Extracellular vesicles containing the transferrin receptor as nanocarriers of apotransferrin

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Abstract
Previous work by our group has shown the pro-differentiating effects of apotransferrin (aTf) on oligodendroglial cells in vivo and in vitro. Further studies showed the remyelinating effect of aTf in animal demyelination models such as hypoxia/ischemia, where the intranasal administration of human aTf provided brain neuroprotection and reduced white matter damage, neuronal loss, and astrogliosis in different brain regions. These data led us to search for a less invasive and controlled technique to deliver aTf to the CNS. To such end, we isolated extracellular vesicles (EVs) from human and mouse plasma and different neuron and glia conditioned media and characterized them based on their quality, quantity, identity, and structural integrity by western blot, dynamic light scattering, and scanning electron microscopy. All sources yielded highly pure vesicles whose size and structures were in keeping with previous literary evidence. Given that, remarkably, EVs from all sources analyzed contained transferrin receptor 1 (TfR1) in their composition, we employed two passive cargo-loading strategies which rendered successful EV loading with aTf, specifically through binding to TfR1. These results unveil EVs as potential nanovehicles of aTf to be delivered into the CNS parenchyma, and pave the way for further studies into their possible clinical application in the treatment of demyelinating diseases.

Keywords
dynamic light scattering, exosomes, extracellular vesicles, scanning electron microscopy, size-exclusion chromatography, transferrin, transferrin receptor 1

1 INTRODUCTION

Extracellular vesicles (EVs) are lipid bilayer-enclosed structures originated from the intracellular endocytic trafficking pathway or from the plasma membrane which are released into the extracellular space by a variety of cells (György et al., 2011; Harding, Heuser, & Stahl, 1983; Pan, Teng, Wu, Adam, & Johnstone, 1985). The lipid composition of EVs reveals enrichment in cholesterol, sphingomyelin, and ganglioside GM3 levels (Sktoland, Sandvig, & Llorente, 2017; Wubbolts et al., 2003). Furthermore, their membranes contain tetraspanin proteins, including CD63—a protein accumulating in multivesicular bodies—CD9/CD81—mainly at the plasma membrane, and endosome membrane proteins, flotillin and ALIX, which have been used as EV markers (Kowal et al., 2016; Park et al., 2010). EVs can be secreted by most cell types in vitro and are detectable in different biological fluids such as blood (Harding, Heuser, & Stahl, 1984; Sanz-Rubio et al., 2018), cerebrospinal fluid (CSF) (Soares

Abbreviations: aTf, apotransferrin; CNS, central nervous system; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; OL, oligodendrocyte; OPC, oligodendrocyte progenitor cell; ARID, research resource identifier; Tf receptor, TfR1; Tf-TfR, Texas red-labeled Tf.

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and is mostly expressed in the liver. In the CNS, aTf is produced by oligodendrocytes and is vital for normal brain development. Our group has previously demonstrated the pro-differentiating effects of aTf in vitro (Escobar Cabrera, Bongarzone, Soto, & Pasquini, 1994; Escobar Cabrera, Zakin, Soto, & Pasquini, 1997; Marta et al., 2000) as well as in vivo (Paez, Marta, Moreno, Soto, & Pasquini, 2002). We have also reported that a single intracranial injection of aTf stimulates remyelination after cuprizone-induced demyelination (Adamo et al., 2006) and prevents hypomyelination produced by iron deficiency (Rosato-Siri et al., 2010). Furthermore, using a hypoxic-ischemic model in rats, we have shown that aTf injected intracranially reduces white matter injury (Guardia Clauí, Paez, Campagnoni, Pasquini, & Pasquini, 2010), whereas human aTf administered intranasally provides neuroprotection to neonate animals (Guardia Clauí, Paez, Campagnoni, Pasquini, & Pasquini, 2012).

The use of EVs with a clearly defined active therapeutic cargo such as aTf entails the need of a surface marker to ensure targeting at recipient cells. However, EV isolation as nanovehicles and their targeting at specific cell types affected in the CNS remain a challenge. Although shown to stimulate remyelination in animal models, intracerebral administration of aTf might not be adequate for clinical treatments. For these reasons, intranasal administration might be regarded as a highly promising non-invasive route for the treatment of chronic CNS disease, with several studies underscoring the potential of loaded EVs (Haney et al., 2015; Long et al., 2017; Zhang et al., 2011). Moreover, aTf encapsulation in EVs reduces potential immunogenicity, prevents its degradation by proteases and allows it to cross the BBB without serious systemic side effects. Therefore, the development of a minimally invasive technique to intranasally administer aTf-loaded EVs targeted at oligodendrocytes might prove to have clinical significance.

