Journal of Affective Disorders Reports 1 (2020) 100003

Contents lists available at ScienceDirect

Journal of Affective Disorders Reports

journal homepage: www.elsevier.com/locate/jadr

Research Paper

# Search of brain-enriched proteins in salivary extracellular vesicles for their use as mental disease biomarkers: A pilot study of the neuronal glycoprotein M6a

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ARTICLE INFO

*Keywords:*
Biomarker candidates
Mood disorders
Extracellular vesicle proteome
Patients
Antidepressant treatment
Depression and anxiety

ABSTRACT

*Background:* Mental disorders affect millions of people worldwide. Their etiology is complex and the fact that the main effects occur in the brain hampers biochemical diagnosis. Therefore, biomarker finding in peripheral fluids such as serum or saliva is desirable. Here, we searched for biomarkers in salivary extracellular vesicles (EVs). Then, we focused on the protein M6a, related to neuronal connectivity and associated with several mood disorders to study its usefulness in saliva for the diagnosis of depression and anxiety.

*Methods:* Biomarker candidates were searched by proteomic analysis of human salivary EVs. M6a presence in salivary EVs was validated by transmission electron microscopy and Western blot. M6a levels were measured by ELISA in saliva samples of 88 individuals classified as control, depressed or anxious.

*Results:* We identified ten protein candidates in salivary EVs: OLIG2, PMP2, CNP, CAMK2A, SLC25A22, MLLT11, HTR2A, MAPT, ATP2B2 and M6a, all associated with emotional disorders. Salivary M6a levels positively correlated with the scores for the perceived stress scale in individuals diagnosed with depression. Furthermore, saliva samples of depressed patients treated with serotonin reuptake inhibitors (SSRI) or benzodiazepines differed in their M6a levels with respect to untreated patients.

*Limitations:* The main limitation of this study lies in the low number of patients involved, which warrants replication.

*Conclusions:* Salivary EVs contain promising biomarker candidates for mental disorders. Further studies will help validate them for their potential use in diagnosis. Our results lead us to propose M6a as a workable molecule to take into account as a possible stress biomarker.

## 1. Introduction

Mental disorders affect hundreds of people around the world and have a profound impact on productivity, motivation and outcome of several chronic diseases. Although there are many subtypes and more than one etiology, it is generally accepted that chronic stress is a key factor in their onset (McEwen, 2008; Schneiderman et al., 2005). Mood disorders can affect adults, adolescents and children and have a high rate of relapse and recurrence. Several studies show that prompt treatment and follow-up result in better outcomes, making early detection of afflicted patients critical (Duval et al., 2006). At present, the diagnosis and treatment of mental disorders is still based on the subjective clinical evaluation of symptoms and not on a biochemical assessment. There are virtually no commercial kits available for mood-disorder diagnosis. Although many studies have analyzed different biological markers (biomarkers), none was conclusive regarding how biologic information can be used to enhance diagnosis, treatment and prognosis (Dhama et al., 2019; Rodriguez Cerdeira et al., 2017; Strawbridge et al., 2017). A stress biomarker would indicate that an individual is not in physiological comfort and that different mechanisms are activated to restore homeostasis (Dhama et al., 2019; McEwen et al., 2015). Beyond HPA axis activation markers, such as cortisol, immune system molecules and autonomic nervous system hormones and neurotransmitters have been used as biomarkers (Dhama et al., 2019; Nater et al., 2013). The main disadvantage of such molecules is the need for invasive blood sampling.

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https://doi.org/10.1016/j.jadr.2020.100003
Received 13 July 2020; Received in revised form 2 September 2020; Accepted 16 September 2020
Available online 20 September 2020

Therefore biomarkers from easy accessible fluids are gaining popularity. Saliva sampling methods, as compared to blood sampling, are less invasive, simpler, safer, less stressful and do not require any particular training or equipment (Han et al., 2018; Ivkovic et al., 2015; Katsani and Sakellari, 2019). Most compounds that appear in blood can also be identified in saliva, although at different concentrations (Loo et al., 2010). In addition, almost 40% of the proteins that have been suggested to be candidate markers for diseases can be found in saliva (Katsani and Sakellari, 2019). However, biomarker detection in saliva is hindered by the presence of proteins such as alpha-amylase that may mask other proteins with low expression (Deutsch et al., 2008; Han et al., 2018). Extracellular vesicles (EVs) could overcome this disadvantage acting as reservoirs of molecules and allowing their detection, which would otherwise be overlooked due to their dilution in the whole fluid. EVs are lipid bilayer-delimited particles that carry proteins, nucleic acids and lipids from their original cell in both physiological and pathological conditions. Thus, EVs participate in intercellular communication (Boukouris and Mathivanan, 2015).

Brain EVs can reach the oral cavity and can be detected in saliva (Cheng et al., 2019). EVs have been used in the diagnosis of diseases such as Alzheimer's and Parkinson's, prion disease, traumatic brain injury and glioma (Cheng et al., 2019; Han et al., 2018; Saman et al., 2012; Stern et al., 2016), making salivary EVs an attractive source to look for biomarkers related to brain disorders.

