019). For MDD, two such studies have been conducted recently, one of which was a case study (Wei et al., 2020; Y. Zhang et al., 2018). In the present study, we investigated the serum EV miRNome in MDD and matched healthy Control subjects, with the aim of identifying possible EV miRNA biomarkers of MDD and then investigating the ontological pathways via which they may exert pathophysiological effects in the periphery or the CNS.

2. Methods

2.1. Participants

The study was approved by the Ethics Committee of the Canton of Zurich and all subjects gave written informed consent. Patients with a diagnosis of major depressive disorder (MDD, n = 42) were recruited from outpatient and inpatient units of the Psychiatric Hospital, University of Zurich (Zurich, Switzerland) and affiliated institutions, while healthy matched Controls (n = 18) were recruited from the community via advertisement. MDD diagnosis or absence thereof were confirmed by conducting the Mini-International Neuropsychiatric Interview (Sheehan et al., 1998), and all MDD patients met the DSM-IV-TR criteria for MDD (American Psychiatric Association, 2000). Continuous depressive symptoms of all participants were scored using the Beck's Depression Inventory (BDI), and MDD patients were further assessed using the Hamilton Depression Scale (HAM-D). The inclusion age was 18–65 years. The mean age of MDD patients was 35.7 years and that of Control subjects was 32.9 years. The percentage of females was 60% in MDD and 56% in Controls. MDD subjects had an average HAM-D score of 18.3 and an average BDI score of 25.8. Average MDD duration at the time of sample collection was 6.8 years. The majority (81%) of MDD subjects were receiving antidepressant medication at the time of sample collection. The demographic and clinical characteristics of subjects are summarized in Table 1. Blood was drawn between 8 and 10 am, and participants were instructed to fast for at least 8 h prior to blood draw. Blood samples were collected into 7.5 ml serum collection tubes, stood at room temperature for 10 min and centrifuged at 2000 g and 4 °C for 20 min. Serum aliquots of 200 μl were transferred into 0.5 ml microcentrifuge cryotubes and stored immediately at − 80 °C. Additional details regarding study participants and sample collection procedure are provided elsewhere (Klaus et al., 2021).

2.2. EV isolation and RNA extraction

Extracellular vesicles (EVs) were isolated from one 200 μl serum aliquot per subject using size exclusion chromatography (SEC) (Stranska et al., 2018). Briefly, samples were thawed at 37 °C and centrifuged at 3000 g and room temperature for 10 min. The supernatant (170 μl) was applied onto a SEC column (qEV single 35 nm, Izon Science) and eluted by progressive addition of freshly filtered phosphate buffered saline (PBS), which allowed for separation and collection of five distinct 200 μl fractions containing EVs. The presence of EVs and absence of contaminants in the purported EV-rich fractions were validated using several complementary methods, namely particle count, transmission electron microscopy, western blotting, and low density lipoprotein assay, according to the Guidelines of the International Society for Extracellular Vesicles (Théry et al., 2018). For details see Supplementary Material. Each pool of EV-rich fractions was ultra-filtrated to a volume of 200 μl using an Amicon Ultra-4 10 K Centrifugal Filter Device (Merck) and then 600 μl lysis buffer (Norgen Biotek) were added. Lysed samples were frozen immediately on dry ice and stored at − 80 °C until RNA extraction. RNA was extracted using the Plasma/Serum Purification Mini Kit

Table 1
Demographic and clinical characteristics of MDD subjects and healthy controls.

Parameter	Controls (n = 18)	MDD (n = 42)
Age Years: min – max (mean)	23 – 58 (32.9)	20–57 (35.7)
Gender: % Female	56	60
AD treatment: % SSRIs/Other/None	0/0/100	43/38/19
Disorder Duration: Years min – max (mean)	N/A	0.08 – 37 (6.8)
BDI score: min – max (mean)	0 – 9 (1.3)	4 – 45 (25.8)
HAM-D score: min – max (mean)	N/A	4 – 33 (18.3)

AD: antidepressant; SSRIs: selective serotonin reuptake inhibitors; BDI: Beck's depression inventory; HAM-D: Hamilton depression rating scale; N/A: non-applicable.

(Norgen Biotek) according to the manufacturer's instructions (title of instruction protocol: Exosomal RNA Purification from Exosomes Already Purified via Ultracentrifugation, Exoquick, Filtration or any other Precipitation Method). RNA concentration and size distribution were assessed using the Agilent RNA 6000 Pico kit and a 2100 Bioanalyzer system (Agilent Technologies). Isolated RNA samples were stored at − 80 °C until library preparation.

2.3. MicroRNA sequencing

Small-RNA libraries for multiplexed sequencing were prepared from the EV RNA samples using the QIAseq miRNA Library kit (Qiagen) according to the manufacturer's instructions. Library concentration and quality were assessed using a TapeStation High Sensitivity DNA system (Agilent Biotechnologies). One sample (MDD patient) did not pass the quality control. Using an equal amount of cDNA per sample library, the cDNA libraries were pooled, and sequencing was conducted on an Illumina HiSeq2500 System with a sequencing depth of 5 million reads per sample and sequencing configuration single-end 100 bp. The raw sequencing data were uploaded onto the GeneGlobe Data Analysis Center (Qiagen) for pre-processing and quantification. First, 3' adapters and low-quality bases were trimmed using Cutadapt. Next, the insert sequences and unique molecular identifiers (UMIs) were identified. Read mapping was performed according to a sequential alignment strategy using Bowtie: Firstly, reads were mapped to miRBase Mature, miRBase Hairpin, Non-coding RNA, mRNA, and other RNAs, to identify perfect matches; second, mapping to a species-specific miRBase mature database was conducted (1–2 mismatches tolerated); third, all remaining unmapped sequences were aligned to the human genome (Genome Reference Consortium GRCh38) to identify possible novel miRNA molecules. All reads assigned to each specific miRNA were quantified and the associated UMIs aggregated to count unique molecules. To assess whether any systematic amplification bias was introduced during library preparation, Spearman's rank-order correlation analysis of total read counts versus total UMIs was conducted; high correlations (rho = 0.99–1) demonstrated an absence of bias.