In this context, our work has focused on the isolation of EVs from plasma and different conditioned media, with special emphasis on those of human plasma. EV preparations were characterized in terms of identity, size, yield, and structural integrity. The presence of the Tf receptor 1 (TfR1) in EVs isolated from different sources and the binding of aTf to its receptor make EVs suitable vehicles for aTf and paves the way for their use as nanocarriers of aTf in different demyelination models.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Experiments complied with the guidelines for animal use and care of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, approved by Council Directive Res.250/2014. No randomization methods were applied. No blinding was performed. This study was not pre-registered. Sample size was determined using literary evidence in the field (Boing et al., 2014; El-Andaloussi et al., 2012; Haney et al., 2015; Théry et al., 2006). All experiments were conducted in compliance with the ARRIVE guidelines.

2.2 | Materials and reagents

Human aTf, poly-L-lysine, and Sepharose CL-2B were obtained from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium (DMEM)/F12 and human transferrin conjugated to Texas red (Tf-TR) were purchased from Life Technologies. Fetal calf serum (FCS) was obtained from Natocor. Cartridges of 12 mL were purchased from Applied Separations and PVDF transfer membranes were from Millipore, whereas ECL Plus Western Blotting Detection reagents were purchased from GE Healthcare. Antibodies used were as follows: mouse anti-Alix (RRID: AB_10899268), mouse anti-CD63 (ab213090), mouse anti-CD63 (ab193349), and rabbit anti-Calnexin (CNX) (RRID: AB_1310022) were purchased from Abcam and chicken anti-seroTf (RRID: AB_1081485) was from Genetex, whereas mouse anti-TFR1 (RRID: AB_86623) was obtained from...
Thermo Fisher Scientific. Human anti Tf (RRID: AB_2287232) was from ICN Biomedicals. Horseradish peroxidase-conjugated secondary antibodies used for immunoblotting were obtained from Jackson Immuno Research Laboratories. All other chemicals were analytical grade reagents.

### 2.3 Cells and plasma EVs sources

The mouse cell line Neuro-2A (N2a) derived from a spontaneous neuroblastoma in A/J mice (ATCC; CCL-131), and the rat oligodendroglioma OLN-93 cell line (a generous gift from Dr C. Richter-Landsberg, Richter-Landsberg & Heinrich, 1995), neither listed as a commonly misidentified cell line by the International Cell Line Authentication Committee, were cultured in DMEM/F12 (1:1 v/v) supplemented with 10% (v/v) FCS, 5 mg/mL streptomycin and 5 U/mL penicillin at 37°C in a humidified atmosphere containing 5% CO2. The medium was replaced every 2 or 3 days until reaching 70% confluence.

The conditionally immortalized oligodendrogial cell line N20.1, kindly provided by Dr A.T. Campagnoni (Verity, Bredesen, Vonderscher, Handley, & Campagnoni, 1993) and not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee, was grown to confluence in DMEM/F12 with 10% FCS at 34°C (permissive temperature). The medium was replaced by DMEM/F12 with 1% FCS and the temperature shifted to 39°C (non-permissive temperature) for 7 days. Cells were maintained up to eight passages, cryopreserved regularly and authenticated according to ATTC recommended tests, through morphological observation on an optical microscope and Hoechst assays to control mycoplasma contamination.

Primary cultures of astrocytes from newborn P0-P3 Wistar rats (Jackson Laboratory, RRID: RGD_13508588) were performed according to McCarthy and de Vellis (McCarthy & Vellis, 1980). Animals were decapitated with scissors and, after the removal of the meningeal membranes, cerebral hemispheres were mechanically dissociated by gentle repetitive pipetting in a mixture of DMEM/F12 (v/v) supplemented with 10% FCS, 5 mg/mL streptomycin and 5 U/mL penicillin. The cell suspensions were seeded in poly-L-lysine-coated 75 cm² tissue culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO2. The medium was replaced every 2 or 3 days until reaching 70% confluence. After 14 days in culture and once cells reached confluence, the microglial subpopulation was discarded after a first shake at 140 rpm during 1 h. The remaining culture was then shaken overnight at 250 rpm at 37°C, the suspended cells were discarded, and the remaining astrocytes were kept in DMEM/F12 with 1% FCS.

Human and mouse plasma were obtained from blood samples from healthy donors recruited from the laboratory personnel (n = 5; both genders; age: 25–35 years; BMI: 24–33), or mouse (25–40 g; 45–50 days old) obtained from Jackson Laboratory (RRID: MGI:5656552), and collected into tubes containing citrate dextrose solution (BD Biosciences Pharmingen). Plasma was obtained by two centrifugation cycles at 3,000 g for 10 min at 4°C, keeping in both cases the supernatants. For plasma EV isolation, a total of 20 adult mice were used. For primary culture, a total of 10 P0-P3 rats were used.

Animals were maintained in transparent plastic cages with a maximum of five animals per cage, with access to drinkable water ad libitum. Watering devices, such as drinking tubes and automated water delivery systems, were checked frequently to ensure appropriate maintenance, cleanliness, and operation.