Psychiatric disorders appear to be the result of the disruption of homeostatic mechanisms that maintain synaptic plasticity (Duman and Aghajanian, 2012) which implies changes in genes related to such pathways, including the gene for the neuronal glycoprotein M6a. M6a participates in neuronal differentiation, filopodium/spine formation and synaptogenesis and is also a stress-regulated protein (Alfonso et al., 2006, 2005, 2004; Brocco et al., 2010; Formoso et al., 2015). Moreover, the changes observed in M6a levels in the hippocampus of stressed animals were similar to those observed in the serum of such animals (Monteleone et al., 2017). In humans, M6a mRNA is downregulated in the hippocampus of depressed suicides (Fuchsova et al., 2015) and a duplication of M6a gene was associated with mental retardation (Gregor et al., 2014). Polymorphisms in M6a gene have been associated with schizophrenia (Boks et al., 2008), claustrophobia (El-Kordi et al., 2013) and bipolar disorder (Greenwood et al., 2012; Khalid and Sezerman, 2020). Remarkably, M6a has been identified by us and others in EVs (Gonzales et al., 2010; Lazar et al., 2015; Monteleone et al., 2017).

In this work, we aimed to find new biomarkers for mood disorders by proteomic analysis of extracellular vesicles isolated from human saliva. Then, in a pilot study, we evaluated the usefulness of one of the candidates, the glycoprotein M6a, as biomarker for depression and anxiety and treatment efficacy.

## 2. Methods and materials

### 2.1. Extracellular vesicle isolation

Whole saliva (15 ml) was collected by drooling in a sterile Falcon. EVs were isolated as previously described (Théry et al., 2006) with slight modifications. Collected saliva was diluted by half in PBS and centrifuged at 2,600 × *g* for 30 min at 4 °C to pellet cells, debris and bacteria. Supernatant was transferred to clean tubes and ultracentrifuged at 120,000 × *g* for 120 min at 4 °C. Pellets containing extracellular vesicles were resuspended in sterile PBS. Characterization of EVs was done by transmission electron microscopy (TEM) and Western blot (see below).

### 2.2. Proteomics

LC-MS/MS analysis of total salivary EVs were done at ITSI service (ITSIBiosciences, Johstown, PA). The received protein list was ordered according to the NSAF, which allows the comparison of abundance of individual proteins in multiple independent samples.

### 2.3. Bioinformatics tools

Uniprot, Human Protein Atlas and Genecard databases were used to complete protein name or tissue and expression sites. The enrichment in EV proteins was checked by comparison with ExoCarta Top100 list (http://www.exocarta.org, (Mathivanan and Simpson, 2009)). Enrichment of Gene Ontology (GO) terms (Gene Ontology Consortium, 2015) was carried out with Panther Classification system http://www.pantherdb.org/ (Thomas et al., 2003).The brain-specific proteome consisting of the 419 brain-enriched genes was downloaded from the Human Protein Atlas Database (Uhlen et al., 2017) (Human Protein Atlas available from www.proteinatlas.org). Venn diagrams were created with Venny (Oliveros, n.d.). The disease related pathways were analyzed with the ToppGene Suite portal (Chen et al., 2009).

### 2.4. Transmission electron microscopy (TEM)

TEM was performed at the LANAIS-MiE-IBCN (SNM-CONICET) facility (Buenos Aires, Argentina). Sample preparation was done according to Théry 2006 (Théry et al., 2006). EVs were fixed with 4% EM-grade PFA in TEM-buffer (50 mM HEPES, pH 6.5) and absorbed in GO coated copper grids (200 mesh). Samples were post-fixed in 1% EM-grade glutaraldehyde in TEM-buffer and rinsed with deionized water, then stained with 2% Uranyl acetate. Micrographs were acquired in a Carl Zeiss EM 109T transmission electron microscope operating at 80 kV and equipped with a Gatan ES1000W (11 Mpixel) digital camera.

For immunogold stain, M6a antibody (MBL International, Woburn, MA) was used and complexes were detected using 18 nm gold conjugated anti-rat IgG antibody (Jackson ImmunoResearch, WestGrove, PA, USA).

### 2.5. Western blot and antibodies

Primary antibodies used were: anti-M6a: polyclonal rabbit antibody against C-terminus of M6a developed in our laboratory (1/500); anti-flotillin-1 monoclonal mouse antibody (1/1000, BD Bioscience, #610820) and anti-CD9, clone B2C11 (1/250, DSHB Hybridoma Product B2C11, deposited by P.H. Patterson). Detection was done with an Odyssey infrared imaging system (LI-COR Biotechnology, Nebraska, US). Anti-rabbit IgG or anti-mouse IgG IRdye 680 LI-COR P/N 925-68021 and P/N 925-68020 were used.

Blot images were digitally processed with Image J software (http://rsb.info.nih.gov/ij/) to show each band more clearly. In all cases, processing was applied equally across the entire image. Original blot images without digital processing are shown in Supplementary Fig 1.

### 2.6. Participants and collection of saliva

The work described below has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

#### 2.6.1. Ethics approval and consent to participate

Procedures involving human participants were evaluated and approved by the Research Committee and the Bioethics Committee of the San Juan de Dios Hospital House (Protocol CEI 004/2017, approved on April 14th 2017). Participants were informed about the purpose of the investigation and were asked to sign a consent form.

**Table 1**
*Overview summary of participants.* Median (Q1-Q3) is reported in each case.

| | Female | Male |
|---|---|---|
| ***N*** | 62 | 26 |
| **Age** median (IQR) | 59 (39–67) | 62 (37–68) |
| **Individuals under pharmacological treatment (n)** | 31 | 8 |
| **Years of treatment** median (IQR) | 2.3 (0.2–5.3) | 2.6 (0.6–4.8 ) |
| **Depressed (n)** | 24 | 7 |
| **Anxious (n)** | 26 | 13 |
| **Control (n)** | 12 | 6 |

Other than saliva collection, there were no study-related consequence interventions.