Downstream expression analyses were conducted in R. MiRNAs with less than 10 counts in $\geq$ 50% of the samples in one of the two experimental groups were considered to be at background noise level and discarded. Spearman's rank-order correlations were calculated for each subject's miRNA counts versus the group mean miRNA counts, to assess within-group homogeneity and identify possible outliers. Subjects whose correlation coefficients were below the 25th percentile of all subjects' correlation coefficients were considered outliers and excluded from the downstream analysis; this applied to three MDD and two Control subjects. Following outlier subject exclusion, cluster analysis of individual EV miRNomes was conducted using a non-metric multidimensional scaling approach: this identified the absence of separation according to the variables gender and medication (Fig. S2a, S2b), which accounted for 0.7% and 1.46% of the total variance of the EV miRNome, respectively; on the basis of these results, gender and medication were not included as co-variables in the downstream differential expression analysis.

2.4. Statistical analysis

EV miRNome differential expression analysis was implemented using the Bioconductor package *EdgeR*. Firstly, aggregate counts were normalized to the trimmed mean of the M values (TMM). Differential expression analysis was then conducted using the quasi-likelihood F-test. Criteria thresholds for differential expression were p value < 0.05 and $\log_2$ fold change ≥ 0.5 or ≤ -0.5. In addition, adjusted p values were calculated using the false discovery rate according to the Benjamini-Hochberg method. Power analysis was conducted retrospectively using the R package *ssizeRNA* (Bi & Liu, 2016): pi_0 was set at 0.9, power at 0.8, and FDR at ≤ 0.05; the analysis was conducted with both the whole dataset and the dataset after outlier subject removal (2 CON and 3 MDD subjects). All other statistical analyses were conducted using GraphPad Prism v.7. For those miRNAs that were up- or down-regulated in MDD versus Control subjects, receiver-operating characteristic (ROC) analysis was conducted. ROC analysis was conducted using the z-scored miRNA expression values for each differentially expressed miRNA to control for differences in absolute expression values between miRNAs. The area under the ROC curve (AUC) and corresponding p values were used to assess the diagnostic power of MDD-Control separation. Separately for up- and down-regulated miRNAs, starting with the miRNA with the highest diagnostic power, each subject's z-scores for two or more miRNAs were averaged and ROC analysis was run with these averaged z-scores, to determine whether a miRNA panel could be identified with higher diagnostic power than that of the most diagnostic single miRNA. Furthermore, the means of the z-scores of the miRNAs contributing to the panel of up-regulated miRNAs and of those contributing to the panel of down-regulated miRNAs, were compared between MDD and Control subjects using an unpaired two-tailed Student's *t*-test. Finally, in MDD patients, association between BDI or HAM-D scores and the mean z-scores of the miRNAs in each panel was assessed using Pearson's product-moment correlation.

2.5. Pathway analysis

For up-regulated and down-regulated miRNAs separately, the predicted target genes were identified using the database miRWalk (v. 3.0, Sticht et al., 2018); only interactions between miRNAs and mRNA 3' UTRs were considered. Only those genes that were predicted as targets by both miRWalk and the miRDB database (Chen & Wang, 2020) were retrieved. Standard enrichment analysis with respect to pathways was then performed on these predicted target genes according to the standard method based on the Fisher's exact test (Subramanian et al., 2005). Pathways were retrieved from the database Kyoto Encyclopedia of Genes and Genomes (KEGG). Significantly enriched pathways were defined as those with a p value < 0.05 following Benjamini-Hochberg method adjustment and grouped into broader functional categories based on the KEGG classification. Cancer-related pathways were excluded from the analysis to overcome the strong bias in the database towards these pathways compared with other types of diseases, including psychiatric disorders.

2.6. RT-qPCR

For validation purposes, expression levels of three selected miRNAs – miR-4433b-5p, miR-16–5p, and miR-625–3p – were also assessed using quantitative reverse transcription PCR (RT-qPCR). These miRNAs were selected on the following basis: (1) The primers (Qiagen) could be validated in-house with respect to sensitivity and specificity. (2) In the case of miR-4433b-5p, it was up-regulated in MDD in miRNA-Seq. (3) miR-16 is one of the most abundant mammalian miRNAs (Landgraf et al., 2007). Although miR-16 is commonly applied as an endogenous control in miRNA studies (Donati et al., 2019), it was not in this study because in other studies we conducted, we observed that it is differentially expressed in different groups e.g. multiple sclerosis patients versus control subjects. Per sample, 4.5 μl RNA were used for reverse transcription, conducted using the miRCURY LNA RT kit (Qiagen) according to the manufacturer's instructions. A synthetic spike-in miRNA, UniSp6 (Qiagen), was added to the solution to normalize any potential difference in reaction efficiency between samples. The cDNA samples were stored at − 20 °C until further processing. The qPCR reaction was prepared using the miRCURY LNA SYBR PCR Kit and miRNA-specific primers (Qiagen) and run using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad); samples were run in triplicate for each miRNA and in duplicate for Unisp6. Data were analysed using the CFX Maestro software (Bio-Rad) and the quantile normalization approach (Mar et al., 2009) was implemented to identify outlier technical

