Syntaxin-1a and SNAP-25 Expression Level is Increased in the Blood Samples of Ischemic Stroke Patients.

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Research Article

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Abstract

The interest in peripheral blood biomarkers is growing exponentially in several neurological disorders including Ischemic Stroke (IS). The identification of neurological biochemical signs through blood sample analyses would be revolutionary allowing a better pathology diagnosis giving also information on potential recovery after specific treatments. Indeed, the increased permeability of the blood-brain barrier, following a brain infarct, allows the detection of synaptic proteins in the blood flow. In this work, we analyzed the expression levels of two synaptic proteins belonging to the Soluble N-ethylmaleimide-Sensitive Fusion Protein Attachment Protein Receptor (SNARE) family, Syntaxin (STX)-1a and Synaptosomal Associated Protein, 25 kDa (SNAP-25), in Peripheral Blood Mononuclear Cell (PBMC), serum and in Neuronal Derived Extracellular vesicles (NDEs) of IS patients, age and sex matched healthy control (HC) and younger HC (Y-HC). Interestingly, we found, for the first time, that STX-1a protein is present in the cytoplasm of PBMC. Moreover, both protein, STX-1a and SNAP-25, levels were significantly augmented in all IS patient's blood fractions (serum, PBMCs and NDEs) compared to control subjects. Interestingly, the STX-1a blood levels always correlated with the IS clinical scales National Institutes of Health Stroke Scale (NIH-SS) and the modified Barthel Index (BI). These results prompted us to speculate that STX-1a and SNAP-25 hematic fluctuations depict the brain damage after an ischemic attack and that their hematic detection could represent a novel and accessible IS biomarkers.

Introduction

Ischemic Stroke is a severe pathology originating from a thrombolytic or embolic event that reduces blood supply to the brain [1]. It represents the third leading cause of death in the industrialized countries (40-60% of IS patients die within 5 years after the ischemic event) and the second most common origin of dementia [2, 3]. The current therapies and patients management are not sufficient to increase the lifetime expectancy after stroke [4, 5]. One of the main obstacles encountered in ordinary therapies is certainly be given by the heterogeneous nature of the pathology and the patient's individual variability. Indeed, the possibility to tailor specific therapies for each patient is a medical unmet need [6].

The brain tissue affected by stroke presents two distinct areas of damage that can be identified as the ischemic core, in which the blood flow is completely absent and neuronal death occurs within a few minutes, and the penumbra, in which the blood flow is moderately reduced and the brain tissues, although functionally impaired, is still semi-viable [7]. Neurons located in the ischemic core region undergo death, mainly through necrosis mechanisms including the lack of ATP (Adenosine Tri-Phospahte), increased concentrations of ions and glutamate, and tissue acidosis [8]. By contrast, neurons in the ischemic penumbra undergo a similar fate if blood flow (and therefore, oxygen and glucose supply) is not restored within a short time. Neurons in the penumbra area die because of the strong activation of the apoptotic pathway driven by the augmentation of Ca2+ into cells that causes cells death for the excess of glutamate released [9].

The over-flow of glutamate and its persistence in the synaptic cleft induces a cascade of biochemical events, known as ‘excitotoxicity’, which includes a prolonged activation of glutamate receptors and forms a vicious circle between elevated concentrations of intracellular Ca2+ ions and aberrant glutamate release, worsening at the end the effect of the ischemic event and leading to neuronal cells loss [10].

The release of neurotransmitters, including glutamate, relies on a well-studied molecular mechanism which involves a group of proteins, implicated in the synaptic release of glutamate, that form a complex called SNAREs. Indeed, it is reported that the disruption of the SNARE complex formation induces the neurotransmitter release disorder especially by cleaving SNAP-25, a key SNARE complex protein [11-14].

Another essential partner for the formation of SNARE complex is STX-1a, a protein anchored to the presynaptic membrane, which it requires a switch from closed to open conformation to bind SNAP-25 and participate in the release of neurotransmitters [15].