*2.6.1.1. Healthy subjects.* Twenty-five volunteers self-identified as healthy (14 women, 11 men) between 25–75 years old collected saliva using a commercially available device (Salivette®; Sarstedt, Rommelsdorf, Germany). They were requested not to eat or collect the sample immediately after brushing teeth or after any activity that may cause gums to bleed. Salivette® devices were centrifuged at 1,000 × *g* for 2 min upon reception according to manufacturer's instructions. To prevent degradation of critical biological molecules (e.g., RNA and proteins) due to freeze and thaw cycles, the clear effluent was divided in 50 $\mu$l working aliquots and stored until use at −20°C in a freezer designated for human samples storage only. Saliva samples were used for M6a determination according to ELISA kit #MBS9312190 manufacturer instructions. (MyBioSource, San Diego, CA, US). Cortisol levels in these samples were determined by ECL (División de Endocrinología – CEDIE, Hospital de Niños "Dr. Ricardo Gutiérrez", CABA, Argentina).

*2.6.1.2. Patients and control individuals.* Eighty-eight individuals were recruited (62 women, 26 men). Further details of the cohort are shown in Table 1. After a directed anamnesis and clinical examination and based on the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V) (American Psychiatric Association, 2013) subjects were classified in 5 groups: depressed (if they had Major Depressive Disorder, MDD), anxious (if they have Generalized Anxiety Disorder, GAD), depressed or anxious under medical treatment or controls. Two physicians from the San Juan de Dios Hospital did the assessments. Depressed individuals were mild or moderate in severity. Depressive and anxious patients under medical treatment were later classified according to the drug they received: Selective Serotonin Reuptake Inhibitors (SSRI: Fluoxetine, Escitalopram, Paroxetine) or Benzodiazepines (Alprazolam, Clonazepam). Individuals with cognitive disorders, treated with glucocorticoids, with recent surgery proceedings and pregnant or lactating women were excluded.

The following psychometric test were administered to all individuals: PSS, Perceived Stress Scale which is a validated measure to assess the level of subjective life stresses (Cohen et al., 1983); the Beck Depression Inventory (BDI) which is a widely used questionnaire assessing anxiety severity and hospital anxiety and depression scales (HADS-A and HADS-D), which focus on the evaluation of the cognitive aspects of anxiety and depression (Bjelland et al., 2002).

### 2.7. Statistical analysis

Statistical analysis and graphs were carried out with GraphPad Prism Version 6.01. All data were subjected to normality and equal variance testing using IS version 2010 (Infostat software, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Statistical significance was determined by Mann-Whitney *U* test. Non-parametric one-way analysis of variance (Kruskal-Wallis) test followed by Dunn's multiple comparison tests was used to compare more than 2 groups. Correlations were assessed using Spearman's correlation coefficient ($r_s$). For all tests, a $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Salivary EVs contain several brain proteins related to psychiatric disorders

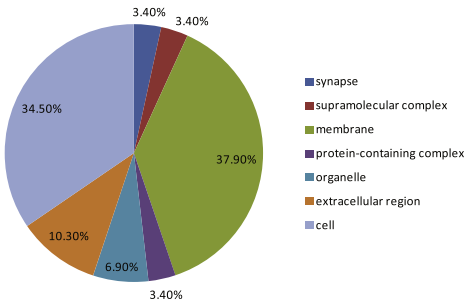
For candidate biomarker discovery in saliva, we used an EV-based strategy. Extracellular vesicles (EVs) from a pool of saliva samples from four healthy donors (women and men) were isolated and subjected to proteomic analysis. Among the identified proteins (4380), we found, 44 of the considered TOP100 EV markers (proteins often identified in EVs) from the Exocarta database (Supplementary Fig 2). This result further confirms that the sample analyzed contained EVs.

Then, to identify the most frequently detected proteins, we set a cut-off in a Normalized Spectral Abundance Factor (NSAF) of $10^{-6}$ and obtained a list of 498 proteins. The cellular components, biological processes and molecular functions of these proteins were analyzed with the PANTHER classification system (Thomas et al., 2003). Most of them fell in the category "cell" (34.5%, Fig. 1A). The rest was classified as extracellular (10.3%), membrane (37.9%), organelle (6.9%), synapse (3.4%) or involved in protein complexes (3.4%, Fig. 1A). EV proteins were involved in 11 types of biological processes (Fig. 1B), mainly biological regulation and signaling (27.3 and 12.7% respectively). In terms of molecular functions, the proteins were mainly associated to binding and catalytic activity categories (32.6 and 26.1% respectively; Fig. 1C). Besides their diversified biological processes and molecular functions, these proteins have been involved in more than ten different pathways. Several proteins were in the glutamate receptor pathway, inflammation mediated by chemokine and cytokine signaling pathway and serotonin receptor pathway (Fig. 1D).