replicates. For statistical analysis, normalized quantification cycle (Delta Cq) values were used, calculated as the difference between the mean Cq of the target miRNA and the mean Cq of Unisp6, and unpaired two-tailed Student's *t*-tests were conducted for MDD versus CON. Pearson's product-moment correlation analysis between normalized miRNA-Seq counts (CPM) and delta Cq values was conducted, separately within CON and MDD subject groups. Statistical analysis was conducted using GraphPad Prism v7.

3. Results

3.1. Differential expression analysis of serum EV miRNAs

Per sample, 1212–2205 mature miRNAs (mean = 1823) were identified in the serum EVs from MDD and Control subjects. Differential expression analysis ($p < 0.05$, $\log_2$ fold change ≥ 0.5 or ≤ -0.5) of the serum EV miRNome in MDD versus Control identified 33 EV miRNAs (Table 2). Of these, 17 were up-regulated in MDD subjects, with an expression fold increase of 1.4–2.5, and 16 were down-regulated in MDD subjects, with an expression fold decrease of 0.7–0.3. Based on adjusted p values, 0 miRNAs were significantly up-regulated and 3 miRNAs were significantly down-regulated (Table 2). Power analysis conducted at FDR ≤ 0.05 identified a power of 0.7 for the current sample size of N = 18 controls; achieving a power of 0.8 would have required a sample size of N = 29 controls, or N = 28 controls following outlier exclusion.

RT-qPCR was conducted for three EV miRNAs of interest. For each of these, correlation analysis revealed agreement between normalized miRNA-Seq counts (CPM) and RT-qPCR delta Cq values in both MDD samples and Control samples (Table S1; Fig. S3). Moreover, the MDD up-regulated EV miRNA miR-4433b-5p had lower mean delta Cq values in MDD compared with CON subjects, although the difference did not reach statistical significance (p = 0.1) (Table S1).

Table 2
Differentially expressed serum EV miRNAs in MDD subjects versus matched healthy Controls.

miRNA ID	CON	MDD	p value	FDR	$\log_2$FC	FC
Up-regulated						
hsa-miR-1207–5p	65	133	0.005	1	1.0	2.0
hsa-miR-219b-5p	187	414	0.008	1	1.1	2.2
hsa-miR-7152–5p	181	297	0.01	1	0.7	1.6
hsa-miR-615–5p	95	167	0.01	1	0.9	1.8
hsa-miR-1587	87	128	0.02	1	0.6	1.5
hsa-miR-1471	321	810	0.02	1	1.3	2.5
hsa-miR-6887–3p	331	459	0.03	1	0.5	1.4
hsa-miR-4433a-3p	55	99	0.03	1	0.8	1.8
hsa-miR-4433b-5p	477	707	0.03	1	0.6	1.5
hsa-miR-5192	184	328	0.03	1	0.8	1.8
hsa-miR-3667–5p	113	157	0.03	1	0.5	1.4
hsa-miR-4776–3p	206	347	0.04	1	0.8	1.7
hsa-miR-205–5p	541	835	0.04	1	0.6	1.5
hsa-miR-1236–5p	111	165	0.04	1	0.6	1.5
hsa-miR-769–5p	238	332	0.05	1	0.5	1.4
hsa-miR-760	210	319	0.05	1	0.6	1.5
hsa-miR-557	119	165	0.05	1	0.5	1.4
Down-regulated						
hsa-miR-6735–3p	225	73	< 1e-16	< 1e-16	-1.7	0.3
hsa-miR-6833–5p	670	191	< 1e-16	< 1e-16	-1.8	0.3
hsa-miR-615–3p	273	126	2.00E-05	0.04	-1.1	0.5
hsa-miR-8052	378	250	3.10E-04	0.6	-0.6	0.7
hsa-miR-34c-3p	268	189	3.60E-04	0.7	-0.5	0.7
hsa-miR-4433a-5p	174	119	0.003	1	-0.6	0.7
hsa-miR-134–5p	746	542	0.003	1	-0.5	0.7
hsa-miR-6798–5p	122	73	0.003	1	-0.7	0.6
hsa-miR-4448	720	486	0.005	1	-0.6	0.7
hsa-miR-5589–5p	245	149	0.01	1	-0.7	0.6
hsa-miR-3677–5p	113	64	0.01	1	-0.8	0.6
hsa-miR-6744–3p	101	64	0.02	1	-0.6	0.6
hsa-miR-6086	126	89	0.02	1	-0.5	0.7
hsa-miR-196a-3p	146	103	0.02	1	-0.5	0.7
hsa-miR-23a-5p	833	555	0.04	1	-0.6	0.7
hsa-miR-128–2–5p	112	79	0.05	1	-0.5	0.7

Expression levels of CON and MDD miRNAs expressed as mean: counts per million normalized to the TMM; p value: nominal p value; FDR: p values calculated using the false discovery rate (FDR) according to the Benjamini-Hochberg method; logFC: fold change in logarithmic base 2 scale for MDD versus CON; FC: absolute fold change for MDD versus CON.