### 2.4 Isolation of EVs from blood plasma with size-exclusion chromatography (SEC)

SEC was performed as described previously (Böing et al., 2014). Human or mouse plasma of 2 mL was loaded into gravity-eluted columns of 12 mL cartridges containing Sepharose CL-2B matrix and fractions were eluted with degassed PBS: citrate 0.32%. The first four fractions of 1 mL were collected, pooled, and ultracentrifuged at 100,000 g for 70 min at 4°C. The resulting pellets were lysed with RIPA extraction buffer (NaCl 150 mM, EDTA 5 mM, Tris 50 mM, NP-40 1%, SDS 0.1%) supplemented with a protease inhibitor cocktail for 5 min on ice or in PBS, depending on the assay. Additionally, a dilution of plasma sample in RIPA buffer was prepared at the same time and used in parallel with EVs as control for immunoblot analysis.

### 2.5 Isolation of EVs from conditioned medium by differential centrifugation

Following the different cell culture conditions previously described, N2a, N20.1, OLN-93 cells, and astrocytes from primary cultures were grown to 70% confluence, washed twice with PBS and replaced with serum-free medium for 24–72 h. The conditioned media were collected and centrifuged during 10 min at 300 g in order to remove the remaining cells. The supernatants were then subjected to 2,000 g centrifugation to remove dead cells. The resulting supernatants were collected and centrifuged at 10,000 g for 30 min at 4°C in order to pellet the cell debris and organelles. Finally, the supernatants were again subjected to high speed centrifugation at 100,000 g for 70 min at 4°C to pellet EVs. The pellet was washed with 1 mL PBS to eliminate the contaminating proteins and ultracentrifuged again at 100,000 g for 70 min at 4°C. In all cases the resulting pellet were lysed with RIPA extraction buffer supplemented with a protease inhibitor cocktail for 5 min on ice or in sterile PBS, depending on the assay. Additionally, cellular lysates were prepared at the same time and used in parallel with EVs as control for immunoblot analysis.

### 2.6 Immunoblot analysis

EVs obtained from conditioned media were harvested in 100 μL of ice cold lysis buffer (20 mmol/L Tris-HCl (pH: 8), 1% Nonidet P-40, 10% glycerol, 137 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L aprotinin, 0.1 mmol/L sodium vanadate, and 20 mmol/L of sodium fluoride). Protein content in EV samples and cell lysates was...
determined by Pierce BCA Protein Assay Kit (Life Technologies), and the samples were adjusted with loading buffer containing 2% sodium dodecyl sulfate (SDS), 5% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue, boiled for 5 min. Aliquots containing 20–40 µg protein were resolved by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat-dried milk in 0.1% Tween 20 in TBS for 1 h at 25°C and incubated with an appropriate primary antibody overnight at 4°C (CD63 1:600; Alix 1:1000; TFR1 1:1000; Tf 1:1000; CNX 1:1000). After being washed three times for 10 min with TBS Tween 0.1%, membranes were incubated with the corresponding secondary antibodies (1 : 20000), and bands were visualized by Pierce™ ECL Western Blotting Substrate (Thermofisher).

2.7 | Size and size distribution analysis

The average size (Z-average), the hydrodynamic diameter (Dh), the size distribution (polydispersity index, PDI), and zeta potential (Z-potential) of different EV sources were assayed by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments) at a scattering angle of 173°. The Nano-ZS contains a 4 mW He-Ne laser operating at a wavelength of 633 nm, a digital correlator ZEN3600 and Non-Invasive Back Scatter (NIBS®) technology. For the preparation of each EV sample, 1 mL plasma was isolated and resuspended in 10 µL of the original fresh sample and then diluted to 1000 µL of final volume using PBS (1:100). All the samples were analyzed at 4 and 25°C. Refractive index (RI) was 1.48 and viscosities between 1.568 and 1.572 cP (4°C) and 0.8866 and 0.8885 cP (25°C). Results are expressed as mean ± SD of three independent samples prepared in identical conditions. Data for each single specimen were the result of at least six runs.

2.8 | Scanning electron microscopy (SEM)

After the first ultracentrifugation step, the pellets of all samples were fixed in 50 µL paraformaldehyde (PFA) 4% for 15 min at 4°C. The solution was then washed with 1 mL Milli-Q water and ultracentrifuged at 100,000 g for 70 min at 4°C, and the resulting pellet was resuspended in 50 µL of Milli-Q water. Fresh samples of 20 µL were put on a silica substrate, dried, metalized, and observed with a Zeiss Supra Electron microscope. Six fields per experimental n were chosen randomly and EV size was determined using ImageJ software, first calculating the area of each individual EV to obtain the diameter.

2.9 | Loading of aTf into EVs

Molecules can be incorporated into EVs using different approaches that result in different loading efficiency and molecule stability (Luan et al., 2017).

2.9.1 | Passive loading of aTf into human plasma EVs

For aTf loading into human plasma EVs, we first verified the presence of TFR1 in EVs. Upon detection of TFR1, EVs isolated from 2 mL of human plasma isolated by SEC were incubated with 100 µg/mL aTf overnight at 25°C and loading efficiency was evaluated by western blot and fluorometry.