To investigate if there were brain proteins among salivary EV proteins, our 498-protein list from the EV proteome was compared with the list of brain-enriched proteins (mRNA levels 5-fold or higher in the brain than other tissues) from the Human Protein Atlas database. Ten proteins were identified: 5-Hydroxytryptamine Receptor 2A (HTR2A), Calcium/Calmodulin Dependent Protein Kinase II Alpha (CAMK2A), Plasma Membrane Calcium-Transporting ATPase 2 (ATP2B2), Oligodendrocyte Transcription Factor (OLIG2), Peripheral Myelin Protein 2 (PMP2), 2′,3′-Cyclic Nucleotide 3′ Phosphodiesterase (CNP), Glutamate Solute Carrier family 25 member 22 (SLC25A22), Microtubule-Associated Protein Tau (MAPT), Myeloid/Lymphoid Leukemia Translocated To 11 (MLLT11) and Glycoprotein M6A (GPM6A) (Fig. 1E-F, Supplementary Fig 3). ToppGene analysis indicated an association of candidates with psychotic disorders (Fig. 1G). The aforementioned biological and molecular functions coupled to the ToppGene prioritization suggest that salivary EVs carry brain proteins related to neuropsychiatric diseases.

As mentioned, the neuronal glycoprotein M6a was found among them. Since our group has extensively studied the role of M6a in neuronal architecture and in several animal models to induce depression-like symptoms, we centered our study on this glycoprotein in the next experiments.

A

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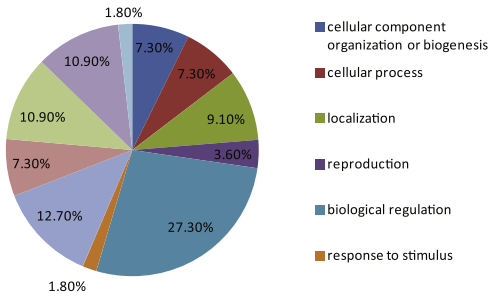


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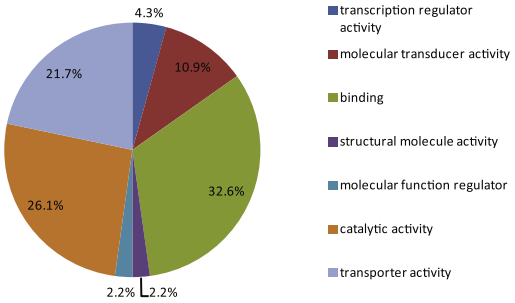


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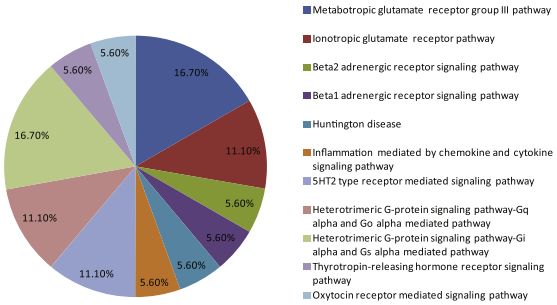


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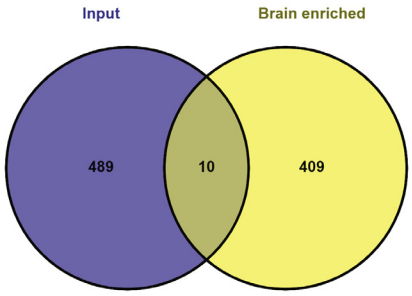


F

| Brain enriched | |
|---|---|
| **GPM6A** | HTR2A |
| CAMK2A | ATP2B2 |
| OLIG2 | PMP2 |
| CNP | SLC25A22 |
| MAPT | MLLT11 |

G

| Disease | pValue | Bonferroni |
|---|---|---|
| Muscle Rigidity | 6.50E-06 | 2.94E-03 |
| Psychotic Disorders | 1.54E-05 | 6.99E-03 |
| Abnormal behavior | 3.20E-05 | 1.45E-02 |
| Delusions | 5.77E-05 | 2.62E-02 |
| Mild cognitive disorder | 8.60E-05 | 3.89E-02 |
| Unipolar Depression | 8.87E-05 | 4.02E-02 |

**Fig. 1.** *Saliva contains brain-enriched proteins with potential use as mood-disorder biomarkers.* PANTHER generated pie charts (A) Cellular component. (B) Biological process. (C) Molecular process. (D) Pathways. (E) Venn diagram showing overlap between the Human Protein Atlas 419 brain-enriched proteins and the salivary EV proteins with NSAF > $10^{-6}$. (F) List of the ten identified candidates. (G) ToppGene Suite analysis of the ten candidates shows that they are related to several cognitive disorders.