3.2. Diagnostic power analysis of dysregulated miRNAs

To assess the diagnostic power of differentially expressed miRNAs in terms of demarcating MDD from Control subjects, ROC analysis was conducted. For each miRNA in Table 2, normalized counts were z-score transformed. ROC analysis for individual miRNAs identified three up-regulated miRNAs with significant diagnostic power (Fig. 1A): miR-1587 (AUC = 0.81, 95% CI = 0.63 – 0.98, p = 0.0004), miR-1207–5p (AUC = 0.7, p = 0.02), and miR-6887–3p (AUC = 0.67, p = 0.05). As a further step, we investigated for panels of EV miRNAs that yielded higher diagnostic power than the individual EV miRNAs. For this, the latter were ranked based on their z-score ROC AUC and p values. A panel comprising the four up-regulated miRNAs miR-1587, miR-1207–5p, miR-6887–3p and miR-4433b-5p, had a higher diagnostic value than the most diagnostic individual miRNA (AUC = 0.84, 95% CI = 0.71–0.96, p = 0.0001; Fig. 1A), whilst inclusion of additional up-regulated miRNAs did not yield further increase. Furthermore, the per subject means of the z-scores of these four miRNAs were significantly higher in MDD than Control subjects ($t\,(52) = 3.46$, $p < 0.002$; Fig. 1B). For down-regulated miRNAs, ROC analysis identified four miRNAs with significant diagnostic power for MDD (Fig. 1C): miR-196a-3p (AUC = 0.76, 95% CI = 0.62 – 0.89, p = 0.003), miR-134–5p (AUC = 0.72, p = 0.01), miR-4433a-5p (AUC = 0.71, p = 0.02), and miR-6735–3p (AUC = 0.71, p = 0.02). A panel of the six miRNAs miR-196a-3p, miR-134–5p, miR-4433a-5p, miR-6735–3p, miR-8052 and miR-34c-3p, had a higher diagnostic value than the most diagnostic individual miRNA (AUC = 0.87, 95% CI = 0.75 – 1.0, $p < 0.0001$; Fig. 1C), whilst inclusion of additional down-regulated miRNAs did not yield further increase. The per subject means of the z-scores of these six miRNAs were significantly lower in MDD than Control subjects ($t\,(52) = 5.03$, $p < 0.0001$; Fig. 1D). Separate correlation analyses were then conducted between the mean z-score of the miRNAs in each of the up-regulated and down-regulated panels and the BDI and the HAM-D score of MDD patients; these indicated an absence of correlation ($r = -0.2$ to 0.06, $p > 0.2$). Lastly, we assessed the diagnostic power of the ratio of the normalised expression values (CPM) of miR-1587–5p/miR-196a-3p, i.e. the up-regulated and down-regulated miRNAs with the highest diagnostic power. This ratio had a significant diagnostic power (AUC = 0.78, 95% CI = 0.64 – 0.92, $p < 0.01$; Fig. 1E), and ratio values were significantly higher in MDD than in Control subjects ($t\,(50) = 2.69$; p = 0.01; Fig. 1F).

3.3. Target prediction and pathway analysis of dysregulated miRNAs

Target mining and pathway analysis of the differentially expressed EV miRNAs were then conducted to obtain insights into their biological functions as well as their potential relevance to the pathophysiological mechanisms of MDD (Table 3). For the 17 up-regulated EV miRNAs, target mining identified 2449 predicted target transcripts. Enrichment analysis of these targets identified 17 significantly associated KEGG pathways, involving 230 of the target transcripts. Regarding the functional annotation of these KEGG pathways, there was a significant enrichment of *Nervous system* pathways, including those related to neurotransmission (dopaminergic and glutamatergic signalling), synaptic plasticity (long-term potentiation), and brain development (axon guidance). *Signal transduction* pathways were also significantly overrepresented as were those of *Substance dependence*. For the 16 down-regulated EV miRNAs, target mining identified 1415 predicted target transcripts. There were three KEGG pathways that were significantly associated with these target transcripts, involving 69 of the target