2.9.2 | Passive loading of aTf into OLN93 EVs

To obtain aTf-loaded EVs, when the cells reached 70% confluence, the medium was depleted from FCS and cells were treated with either vehicle or 100 µg/mL of aTf for 30 min, which is the time when Tf reaches its maximum cytoplasmic concentration (Pérez, Fernandez, & Pasquini, 2013). Subsequently, 48–72 h later, culture media were collected and EVs were isolated by differential centrifugation and ultracentrifugation methods.

2.10 | Ligand-binding assays of EV-TFR1-Tf

2.10.1 | Competition assays

**Competition assay I**

EV samples obtained from 2 mL plasma (yielding ~60 µg protein) and isolated by SEC as described earlier were resuspended in PBS or 100 µg/mL Tf-TR and incubated overnight with continuous shaking at 25°C. The incubated samples were ultracentrifuged twice at 100,000 g during 70 min at 4°C to eliminate non-bound fluorescent protein and supernatant samples were also collected. Aliquots of samples incubated with Tf-TR were incubated with a high concentration of 400 µg/mL non-fluorescent human aTf overnight with continuous shaking at 25°C. After incubation, samples were ultracentrifuged twice again and a supernatant sample was collected. The remaining pellet was resuspended in RIPA and fluorescence was measured in a Perkin Elmer LS55 fluorometer (Perkin Elmer Ltd.).

**Competition assay II**

EV samples obtained from 2 mL plasma per condition and isolated by SEC as described earlier were resuspended in PBS or 400 µg/mL both native and denatured Tf and incubated overnight with continuous shaking at 25°C. The incubated samples were ultracentrifuged twice at 100,000 g during 70 min at 4°C to eliminate non-bound protein and supernatant samples were also collected. Aliquots of samples incubated with Tf were incubated with a low concentration of 100 µg/mL Tf-TR overnight with continuous shaking at 25°C. After incubation, samples were ultracentrifuged twice again and the supernatant sample was collected. The remaining pellet was resuspended in RIPA and fluorescence was measured in a Perkin Elmer LS55 fluorometer (Perkin Elmer Ltd.).
2.10.2 | Incubation of EVs with different Tf-TR concentrations

EV samples obtained from 2 mL plasma per condition and isolated by SEC as described earlier were resuspended in PBS, different Tf-TR concentrations (10, 20, 100, 200 μg/mL) and incubated overnight with continuous shaking at 25°C. The incubated samples were ultracentrifuged twice at 100,000 g during 70 min at 4°C to eliminate non-bound protein and supernatant samples were also collected. The remaining pellet was resuspended in RIPA and fluorescence was measured in a Perkin Elmer LS55 fluorometer (Perkin Elmer Ltd.).

2.10.3 | Incubation of TF-TR with different amounts of EVs

EV samples obtained from 2 mL plasma per condition were isolated by SEC as described earlier. Different amounts of EVs (30, 60, 90, 120 μg) were resuspended in PBS or 100 μg/mL TF-TR overnight with continuous shaking at 25°C. The incubated samples were ultracentrifuged twice at 100,000 g during 70 min at 4°C to eliminate non-bound fluorescent protein and supernatant samples were also collected. The remaining pellet was resuspended in RIPA and fluorescence was measured in a Perkin Elmer LS55 fluorometer (Perkin Elmer Ltd.).

2.11 | Statistical analysis

Graph-Pad Prism 7 software was used for data analysis. Results are presented as the mean of at least three independent EV isolation experiments ± standard error of the mean (SEM). Normality of data was evaluated using Shapiro–Wilk normality test. No data points were excluded from the analysis. Comparisons were performed using unpaired one tailed Student’s t test and one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests, where appropriate. A p value under .05 was considered statistically significant.

3 | RESULTS

3.1 | Isolation and characterization of EVs from plasma, cell lines, and cell culture isolated cells

Using different strategies, we characterized EVs isolated from both human and mouse fluids and different conditioned media. The main objective of the isolation and characterization process was to obtain EVs which function properly as nanovehicles, focusing on EV identity, yields, quality, purity, and structural integrity.

For the isolation of fluids, protocols based on differential centrifugation can result in the co-isolation of complexes associated with non-vesicular materials. In order to overcome this obstacle, we isolated vesicles from plasma using SEC in order to efficiently separate the vesicular fractions from the protein fractions. The amount of protein from the plasma fractions measured and each fraction was analyzed by western blot to detect the typical CD63 marker of the vesicular route (Figure 1a).

We found that fractions 2–4 expressed CD63 and succeeded in separating the vesicular fraction from protein content, especially albumin. For all sources, we determined the identity of the vesicles by western blot (Figure 1b) using the Alix endosomal pathway marker and observed a significant enrichment in the vesicular fractions with respect to plasma or the respective cell lysates of each source. Particularly, in EVs from in vitro secretory cells, the level of Alix enrichment was high. Simultaneously, we conducted analyses of proteins which should be absent in EVs and which are associated with compartments other than plasma membrane or endosomes, such as the intracellular endoplasmic protein CNX. The presence of CNX in the EV fraction is also an indicator of cell lysis, a condition in which EVs should not be isolated not only because of the contaminating components from other intracellular compartments, but also because of the physiological state of the cells. In this sense, we observed the absence of CNX in the fractions corresponding to the EVs.