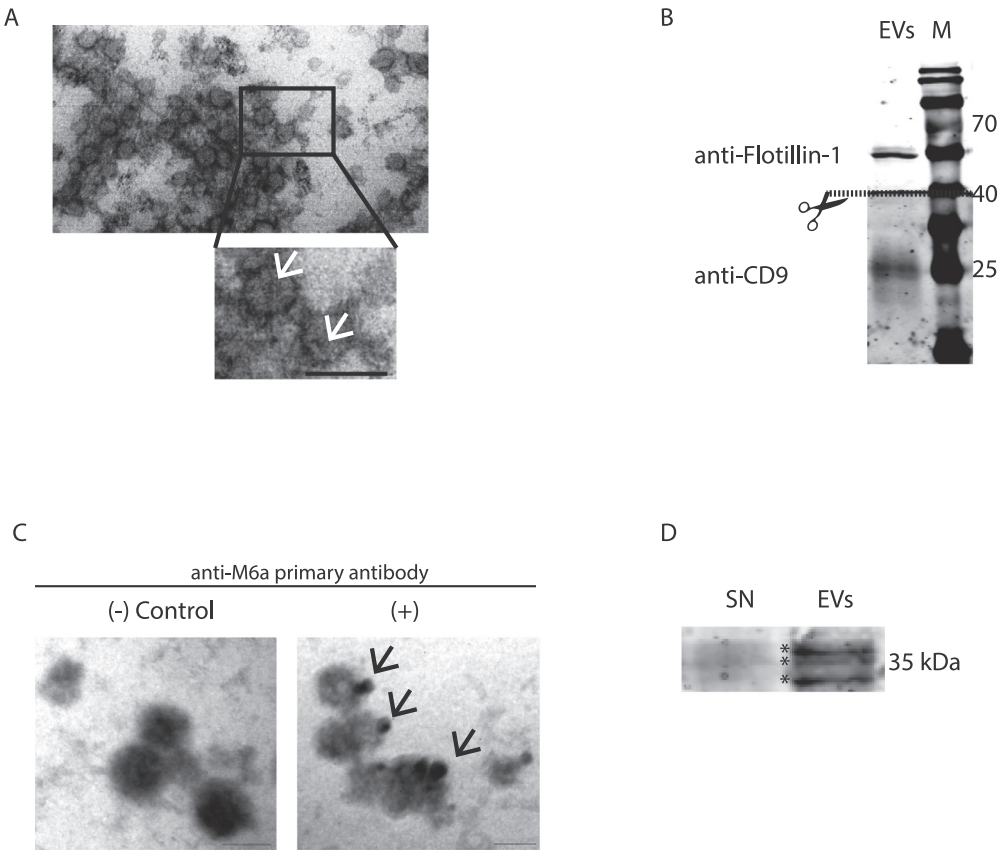


**Fig. 2.** *M6a can be detected in salivary EVs.* (A) TEM representative images of EVs isolated from saliva. In the magnification, round shaped vesicles with the classical cup shaped morphology (indicated with arrows) can be observed. This indicates that intact vesicles were isolated rather than cell debris or bacteria. Scale bar 100 nm. (B) Western blot of extracellular vesicles showing classical markers Flotillin-1 and CD9. Membrane was cut at ~40 kDa. Upper membrane was reacted with anti-Flotillin-1 antibody and lower membrane with anti-CD9 antibody. M: molecular weight marker. (C) Immunogold labeling of M6a in the isolated EVs. Arrows show the immunoreactive complexes. These are not seen when primary antibody is omitted. Scale bar 100 nm. (D) Western blot of M6a in EVs. EVs, as well as the supernatant from the final step (SN) were loaded on a 12% SDS gel. Same amount of protein was loaded in each lane. As expected, no signal was detected in the supernatant (SN) of the isolation protocol. M6a signal was easily detected in EVs. Multiple M6a bands are indicated by stars.

### *3.2. M6a can be detected in salivary EVs (Validation of proteomics)*

Extracellular vesicles from saliva were obtained by differential centrifugation. Transmission electron microscopy (TEM) was used to evaluate EVs morphology. Under TEM, a round morphology was observed, several displaying a cup-shaped appearance characteristic of EVs (Fig. 2A). To further characterize EVs, we analyzed the presence of representative membrane markers, such as CD9 (~25 kDa) and Flotilin-1 (~48 kDa). These markers were identified by proteomics and therefore were validated by Western blot. Fig. 2B shows the presence of both markers in EVs.

Next, we assessed the presence of M6a in salivary EVs by immunogold labeling. Isolated EVs showed M6a immunoreactive complexes (arrows) not detected when the primary antibody was omitted (control, Fig. 2C). Western blot analysis showed that while no signal was detected in the supernatant of 120,000 × *g* ultracentrifugation (SN), reactivity against 35–40 kDa bands (the expected sizes for M6a) was easily observed in EVs (stars in Fig. 2D). Multiple M6a bands are normally observed due to posttranslational modifications. Altogether these results confirm M6a presence in salivary EVs.

### *3.3. M6a can be detected in whole human saliva*

Since a simple method of diagnosis ideally requires minimum sample processing, we next evaluated if M6a could be detected in whole saliva. M6a levels were determined in saliva samples donated by 25 healthy volunteers (11 men, 14 women between 25–75 years old). Donors were not under any antidepressant treatment and showed cortisol levels within the normal range according to the test applied (morning range 3.3–34.8 nmol/l; night range 1.7–7.7 nmol/l) (Supplementary Fig 4). M6a levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Fig. 3A). M6a levels were 25.4 ng/ml (10.6–53.0) for men and 15.3 ng/ml (3.9–57.8) for women.

**Table 2**
*Scores obtained for each test in control, depressed and anxious patients.* Median (Q1-Q3) is indicated for each case. Non-parametric ANOVA followed by Dunn's multiple comparison tests were used. Asterisks indicate statistical differences compared to control values. HADS D $p = 0.002$; HADS A $p = 0.0094$; PSS $p = 0.0022$ (Dunn's multiple comparison test C vs A; C vs D); BECK $p < 0.0001$ (Dunn's multiple comparison test C vs A; C vs D).

| | Control | Depressed | Anxious |
|---|---|---|---|
| **HADS-D** | 2.0 (1.0–4.5) | 7.0 (5.0–12.0)** | 4.5 (2.3–9.0) |
| **HADS-A** | 6.5 (4.0–8.3) | 7 (5.0–9.0) | 10.5 (6.3–12.8)* |
| **PSS** | 10.5 (7.8–16.3) | 23.0 (19.0–29.0)** | 19.5 (13.5–25.0)* |
| **BECK** | 5.0 (2.0–6.0) | 22.0 (10.0–23.0)*** | 12.0 (8.3–17.5)** |

M6a mean levels did not differ between men and women. Therefore, in further analysis men and women were studied together. Healthy candidate M6a levels ranged between 0–110 ng/ml.