Few evidence highlighted the involvement of both STX-1a and SNAP-25 in IS, but still, further research is needed to understand their contribution to this pathology. Indeed, the protein level of STX-1a is markedly up-regulated in the cortex of a rat model of IS, probably suggesting the attempt of the brain to preserve the neuronal synaptic function after cerebral ischemia [16]. The correlation between SNAP-25 and IS is not clear: the mRNA (Messanger Ribonucleic Acid) levels of SNAP-25 has been found increased in the mossy fiber layer of gerbils up to 7 days after induced transient ischemia lasted for 5 minutes [17] while, another study on rat models indicates that IS induced its protein reductions [18].

Several proteins such as GFAP (Glial Fibrillary Acid Protein), S100β (S100 calcium-binding protein B), NSE (Neuron Specific Enolase), Lp-PLA 2 (Lipoprotein-associated Phospholipase A2), MMP-9 (Matrix Metalloproteinase-9), D-dimer and HSP70 (Heat Shock Protein 70) [19, 20], has been proposed as clinical biomarkers for IS, but none of them has reached successfully the clinical usage. Alterations of synaptic proteins levels, instead, have been proposed as pathological biomarkers for Alzheimer’s disease [21], therefore, the detection of these proteins in human biological fluid samples has raised scientific interest.

Recently, the extracellular vesicles isolated from blood samples of patients have been indicated as a good biological sample to identify pathological biomarkers [22]. Extracellular vesicles, and in particular exosomes, which are endosome-derived small membrane vesicles (30-150 nm size) carry biological active molecules (such as genetic material, proteins and lipids), interact with neighboring cells to transmit their cargo from cells to cells, thus playing an essential role in intercellular communication [23, 24]. Brain cells, including neurons, also release extracellular vesicles into the extracellular milieu to be then uptaken by neighboring cells or pass into the cerebrospinal fluid and blood [25, 26]. Recent studies have reported that neuronal derived exosomes, found in the blood of patients, carry substances associated with neurological diseases [27].

Interestingly, it has been demonstrated that STX-1a, known as exclusively neuronal protein, is potentially expressed in human blood cells in which a wide number of syntaxins was found [28]. In addition, the STX-1a mRNA, but not the mRNA for STX-1b and SNAP-25, has been detected in human CD8+ T cells

[29]. On the other hand, SNAP-25 has been assessed in human serum [30] while its expression in PBMCs has never been detected [29, 31]. More recently, both STX-1a and SNAP-25 have been measured in neuronal derived exosomes and proposed as potential biomarkers for neurodegenerative diseases [30, 32]. Anyway, the expression of these two proteins in blood samples of IS patients still has not been described.

Therefore, in this work, we showed the presence of both STX-1a and SNAP-25 in human blood fractions: serum, PBMCs and NDEs. We then analyze their expression levels in the blood components in a clinically characterized cohort of IS patients, HC and Y-HC subjects.

Materials And Methods

Study population

Samples were obtained from IS patients between 11 to 51 days after the ischemic event when they are hospitalized in *Casa di Cura Privata del Policlinico* (CCPP) after the acute ischemic event. A total of 30 IS patients (age mean 70.0 ± 11.5 years, 45-87 years range; 15 men and 15 women), 30 healthy age-matched controls (HC, age mean 72.1 ± 7.3 years, 51-87 years range; 15 men and 15 women) and 15 Y-HC subjects (age mean 32.6 ± 4.0 years, 26-39 years range; 6 men and 9 women) samples were analyzed in this study. IS patient's samples were selected based on the diagnosis with homogenous parameters of gender, age, and treatment, whereas HC subject's samples were chosen based on homogenous parameters of gender and age. IS patients were clinically evaluated and analyzed based on the following scales for stroke outcomes: NIH-SS and BI. The NIHSS quantifies stroke severity based on language, motor function, sensory loss, consciousness, visual fields, extra ocular movements, coordination, neglect, and speech [33], whereas, functional outcomes and daily life activities were measured using the BI scale [34]. Moreover, both IS patients and HC subjects have been analyzed by using CIRS (Cumulative Illness Rating Scale) [35], a typical comorbidity scale, and the MMSE (Mini-Mental State Examination) [36] to evaluate the cognitive function. Samples belonging to IS patients with neurological comorbidity (e.g. Parkinson's or Alzheimer's diseases, major head trauma, neuromuscular diseases etc.) or psychiatric (e.g. psychosis, bipolar disorder) or oncological pathologies in progress have been excluded.