It should be highlighted that, when cultured with DMEM/F12 with FCS without EVs, N20.1 cells were non-viable either at proliferating or differentiating temperatures. Moreover, EV depletion from FCS affected cell phenotype and viability especially when cells were grown at a non-permissive 39°C, which indicates that serum EVs contain fundamental information for the development of this cell line (data not shown). Further experiments will be done in the future in order to clarify this point.

In addition, the amount of EVs produced can vary significantly depending on cell types and physiological state. In the current work, the yield of EV as total protein content obtained from the different cell lines was significantly larger than that of astrocyte...
EVs yield from human and mouse plasma, OLN93 and N2a cell lines, and astrocyte primary cultures. Human and mouse plasma yielded similar amounts of EVs, in both cases higher than those obtained from cell lines (Table 1).

We also confirmed the quality, purity, and integrity of the samples through DLS and SEM. Worth pointing out, factors such as EV dilution of work and temperature can critically affect their quality as nanovehicles. On the one hand, at high concentrations and at temperatures over 4°C, EVs tended to agglomerate and became difficult to handle. On the other hand, frozen samples stored at −80°C for more than 6 months and then thawed rendered measurements outside the expected EV hydrodynamic diameter, which indicates possible breakage and agglomeration because of the freezing and thawing processes (data not shown). In contrast, freshly isolated EVs displayed lower Z-average sizes at 4°C (115–211 nm) than at 25°C (161–374 nm) in all cases, a tendency replicated by Dn. The PDI values were found to be between 0.237 and 0.381 for monomodal EVs and between 0.427 and 0.554 for bimodal samples at 25°C. Also, monomodal size distribution indicated the existence of a single population in the range of sizes expected for EVs, thus ruling out the presence of contaminating residues from the isolation process or EV agglomeration. Moreover, Z-average sizes for Tf-free EVs and Tf-loaded EVs derived from human plasma were 161 and 267 nm at 25°C, respectively. Worth highlighting, Tf incorporation on the surface of EVs did not significantly affect the range of sizes found for EVs and actually rendered a lower negative Z-potential (~26 mV) than Tf-free EVs (~27 mV). The average Z-potential of free Tf at a charge concentration identical to that used for the formation of Tf-loaded EVs was approximately ~14 (±2) mV (Table 2 and 3 and Figure 2).

Finally, SEM revealed uncompromised integrity of the vesicle structures obtained through the two isolation methods and determined the heterogeneity of EV sizes within each source and their average (Figure 3). Of note, even though the EV sources used in this study are morphologically heterogeneous, their EVs had comparable sizes and shapes (Figure 3).

Altogether, these results confirm the successful isolation of EVs from very different and complex sources and their features in terms of identity and integrity to function as suitable nanovehicles.

### 3.2 | EV expression of TfR1 and loading with aTf

Previous studies demonstrated that TfR1 is released in association with vesicles (Pan et al., 1985). Indeed, electron microscopic studies have shown that endocytosis of TfR1 is followed by the formation of multivesicular bodies which, upon fusion with the plasma membrane, release 50 nm buds into the extracellular milieu. TfR1 released in vesicular form is apparently unchanged in its molecular weight, peptide sequence, and capacity for aTf binding (Pan et al., 1985). In agreement with this evidence, all EVs isolated from different conditioned media in this study showed the presence of TfR1 (Figure 4 and Table 4), which exhibited 90 kDa, the size expected for an intact, still membrane-associated monomer. In addition, immunoblotting assays on 1 mL fractions obtained from SEC confirmed TfR1 association to EVs, in agreement with the colocalization of CD63 and TfR1 in fractions 2–4 (Figure 1a).

On the basis of these findings, we analyzed the ability of EVs to load our protein of interest, aTf.

Western blot analyses revealed aTf presence both in EVs isolated from human plasma incubated with aTf and in those secreted by aTf-treated OLN-93 cells, whereas no aTf expression was observed in control human plasma EVs or in vehicle-treated cells (Figure 4a and b).

In turn, EVs incubation with Tf-TR showed higher fluorescence intensity than controls and rendered around 330 ng/mg EVs. In contrast, EV-free PBS incubated with Tf-TR and subsequently processed as EV-containing samples exhibited no fluorescence, which indicates that Tf binding requires the presence of EVs. These results, obtained by means of two different methodologies, indicate that EVs isolated from different sources were able to bind aTf (Figure 4c).