### *3.4. M6a levels positively correlates with stress perception in depressed patients*

Next, we analyzed M6a in saliva samples of patients. Participants were diagnosed by clinicians as depressed, anxious or no depressed nor anxious (control group). Participants completed the Hospital Anxiety and Depression Scales (HADS-A and HADS-D, respectively), the Beck Depression Inventory (BDI-II) and the Perceived Stress Scale (PSS). Overall, scale scores (Table 2) agreed with clinician diagnoses based on clinical examination.

We found no differences in M6a levels in depressed patients or anxious patients compared with control subjects (non-parametric ANOVA $p = 0.3516$) (Fig. 3B)

To find out if any of the scale scores correlated with salivary M6a levels, we carried out Spearman correlation analysis. For depressed pa-

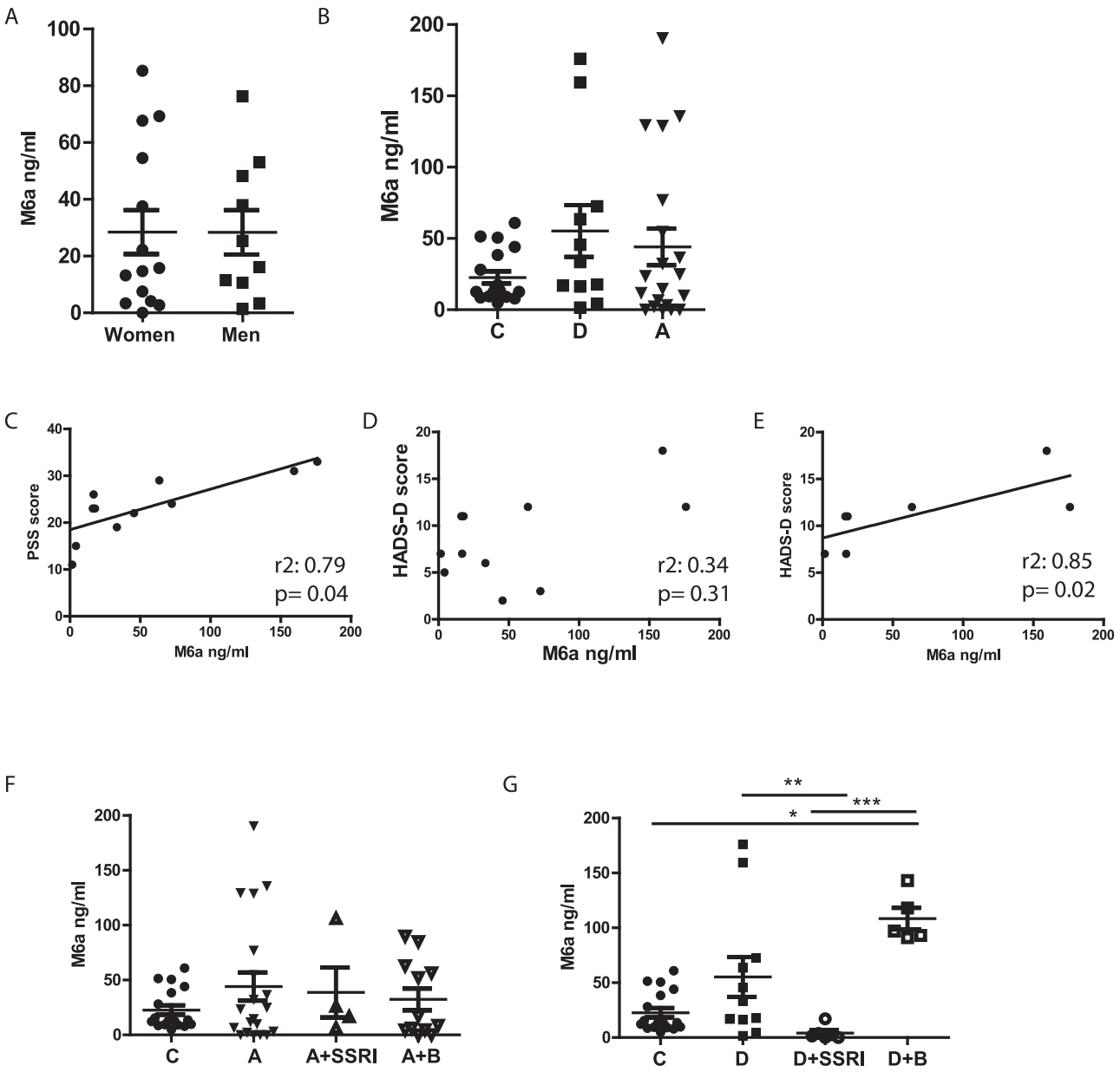


**Fig. 3.** *M6a can be detected in human saliva and correlates with PSS score in patients diagnosed with depression, with HADS-D in patients with score > 7 and is modulated by classic antidepressants such as SSRI and benzodiazepines.* **(**A) ELISA determination M6a levels in saliva in women and men. No differences were detected; therefore men and women were studied together. (B) M6a levels do not differ between control subjects ($n = 18$) and patients diagnosed with anxiety ($n = 20$) or depression ($n = 11$) (Non-parametric ANOVA $p = 0.3516$). (C) M6a positively correlates with PSS score in patients diagnosed with depression (Spearman test, $r^2$=0.79 $p = 0.037$). D) M6a levels do not correlate with HADS-D score in the depressed group (Spearman test $r^2$=0.338, $p = 0.308$). However in borderline or caseness patients (HADS-*D* > 7) a strong positive correlation was detected (Spearman test $r^2 = 0.844$, $p = 0.0168$) (E). (F) No differences were detected for M6a levels in anxious patients under SSRI or B treatment (Non-parametric ANOVA $p = 0.9552$). (G) M6a levels were significantly altered by SSRI and B although with an opposite pattern (Non-parametric ANOVA $p = 0.0002$. Dunn´s multiple comparison test * C vs D+B; *D vs D+SSRI; ***D+SSRI vs D+B). These results show that salivary M6a levels might be modulated by SSRI and benzodiazepines in depressed patients.

tients, we found a positive correlation between M6a levels and the PSS score (Fig. 3C Spearman test, $r^2$ =0.7927, $p = 0.037$). Given the link between chronic stress and depression (Bartolomucci and Leopardi, 2009; Tafet and Bernardini, 2003; Yang et al., 2015), we next analyzed the correlation between the M6a levels found in depressed patients and the HADS-D score. According to HADS-D score, people are classified as normal (0–7), borderline (8–10) or caseness (12–21). Although we did not find correlation between HADS-D score in depressed patients and M6a levels (Fig. 3D, Spearman correlation test $r^2$: 0.3387, $p = 0.3083$), we did find a correlation when patients were classified by the score. HADS-D scores higher than 7 (i.e. borderline and caseness patients) correlated with M6a levels (Fig. 3E, Spearman test $r^2$: 0.8444, $p = 0.0168$).

### 3.5. M6a might be useful to evaluate antidepressant efficacy

We next analyzed the effect of pharmacological treatment on M6a levels. Patients (anxious or depressed) treated with selective serotonin reuptake inhibitors (SSRI) or benzodiazepines (B) were included in the analysis. No differences in M6a levels were observed for patients diagnosed with anxiety under treatment with SSRI or benzodiazepines (Fig. 3F, non-parametric ANOVA $p = 0.9552$).