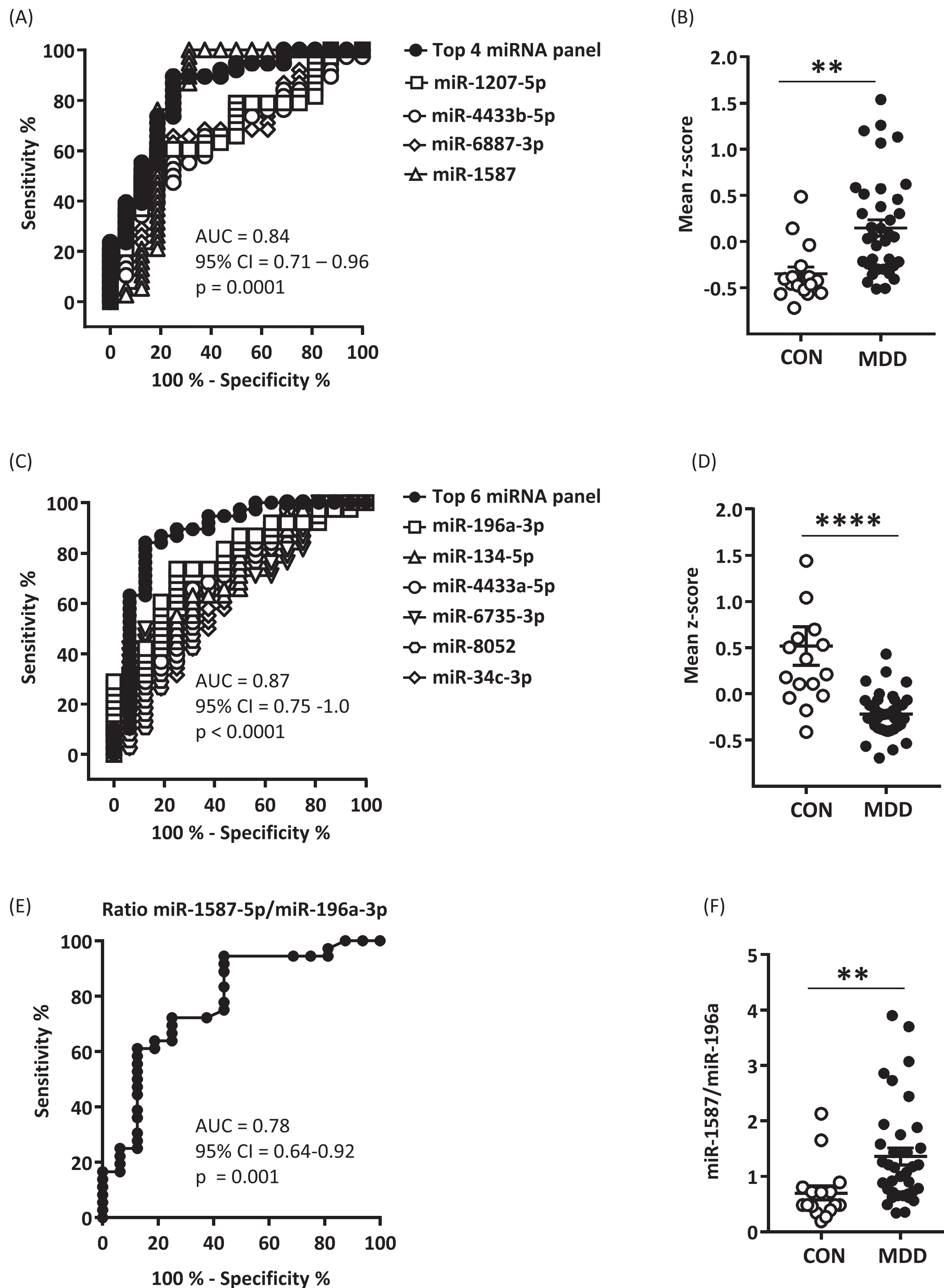

Fig. 1. (A-D): Receiver operating characteristic (ROC) analysis of EV miRNAs that were (A, B) up-regulated or (C, D) down-regulated in MDD (n = 38) versus Control (n = 16) subjects. (A) Using z-score transformation of normalized counts, three up-regulated miRNAs had significant diagnostic power; the mean of these miRNAs and one further miRNA yielded a profile with even greater diagnostic power. (B) Individual subject means ± standard error of the mean (S.E.M.) of the z scores for the four miRNAs given in (A). * * $p < 0.01$, unpaired two-tailed Student's *t* test. (C) Using z-score transformation of normalized counts, four down-regulated miRNAs had significant diagnostic power; the mean of these miRNAs and two further miRNAs yielded a profile with even greater diagnostic power. (D) Individual subject means ± SEM of the z scores for the six miRNAs given in (C). * ** * $p < 0.0001$, unpaired two-tailed Student's *t* test. (E) ROC analysis of the ratio, miR-1587–5p/miR-196a-3p, using normalised expression values (CPM). (F) Individual subject ratio values (and mean ± SEM). * * $p < 0.01$, unpaired two-tailed Student's *t* test.

Table 3
Predicted target genes and associated enriched pathways[a] of 5 most up-regulated and 5 most down-regulated serum EV miRNAs in MDD subjects.