Two different competition assays were conducted to determine whether the binding of Tf to EVs proceeded through its interaction with TfR1 located at the extraluminal phase of the EVs and also whether this interaction was specifically because of the binding of Tf-TR to TfR1. In the first competition assay, EVs showed lower fluorescence intensity values, indicating that bound Tf-TR was displaced from TfR1 in fractions 2–4 (Figure 1a).

| TABLE 1 | EVs yield from human and mouse plasma, OLN93 and N2a cell lines, and astrocyte primary cultures |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **EVs source**                  | **HEK**                        | **N2a**                        | **OLN-93**                      | **Astrocyte primary culture**   |
| Cellular density (cells/mL of CM) | 5.105 cells/mL                 | 6.2.105 cells/mL               | 6.2.105 cells/mL               | 2.0.105 cells/mL               |
| Conditioned medium volume       | 40 ml                          | 40 ml                          | 80 ml                          | 80 ml                          |
| Protein concentration           | 1–4 µg/µl                      | 1–2 µg/µl                      | 1–2.5 µg/µl                    | 0.5–0.7 µg/µl                  |
| Total protein in RIPA's resuspension volume | 160 µg                      | 60–100 µg                      | 60–150 µg                      | 25 µg                          |
| **EVs source**                  | **Human plasma**               | **Mouse plasma**               |                                 |                                |
| Plasma volume                   | 4 ml                           | 4 ml                           |                                 |                                |
| Protein concentration           | 1–2 µg/µl                      | 1–3 µg/µl                      |                                 |                                |
| Total protein in RIPA’s resuspension volume | 120 µg                      |                                 |                                 |                                |
sites in the EVs, whereas subsequent incubation with low concentrations of labeled Tf-TR rendered no fluorescence signal regarding controls. In turn, saturating concentrations of denatured non-labeled aTf followed by incubation with low concentrations of labeled Tf-TR did reveal fluorescence signal regarding controls. These findings indicate that the loading of Tf into EVs occurred through the specific binding of Tf-TR to its receptor TfR1 in the extraluminal phase of EVs (Figure 5c and d). In addition, when the amount of EVs in the samples varied between 30 and 120 μg, the amount of Tf-TR remained fairly constant, as indicated by the similarity of EV fluorescence across samples.

**TABLE 2** Dynamic light scattering (DLS) of EVs derived from human and mouse plasma (Hp and Mp), OLN93 and N2a cell lines, and astrocyte primary cultures performed at 4 and 25°C

| EV source     | Temp. (°C) | Z-average (nm) | Dₐ (nm) |  |  |  |  |
|---------------|------------|----------------|---------|  |  |  |  |
|               | 4          | 115 (7)        | 161 (7) | 163 (19) | 100 (0) | – | – | 0.240 (0.027) |
|               | 25         | 121 (7)        | 161 (7) | 179 (5)  | 100 (0) | – | – | 0.299 (0.038) |
| Hp            | 4          | 190 (4)        | 200 (5) | 192 (6)  | 100 (0) | – | – | 0.238 (0.022) |
|               | 25         | 190 (4)        | 200 (5) | 208 (11) | 100 (0) | – | – | 0.248 (0.013) |
| Mp            | 4          | 157 (14)       | 202 (4) | 167 (24) | 100 (0) | – | – | 0.287 (0.045) |
|               | 25         | 157 (14)       | 202 (4) | 239 (52) | 100 (0) | – | – | 0.381 (0.028) |
| N2a           | 4          | 166 (9)        | 180 (5) | 182 (9)  | 100 (0) | – | – | 0.269 (0.063) |
|               | 25         | 166 (9)        | 180 (5) | 196 (7)  | 100 (0) | – | – | 0.237 (0.020) |
| OLN-93        | 4          | 211 (14)       | 374 (84) | 256 (22) | 100 (0) | – | – | 0.331 (0.049) |
|               | 25         | 211 (14)       | 374 (84) | 362 (25) | 66 (12) | 107 (19) | 34 (12) | 0.554 (0.030) |

*Major population.
**Minor population.

**TABLE 3** Z-average size, hydrodynamic diameter (Dₐ), size distribution (PDI), and Z-potential of Tf-free (HpEV) and Tf-loaded EVs (Hp/Tf EV) derived from human plasma (Hp) performed at 25°C by DLS

| EV source     | Z-average (nm) | Peak 1* (nm) (± SD) | % Intensity (± SD) | Peak 2** (nm) (± SD) | % Intensity (± SD) | PDI (± SD) | Z-potential (mV) |
|---------------|----------------|---------------------|--------------------|----------------------|--------------------|------------|------------------|
| HpEV          | 116 (7)        | 179 (5)             | 100 (0)            | –                    | –                  | 0.299 (0.038) | −27 (4)          |
| Hp/Tf EV      | 267 (4)        | 253 (53)            | 89 (4)             | 43 (18)              | 11 (4)             | 0.427 (0.065) | −36 (2)          |

Note: *Major population.
**Minor population.