On the other hand, among depressed patients, we found that M6a levels were significantly lower in those under SSRI treatment in comparison to those without treatment. No differences in M6a levels were observed between SSRI-treated patients and control subjects. Although, it seems to be a difference in M6a levels between untreated depressed patients or treated with B, there is no statistical significance. In addition, M6a levels in depressed patients treated with benzodiazepines were significantly higher than in control subjects (Fig. 3G, non-parametric ANOVA $p = 0.0002$. Dunn´s multiple comparison test * C vs D+B; *D vs D+SSRI; ***D+SSRI vs D+B). These results suggest that M6a levels would be susceptible to change upon pharmacological treatment.

## 4. Discussion

In this work, by proteomic analysis of salivary extracellular vesicles, we have identified ten protein candidates associated with neuropsychiatric diseases that could serve as biomarkers for such diseases. In addition, we have demonstrated that one of those candidates, the protein M6a, can be quantified in whole saliva. Using patient saliva samples, we have shown that M6a levels positively correlated with stress perception in depressed patients. Moreover, differences in M6a levels among antidepressant-treated patients suggest a potential M6a use to evaluate antidepressant treatment efficacy. These auspicious findings highlight the usefulness of analyzing EVs for new candidate biomarkers discovery for mental disorders.

EVs are secreted by virtually all cells in the organism and are a heterogeneous population of vesicles that transport and deliver proteins and nucleic acids to recipient cells. EVs with different content are released into the circulation by cells from healthy and sick subjects. The molecules carried by EVs released by pathological cells can be measured as disease biomarkers (Barile and Vassalli, 2017). EVs can also cross the blood-brain-barrier, thus are an attractive source of biomarkers for brain related disorders (Alvarez-Erviti et al., 2011). Such biomarkers would help clinicians complementing patient evaluation with biochemical parameters. It is well known that although clinicians follow Diagnostic and Statistical Manual of Mental Disorders (DSM) guidelines and use scales, multiple factors such as patient genetic predisposition, individual features, self-medication and even a trend to conceal symptoms hamper diagnosis. This is why easily accessible biomarkers are needed.

EVs can be isolated from different body fluids, including saliva. Saliva sampling is not invasive and its collection is easiest and painless (e.g. in comparison to blood). The salivary glands are integrated into the neuroendocrine system and contain a wide array of biomarkers related to the pathophysiology of several diseases (Jasim et al., 2018). In this work, we focused on those molecules found in salivary EVs related to neuronal connectivity, whose alteration is a hallmark of mental disorders.

We found ten brain-enriched proteins. Among them we found HTR2A, one of the receptors for serotonin, microtubule associated protein Tau (MAPT), SLC15A22 a mitochondrial glutamate carrier, several proteins involved in myelination (OLIG2, PMP2 and CNP), proteins related to pathways involving $Ca^{2+}$ signaling (ATP2B2, CAMK2A) and the myeloid/lymphoid or mixed-lineage leukemia; translocated to 11 (MLLT11). In addition to their specific role (Supplementary Fig 3), candidates are associated with psychotic and depressive disorders. This suggests that molecules underlying the pathogenesis of stress-related disorders could be evaluated in saliva from patients.