miRNA ID	Predicted target genes	Enriched Pathway	Pathway category
Up-regulated MDD > CON			
miR-1207–5p	GRIK3, GLUL, SLC38A3, GNAI2, GRM4, PLA2G4F, GRIN2A, DLG4, PRKACA	Glutamatergic synapse	Nervous system
	GNAI2, CREB3L1, ARRB1, GRIN2A, PPP1R1B, PRKACA	Dopaminergic synapse	Nervous system
	EPHB2, NGEF, GNAI2, LIMK1, SSH2, PAK4	Axon guidance	Nervous system development
	PPP1R12B, CACNB4, GNAI2, MYLK4, CAMK1D, PLA2G4F, MAP2K5, PRKACA	Oxytocin signaling	Endocrine system
	GNAI2, PDE4B, CREB3L1, ARRB1, PRKACA	Parathyroid hormone	Endocrine system
	GNAI2, KCNMA1, PRKACA	Renin secretion	Endocrine system
	CTNNA2, FGF1, CCND3, DLG2, DLG4, SMAD7, APC2	Hippo signaling pathway	Signal transduction
	CREB3L1, IGF1, ULK1, PDPK1, AKT1S1, HNF4A, VASP	AMPK signaling pathway	Signal transduction
	GNAI2, MYLK4, SRF, KCNMA1, CREB3L1	cGMP-PKG signaling pathway	Signal transduction
	ARC, CREB3L1, GRIN2A, PPP1R1B, PRKACA, FOSB	Amphetamine addiction	Substance dependence
	PDE4B, GNAI2, ARRB1, PRKACA	Morphine addiction	Substance dependence
	CREB3L1, ATG13, IGF1, ULK1, PRKACA, AKT1S1	Longevity regulating pathways	Aging
	CACNB4, GNAI2, CREB3L1, SCN4B, PRKACA	Adrenergic signaling in cardiomyocytes	Circulatory system
	CLDN19, MAVS, CLDN4, OAS3, CLDN5	Hepatitis C	Viral infection
	GNAI2, GRIN2A, PRKACA	Circadian entrainment	Circadian Clock
miR-219b-5p	< 3 genes per pathway		
miR-7152–5p	CAMK2D, CAMK2A, GNAQ, GRIN2A	Long-term potentiation	Nervous system
	CAMK2A, GNAQ, GRIA4, GRIN2A	Dopaminergic synapse	Nervous system
	ADCY1, GNAQ, GRIA4, GRIN2A	Glutamatergic synapse	Nervous system
	EPHB2, SRGAP3, CAMK2A, WNT5B, SRGAP1, EFNB3	Axon guidance	Nervous system development
	CAMK2A, GNAQ, CACNB2	Oxytocin signaling	Endocrine system
	GNAQ, PRKG1, KCNMA1, VASP, ATP1B4	cGMP-PKG signaling pathway	Signal transduction
	WNT5A, MOB1B, WNT5B, CCND2	Hippo signaling pathway	Signal transduction
	PDE11A, GABRR2, OPRM1	Morphine addiction	Substance dependence
	CAMK2A, GRIA4, GRIN2A	Amphetamine addiction	Substance dependence
	CAMK2A, GNAQ, CACNB2, BCL2, ATP1B4	Adrenergic signaling in cardiomyocytes	Circulatory system
	CAMK2A, GNAQ, PRKG1, GRIA4, GRIN2A	Circadian entrainment	Circadian Clock
miR-615–5p	< 3 genes per pathway		
miR-1587	LRTOMT, GNG2, GNAL	Dopaminergic synapse	Nervous system
	WNT5A, PLXNA4, NCK1, PPP3R2, SRGAP1	Axon guidance	Nervous system development
	SLC8A1, ATP2B2, PPP3R2	cGMP-PKG signaling pathway	Signal transduction
Down-regulated MDD < CON			
miR-6735–3p	MRPL30, KCNK9, PTCD3, MPZ, C1RL, FURIN, PRPF4B, RHOB, WDCP, SLC30A6	No enriched pathway identified	
miR-6833–5p	EPHA2, STMN1, MAP3K20, FGF2, RASA1, PTPRR, IGF1, IGF1R, TAOK1, ELK1	MAPK signaling pathway	Signal transduction
	FGF2, RASA1, IGF1, IGF1R, RALBP1, BCL2L1, ELK1	Ras signaling pathway	Signal transduction
	GSK3B, ITGA2, IGF1, IGF1R, ELK1	Focal adhesion	Cellular community
miR-615–3p	TOMM7, MEF2A, FCMR, RAB30, POLR3B, SUPT16H, LRPAP1, SZRD1, CBX6, FADS1	No enriched pathway identified	
miR-8052	GNB1, RASAL2, RRAS2, RASGRP4, ELK1	Ras signaling pathway	Signal transduction
	RRAS2, MAPT, RASGRP4, ELK1	MAPK signaling pathway	Signal transduction
	BCL2, ACTN4, ELK1	Focal adhesion	Cellular community
miR-34c-3p	< 3 genes per pathway		

[a] Enriched pathways included are those with $\geq$ 3 predicted target genes of the up- or down-regulated EV miRNA and using a BH-corrected p value of < 0.05.

transcripts. These were MAPK signalling and Ras signalling (*Signal transduction*) and focal adhesion (*Cellular community*). As can be seen in Table 3, there was minimal overlap between the predicted target genes of, and therefore the enriched pathways for, the serum EV miRNAs that were up-regulated and down-regulated in MDD subjects.

4. Discussion

In the present study we compared the serum EV miRNome in MDD and healthy Control subjects, with the aims of contributing to the currently small number of studies on this important subject, identifying potential peripheral EV miRNA biomarkers for MDD, and investigating their putative target mRNA-pathways with regards to pathophysiology. Our results demonstrate modestly altered expression of the serum EV miRNome in MDD subjects. Nonetheless, among the relatively small number of EV miRNAs that were differentially expressed, some had significant diagnostic power for MDD-Control demarcation and therefore biomarker potential. Moreover, pathway analysis of the transcriptional targets of these differentially expressed EV miRNAs revealed enrichment for pathways that are widely considered to be relevant to MDD pathophysiology.