**FIGURE 2** Size distribution of different EV sources by DLS. Z-average size for particle suspension at 4°C and 25°C. n = 2 (independent plasma or conditioned media preparations). Results are presented as the mean of two independent experiments ± standard deviation (SD). (a) Human plasma EVs, (b) mouse plasma EVs, (c) N2a EVs, (d) OLN-93 EVs, (e) astrocyte primary culture EVs
Moreover, when the concentration of Tf-TR in the samples varied but the amount of EVs in the samples was kept constant, the fluorescence ratio also remained constant (Figure 5e and f).

4 | DISCUSSION

Tf is a glycoprotein synthesized mostly in the liver. The main function of Tf is to capture Fe$^{3+}$ released into plasma from intestinal enterocytes or reticuloendothelial macrophages, maintain Fe$^{3+}$ in a redox-inert state, and deliver it into tissues (Anderson & Vulpe, 2009). The binding of iron-laden Tf to cell-surface TfR1 results in endocytosis and uptake of the metal cargo. In the CNS, most Tf is synthesized endogenously by oligodendrocytes and choroid plexus cells, and it is required for iron mobilization within the brain parenchyma and CSF (Espinosa de los Monteros & de Vellis, 1988). In our laboratory, we have demonstrated that aTf can increase cell proliferation in the subventricular zone and promote oligodendrocyte lineage commitment and terminal maturation (Silvestroff, Franco, & Pasquini, 2012). Furthermore, we also reported that a single intracranial injection of aTf prevents hypomyelination produced by iron deficiency in rats (Badaracco, Ortiz, Soto, Connor, & Pasquini, 2008). Collectively, these findings allowed us to define aTf as a trophic factor for the CNS. However, the intracranial injection of aTf might not be practical for clinical treatments, a drawback which requires the development of less invasive techniques capable of delivering aTf to the CNS.

In this sense, the intranasal route emerges as a promising non-invasive way to deliver molecules to the brain as it has many advantages from a clinical point of view; that is, molecules at biologically effective concentrations or even living cells can bypass the BBB and enter the CNS directly, avoiding hepatic and intestinal metabolism without serious systemic side effects (Danielyan et al., 2009; Merkus & Berg, 2007; Thorne & Frey, 2001). We have demonstrated in a rat model of hypoxia–ischemia encephalopathy that intranasally administered radio-labeled aTf can reach distant areas of the CNS, decreasing astrogliosis and neuronal loss, and promoting the survival and maturation of oligodendrogial precursors after demyelination (Guardia Clausi et al., 2012), which suggests intranasal aTf potential to induce remyelination.

EVs have recently risen to stardom as an excellent delivery system because of their distribution over long distances, long circulation half-life, and reduced toxicity (Lai, Yeo, Tan, & Lim, 2013; Mathieu, Martin-Jaular, Lavieu, & Théry, 2011; Tan, Rajadas, & Seifalian, 2013). Several features place EVs as potentially powerful shuttles for the delivery of therapeutic agents to the brain (Aryani & Denecke, 2016). EVs loaded with antioxidant catalase given by intranasal administration have provided significant neuroprotection in an animal model of Parkinson’s disease (Luan et al., 2017). Similarly, curcumin-loaded EVs have also been administered intranasally in mice, decreasing LPS-induced brain inflammation (Zhuang et al., 2011).

Although EVs show several advantages over other nanoparticle systems for carrying therapeutically active molecules, particularly polymer-based nanoparticles and lipid-based systems (Rufino-Ramos et al., 2017), EV isolation and purification methods still pose a challenge and should be improved to render higher yields, which will allow large-scale production and wide-spread use in clinical practice.

In this study, we isolated and properly characterized EVs from human and mouse plasma, rat oligodendroglioma cells OLN93, mouse neuroblastoma cells N2a and rat astrocytes in primary cultures, all of them expressing TFR1 and thus potentially capable of delivering aTf.
to the CNS. TfR1 plays a central role in the transport of iron from the iron-Tf complex into the cell, which makes it ubiquitous in growing cells owing presumably to iron requirements for proliferation (Wang et al., 2008). After incubation, EVs were isolated by differential centrifugation and ultracentrifugation and TfR incorporation was verified by western blot. (c) Passive ex vivo loading of Tf into human plasma EVs. (1) EVs isolated from 2 ml of human plasma by SEC were incubated with 100 μg/mL of aTf overnight at 25°C and loading efficiency was evaluated by western blot. (2) EVs isolated from 2 ml of human plasma by SEC were incubated with 100 μg/mL of Tf-TR overnight in agitation at 25°C. Ratio was calculated using RFU values: EVs-Tf ratio: EV-Tf/(TF + UC1-2), n = 4 (independent plasma preparations). Comparisons were performed using Student’s t test (*p < .05; **p < .01; ***p < .001; ****p < .0001). Results are presented as the mean of at least three independent experiments ± standard error of the mean (SEM).