Since M6a participates in neuronal connectivity, has been related to depression and has been widely studied by our group, we focused on this molecule. First, we confirmed that likewise to serum M6a, salivary M6a is also coupled to EVs. Then, we showed that M6a could be measured in whole saliva, without the need of EV purification. In a pilot study, we assessed M6a levels in patients diagnosed with depression or anxiety and also in control subjects. Although we did not find statistical differences, some relation between M6a and depression was suggested.

Clinical diagnosis of mood disorders is often supported by tests such as PSS and HADS-D. Here, we have found that M6a levels positively correlated with the perceived stress scale exclusively in depressed patients. This reinforces the idea of M6a as a stress-responsive protein whose levels changes when the individual experiences stress. Studies have shown that exposure to stressors increases susceptibility to psychological disorders (Alkadhi, 2013; Meyer et al., 2013). Moreover, repeated or recurrent stress is known to quicken or worsen the mood disorders (Khan, 2016; McEwen, 2009). HPA axis deregulation, a common feature of chronic stress, has been found in patients with severe depressive and psychotic symptoms (Khan, 2016). In the case of depressed individuals, it has been reported that their depressive state can alter responses to stressors, amplifying the stress perception (Kumar et al., 2015). Thus, M6a levels could represent a measure of such exacerbated perception.

We found a positive correlation between M6a levels and the HADS-D scores. HADS D is the most commonly used test for depression screening. Notably, the correlation was only observed in the group of patients with higher scores for HADS-D. This suggests that M6a levels could complement the clinical classification as caseness of depression.

Evidences suggest that impairing serotonin function can cause clinical depression and can also compromise mechanisms involved in maintaining recovery from depression (Cowen and Browning, 2015). We have shown that, in SSRI-treated depressed patients, salivary M6a levels were lower than in untreated patients. SSRI increase synaptic plasticity and restore homeostasis attenuating depression symptoms. Interestingly, administration of tricyclic antidepressants (which inhibit the reuptake of the biogenic amines, mostly norepinephrine and serotonin) to chronically stressed animals reverts stress and M6a mRNA downregulation (Alfonso et al., 2004). Now, our results suggest that, in SSRI-treated depressed patients, M6a might accompany the molecular changes induced by SSRI. On the other hand, the high salivary M6a levels in patients treated with benzodiazepines highlight the differences in the mechanisms of action for SSRIs and benzodiazepines. Benzodiazepines act through $\gamma$-amino butyric acid (GABA) receptors A, facilitating the inhibitory action of GABA. Since reduced GABA levels in plasma and cerebrospinal fluid of depressed patients were reported (Luscher and Malenka, 2012), GABAergic deficits may also contribute to depressive disorders. It has been shown that M6a promotes differentiation of GABAergic neurons (Michibata et al., 2008). It remains unknown if M6a may also participate in the benzodiazepine pathway.

Salivary M6a levels are different between depressed patients and depressed patients under SSRI or benzodiazepine treatment. Therefore, with further studies, M6a levels might be used in clinical trials of patients under SSRI or benzodiazepines treatment. It has been reported that biomarkers could complement clinical assessment by highlighting changes in the levels of biomarkers that occur in parallel or ahead of changes in clinical symptoms, allowing physicians to make adjustments in therapy quickly (Carboni et al., 2019). For example, it has been shown that saliva levels of norepinephrine metabolite3-methoxy-4-hydroxyphenylglycol (sMHPG) are higher in individuals with good response to SSRI compared to non-responders, suggesting that sMHPG could be useful to stratify patients for antidepressant treatment (Egami et al., 2013). Although the results shown here for M6a have the constraint that patients with different treatment duration were included (which adds heterogeneity to the sample), they suggest that M6a could help in the discrimination between treatments administered.

This pilot study of M6a as a biomarker in patients diagnosed with mental disorders has as its main limitation that it is a retrospective study done in a small sample of patients. Thus, evaluation of M6a levels in a larger group as well as a longitudinal study is needed. Although in need of replication, the present results show that salivary EVs carry brain-enriched proteins that could be putative biomarkers of stress-related mental disorders. Of the identified proteins, we focused on M6a and showed that it could contribute to stress perception in depressed patients. In addition, we showed that salivary M6a levels are modulated

in opposite directions by SSRI and benzodiazepines, a result compatible to their different actions on neural plasticity. A possible role for salivary M6a in distinguishing between treatments is also suggested. We hope that this work raises interest in the field of EVs as sources for mood disorder biomarker. We believe it would open a new window for the diagnosis of neuropsychiatric diseases.

## 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.

## Funding

This study was supported by ANPCyT Grants #PICT 2016-01710 and PICT 2017-1736. MCM was awarded with an early career research grant from AJ Roemmers Foundation. The funding sources had no role in study design, acquisition, and interpretation of data or writing of the report.

## Author contributions

Conceived and designed the experiments: MCM, SCB, MAB, and ACF. Performed the experiments: MCM, SCB. Recruited, diagnosed and collected patients samples: LV, NC. Analyzed the data: MCM, SCB, MAB, and ACF. Wrote the paper: MCM, SCB, MAB, and ACF. SCB is a technician and MCM, MAB and ACF are researchers from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET). LV and NC are physicians

## Acknowledgments

The authors thank Dr. V Campo, Dr. MC Vila, Dr. C Arregui and Dr. C Buscaglia for critical reading of the manuscript.

## Limitations

This pilot study of M6a as a biomarker in patients diagnosed with mental disorders has as its main limitation that it is a retrospective study done in a small sample of patients. Thus, evaluation of M6a levels in a larger group as well as a longitudinal study is needed.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jadr.2020.100003.

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