Since an initial study published in 2012 (Belzeaux et al., 2012), some 20 studies have investigated changes in the peripheral miRNome, either the PBMC or the extracellular compartments, in MDD (Yuan et al., 2018). Differential expression has been reported for about 200 circulating miRNAs in MDD compared to control subjects. However, between-study replication is rather limited: about 10 miRNAs have been reported to be up-regulated in more than one study, including miR-132 (Bocchio-Chiavetto et al., 2013; Li et al., 2013; Liu et al., 2016; Su et al., 2015), miR-182 (Gururajan et al., 2016; Li et al., 2015; Li et al., 2013) and miR-1202 (Fiori et al., 2017; Lopez et al., 2014); miRNAs for which there is replicated evidence for down-regulation include miR-636 (Belzeaux et al., 2012; Zhang et al., 2016), and there is replicated evidence for altered expression for several members of the let-7 miRNA family in MDD (Gururajan et al., 2016; Maffioletti et al., 2016). This generally low agreement across studies can be explained in part by differences in sample type, miRNA quantification method, study subject characteristics, and sample size. Focusing on the peripheral EV miRNome might be advantageous for the biomarker study of CNS disorders, including MDD. CNS-derived EVs have been detected in the peripheral circulation and have provided information on on-going pathological processes in the CNS (Galazka et al., 2018; García-Romero et al., 2017; Shi et al., 2014). Given that EV miRNAs are transferred actively to recipient cells and thereby affect their status (Ridder et al., 2014), they do not only constitute disease biomarkers but might also inform about and contribute to disease pathophysiology. Moreover, the development of new EV isolation technologies will provide the possibility to study EVs derived from specific cell types selectively, including CNS cells (Picciolini et al., 2018).

In the present study, the protocol used for analysis of the serum EV

miRNome in small volume samples, i.e. 200 μl, represents a practical advance compared to recent studies, where volumes of ≥ 1 mL were used (Fries et al., 2019; Wei et al., 2020). The average number of mature miRNAs identified was about 1800 per sample. This is high compared to published reports, where around 500 blood EV miRNAs were retrieved consistently (e.g. Liu et al., 2019; Zhao et al., 2020), a difference that could well be explained by the allowance of up to 2 nucleotide mismatches during the mapping process in the present study. Other factors that might also have made a contribution include the recently developed kit for the preparation of small RNA libraries that we used and the subsequent utilisation of the corresponding data analysis pipeline for pre-processing and quantification. Of the 1800 serum EV miRNAs consistently identified, 33 were differentially expressed in MDD compared to Controls; 17 were up-regulated and 16 were down-regulated. Of these, three down-regulated miRNAs maintained significant differential expression after multiple comparison adjustment. Due to the small amount of EV RNA and limited availability of sufficiently sensitive and specific primers, validation analysis of the miRNA-Seq findings using RT-qPCR was restricted to three EV miRNAs. In each case, and analyzing MDD and Control subjects separately, the results obtained by miRNA-Seq and by RT-qPCR were in agreement.

Comparison of the differentially expressed EV miRNAs identified in our study with peripheral circulating extracellular miRNAs reported previously (Lopez et al., 2018; Lopizzo et al., 2019; Yuan et al., 2018) indicated little overlap. Specifically, miR-4476–3p was up-regulated in MDD in both the blood miRNome (Yuan et al., 2018) and in our study, whilst miR-1471 and miR-3667–5p were down-regulated in the blood miRNome (Yuan et al., 2018) and up-regulated in our study, and miR-4448 was up-regulated in the blood miRNome (Yuan et al., 2018) and down-regulated here. This low agreement is reminiscent of that observed between studies of the peripheral PBMC/extracellular miRNA profile in MDD. Comparison of EV RNA and extracellular non-EV RNA profiles in various biofluids would be expected to yield marked differences (Galvanin et al., 2019; Prieto-Fernández et al., 2019; Zhao et al., 2020), as would comparison of the RNA profiles of cells (e.g. PBMCs) and EVs due to specific miRNA loading by EV-releasing cells (Squadrito et al., 2014).

With respect to previous reports on the serum EV miRNome in MDD, of which to our knowledge there are two, we replicated findings for two EV miRNAs, namely up-regulation of miR-205–5p (as in the case study by Zhang et al., 2018) and of miR-769–5p (as in Wei et al., 2020). Interestingly, exosomal miR-769–5p targets transforming growth factor beta receptor 1 (TGFBR1) (Ni et al., 2020), and serum TGFB has been reported to be increased in MDD compared to controls (Davami et al., 2016). Two EV miRNAs, miR-139–5p and miR-144–3p, were dysregulated in both previous studies but not here. This low consensus is likely due to differences in EV isolation and RNA extraction protocols, bioinformatics approach, and clinical characteristics of MDD subjects. Comparing our current findings with the few studies that have investigated peripheral EV miRNAs in other psychiatric disorders, namely bipolar disorder (Ceylan et al., 2020; Fries et al., 2019) and schizophrenia (Du et al., 2019), only miR-4433a-5p was down-regulated here in MDD patients and in bipolar disorder relative to their respective controls (Fries et al., 2019). This suggests that the differential EV miRNA expression levels detected are to some extent specific to MDD and bipolar disorder, respectively.

Moving to the potential of the differentially expressed serum EV miRNAs as MDD biomarkers, the most up-regulated miRNA, miR-1207–5p, and the most down-regulated miRNA, miR-6735–3p, had significant diagnostic power according to ROC analysis. We then identified a panel of up-regulated and a panel of down-regulated miRNAs that each had higher ROC diagnostic power than that of the respective most diagnostic individual miRNAs. It is noteworthy that the diagnostic power of miRNAs was not explicitly related to their fold change or significance level of differential expression.