FIGURE 4 TfR1 detection in different EV sources and passive loading of Tf by two loading strategies. (a) Immunoblot analysis of TfR1 in different EV sources. (b) Schematic representation of passive cargo loading of aTf into OLN-93 EVs. OLN93 cells were incubated in the presence of 100 μg/ml of aTf for 30 min at 37°C. After incubation, EVs were isolated by differential centrifugation and ultracentrifugation and TfR incorporation was verified by western blot. (c) Passive ex vivo loading of aTf into human plasma EVs. (1) EVs isolated from 2 ml of human plasma by SEC were incubated with 100 μg/mL of aTf overnight at 25°C and loading efficiency was evaluated by western blot. (2) EVs isolated from 2 ml of human plasma by SEC were incubated with 100 μg/mL of Tf-TR overnight in agitation at 25°C. Ratio was calculated using RFU values: EVs-Tf ratio: EV-Tf/(TF + UC1-2), n = 4 (independent plasma preparations). Comparisons were performed using Student’s t test (*p < .05; **p < .01; ***p < .001; ****p < .0001). Results are presented as the mean of at least three independent experiments ± standard error of the mean (SEM).

The largest amount of EVs, probably because of the contribution of other circulating cell types.

In agreement with literary evidence (Park et al., 2010), EV samples revealed the expression of characteristic proteins tetraspanin CD63 and intraluminal protein Alix, and, as expected, showed the absence of endoplasmic reticulum protein CNX. In addition, EV Z-average sizes and Dp were also consistent with previous findings (Sokolova et al., 2011).

In turn, PDI is an indicator of the degree of aggregation and size distribution of samples and is usually acceptable below 0.5. In this sense, PDI values were acceptable for all monomodal samples and their ranges were consistent with those previously reported by other authors (Sokolova et al., 2011). In addition, the size distribution analyzed was within the range expected for EVs from other sources, and larger EVs were not visualized. However, we detected smaller vesicles, especially when EVs were obtained from cell lines, which were probably cell-released ectosomes. This result indicates that both methods of isolation yielded EVs matching the EV size range and lacking significant impurities associated to larger EVs.

Z-potential is an estimation of surface charge registered for unloaded or loaded nanoparticles, and it is usually a predictive tool of colloidal stability at 25°C. Nanoparticles with Z-potential values greater than +25 mV or less than −25 mV typically have high degrees of stability, whereas dispersions with a low Z-potential absolute value and close to neutrality will eventually aggregate because of van Der Waal interparticle attractions (Blachman et al., 2020). In this context, the fact that Tf incorporation did not significantly affect the range of EV sizes and even displayed a lower negative average Z-potential than that of Tf-free EVs suggests, first, the successful surface modification of these vesicles with Tf and, second, the great stability of this system as a potential biological nanocarrier.

SEM in turn revealed the structural integrity of EVs but also the size heterogeneity of each population and the purity obtained by both methods. First, the spherical morphology of EVs reflected their structural integrity and sizes were moderately smaller than those determined by DLS, especially for astrocytes and mouse plasma sources. These findings suggest changes in contraction patterns during sample preparations for SEM, whereas hydration sphere phenomena should not be ruled out for samples determined by DLS (Sokolova et al., 2011). Overall, this set of biochemical and biophysical properties will allow to attribute cargo-loading functions to EVs isolated from different sources.

Regarding the route underlying Tf binding, competition assays were carried out on the basis of the following hypothesis: if binding is non-specific to TfR1, both native and denatured protein will be adsorbed non-specifically by the vesicular surface; on the other hand, if Tf binding is specific to TfR1, native Tf will saturate all TfR1-binding sites and will not be displaced upon incubation with Tf-TR at lower concentrations, whereas denatured Tf, in lacking the three-dimensional conformation necessary to bind to the receptor and thus being unable to compete with Tf-TR, will indeed be displaced.

Most interestingly, these assays allowed to verify that a) Tf binding took place in the extraluminal phase of EVs, b) Tf was incorporated into EVs specifically through binding to TfR1, and c) all Tf-binding sites were occupied upon EV incubation.
Altogether, the results obtained suggest that EVs isolated from different sources containing TfR1 can incorporate Tf by two distinct approaches of passive loading. To sum up, EVs have become increasingly relevant because of their function in intercellular communication and a wide range of physiological, pathological and therapeutic roles. Although further studies are in progress to track Tf-loaded EVs and ultimately determine whether they reach oligodendroglial cells in a demyelinating model, the current results pave the way for their potential clinical use as oligodendrocyte-targeted Tf nanovehicles to favor remyelination.
AUTHORS’ CONTRIBUTIONS

Pasquini, JM and Correale, J constructed the hypothesis of research. Mattera, VS, Pereyra Gerber, P, and Verstraeten, SV organized, supervised the course of progress, and took responsibility for research. Pasquini, JM, Correale, J, and Mattera, VS took responsibility for the collection of relevant biological materials, the logical interpretation and conclusion of results, writing the whole or important parts of the study, data management and reporting, and execution of the experiments. These three authors also planned methodology to reach the conclusions. Pasquini, JM and Correale, J took responsibility for the literature review necessary for the study. Pereyra Gerber, P, Gilsoni R, Ostrowski, M, and Verstraeten, SV proof-read and scientifically reviewed the article before submission.

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