Next, we investigated the relationship between dysregulated EV miRNAs and their predicted mRNA targets. We assumed that up-regulated and down-regulated miRNAs would play different regulatory roles, and therefore performed two separate enrichment analyses. With regards to the up-regulated miRNAs, for 16 out of 17 miRNAs we found significant enrichment for at least one pathway, and these belonged to the categories *Nervous system*, *Signal transduction*, *Substance dependence*, *Endocrine system*, *Cardiocirculatory system*, *Aging*, and *Circadian clock*. All 16 miRNAs had putative targets enriched for one or more of the *Nervous system* pathways, dopaminergic synapse, glutamatergic synapse, long-term potentiation, and axon guidance. Each of these miRNAs had putative targets enriched for axon guidance pathways, and evidence exists for involvement of axon guidance genes in the adolescent onset of psychiatric disorders, including MDD (Vosberg et al., 2020). With respect to enrichment for dopaminergic synapse and glutamatergic synapse, this was observed for the predicted targets of 15 and 13 up-regulated miRNAs, respectively. The dopaminergic synapse-pathway mRNAs included genes for G-protein subunits (GNAQ, GNG2, GNAL, GNAI2). Among the glutamatergic synapse-pathway mRNAs were several glutamate receptor genes (GRIN2A, GRM4, GRIA4, GRIK3) and calcium/calmodulin-related genes (CAMK2A, CAMK1D, CAMK2D, and ADCY1). Of course, both the dopaminergic and glutamatergic neurotransmitter systems are implicated in MDD pathophysiology. Enriched *Signal transduction* pathways included cGMP-PKG signalling, AMPK signalling, ErbB signalling, and Hippo signalling. Given the enrichment for pathways related to the glutamatergic synapse, long-term potentiation and cGMP-PKG signalling, effects of up-regulated EV miRNAs on synaptic plasticity can be hypothesized. That disturbed synaptic plasticity is proposed as a major pathophysiology in MDD and reversal thereof as a major mode of antidepressant action, underlines the relevance of these findings (Pittenger and Duman, 2008; Reierson et al., 2011). Whilst reliable protocols for the specific isolation of CNS-derived EVs are still being developed (Picciolini et al., 2018), the significant enrichment of mRNA targets for *Nervous system* pathways certainly suggests that peripheral EV miRNAs can contribute to the regulation of CNS function. Moreover, the up-regulated miRNA miR-219b-5p is reported to have a relatively high tissue-specificity index for brain and spinal cord (https://mirgenedb.org/), supporting the presence of CNS-derived EVs in the peripheral circulation. The miR-219 family has been reported to target the calcium/calmodulin-dependent protein kinase II γ subunit (CaMKIIγ) and to thereby contribute to behavioural dysfunctions associated with reduced N-methyl-D-aspartate (NMDA) receptor function (Kocerh et al., 2009; Santa-Maria et al., 2015). However, functional assays are needed to provide empirical evidence for the involvement of the dysregulated EV miRNAs in the predicted target-mRNA pathways, and of the latter in MDD pathophysiology.

With respect to down-regulated miRNAs, there were fewer predicted target mRNAs compared to up-regulated miRNAs and only three significantly enriched pathways, the *Signal transduction* pathways MAPK signalling and Ras signalling pathway, and the *Cellular community* pathway focal adhesion. The up- and down-regulated EV miRNAs had no enriched pathway in common, and only *IGF1* and *BCL2* were targeted by both an up-regulated and a down-regulated miRNA. This clear-cut separation supports our initial hypothesis of different mRNA-pathway regulatory roles for EV miRNAs in MDD depending on the direction of change in expression.

Given that inflammation increases EV release from immune cells and their trafficking across the blood-brain barrier (Morad et al., 2019; Ridder et al., 2014), and also impacts on the EV miRNA cargo (Dalvi et al., 2017; Li et al., 2018), the absence of enrichment for strictly immune-inflammatory pathways is noteworthy. This might indicate that the chronic low-level, inflammation that occurs in MDD (Hodes et al., 2015) is without major impact on immune-cell EV release. It might also indicate that EV miRNAs are downstream targets, rather than regulators, of the immune pathways, and that any inflammation-driven changes in EV miRNA expression in MDD affect miRNA-mediated regulation of non-immune gene-pathways, including CNS-specific pathways.

In summary, the present study adds significantly to the evidence for changes in the status of the serum EV miRNome in MDD, demonstrating biomarker potential for specific EV miRNAs and identifying pathways relevant to MDD pathophysiology to which they may contribute. The study exemplifies the importance of investigating the EV miRNome to gain increased understanding of MDD aetio-pathophysiology and its treatment. The small overlap with respect to the identity of dysregulated miRNAs reported by the only previous study using similar methods and sample size (Wei et al., 2020) highlights the need for large cohort studies to build on the present study, in order to confirm the presence or absence of consistent EV miRNome dysregulation and biomarker potential in MDD.

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CRediT authorship contribution statement

Nagiua Cuomo-Haymour: Methodology, Investigation, Formal Analysis, Writing – original draft. **Stefan Kaiser:** Resources, Writing – review & editing. **Matthias Hartmann-Riemer:** Resources. Resources. **Karoline Guetter:** Resources. **Federica Klaus:** Resources. **Flurin Cathomas:Erich Seifritz:** Supervision, Resources. **Giorgio Bergamini:** Conceptualization, Methodology, Writing – review & editing. **Giancarlo Russo:** Software, Formal analysis. **Christopher R. Pryce:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bionps.2022.100049.

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