A summary of cases is shown in Table 1.

Table 1

Study population and clinical data. TES = Thrombo-Embolic Stroke; ACS = Anterior Circulation Stroke; PCS = Posterior Circulation Stroke; MMSE = Mini-Mental State Examination; NIH-SS = National Institutes of Health Stroke Scales; BI= Bartel Index; n.c. = not calculated.

Population Characteristics			
	IS patients	**HC subjects**	**Young HC subjects**
N°	30	30	15
Males/Females	15/15	15/15	6/9
Age (mean ± SD; range)	70.0 ± 11.5 (45-87)	72.1 ± 7.3 (51-87)	32.6 ± 4.0 (26-39)
TES (%)	26.7	n.a.	n.a.
ACS (%)	46.7	n.a.	n.a.
PCS (%)	26.7	n.a.	n.a.
MMSE (mean ± SD; range)	25.6 ± 8.8 (0-30)	29.1 ± 1.4 (25-30)	n.c.
NIH-SS (mean ± SD; range)	4.9 ± 2.8 (2-14)	n.a.	n.a.
BI (mean ± SD; range)	59.4 ± 20.0 (25-95)	n.a.	n.a.
Processing Days after IS (mean ± SD; range)	30.3 ± 12.1 (14-51)	n.a.	n.a.

Blood Collection

Serum, plasma and PBMCs samples have been obtained from the venous sampling of Y-HC, HC subjects and IS patients.

Serum samples were collected in tubes with Clot activator and spun at 1500 g for 15’ at R.T. (Room Temperature, at least after 30’ from blood collection and max within 1h).

Plasma samples were collected in ethylenediaminetetraaceticacid-K2 vacuum blood (K2EDTA) pre-coated tubes and spin at 2000 g for 15’ at R.T.

PBMCs samples were collected in K2-EDTA tubes. Whole blood was diluted with the same amount of RPMI 1640 (Roswell Park Memorial Institute culture medium) without glutamine (Euroclone), layered on Ficoll-Histopaque (Ficoll-PlaqueTM Plus, GE HealthCare) and centrifuged at 800 g for 30’ brake off at R.T. PBMCs pellet were collected from the interface and washed 3 times with PBS (Phosphate Buffered

Saline) without calcium and magnesium (Euroclone). Platelets were eliminated by an additional wash and centrifugation at 200 g for 10’ at R.T.

All blood samples were stored at -80°C in the CCPP Biobank and the experiments were performed using frozen PBMCs.

Albumin and IgG removal from serum and plasma samples

Albumin and the major subclasses of IgG (Immunoglobulin G) were removed from serum and plasma by using the commercial kit ThermoScientific^{TM}Pierce^{TM} Removal Kit [37]. Briefly, ≈ 500 µg of serum or plasma diluted in “Binding/Wash Buffer” were loaded onto a spin column previously immobilized by the “Cibacron Blue/Protein A gel” and incubated 10’ at R.T. on an orbital shaker. The samples were pulled down by 1’ of centrifugation at 10000 g at R.T. and the recovered filtrate was re-applied on the resin bed. After an additional 10’ of incubation suited by 1’ of centrifugation at 10000 g at R.T., 75 µL of “Binding/Wash Buffer” were loaded to the column and blood samples, removed from albumin and IgG, were recovered after 1’ of centrifugation at 10000 g at R.T. The column was then washed with 100 µL of “Binding/Wash Buffer” and the flow through recovered by adding 100 µL of Laemmli Loading Buffer 1x and centrifuged 1’ at 10000 g at R.T.

Western Blotting Analysis

After the determination of the protein concentration performed by Coomassie Protein Assay (Thermo Scientific), 100 µg of serum, plasma and PBMCs lysates, 0.5 µg of mouse brain cortex lysates and 50 µg of extracellular vesicles lysates were analyzed by Western Blot (WB). PBMCs, mouse brain cortex and all extracellular vesicles fractions were resuspended in RIPA buffer (Radioimmunoprecipitation Assay Buffer) and sonicated before the determination of protein concentration. 10 µL of the flow through, obtained after the stripping of albumin and IgG from serum or plasma, were loaded for WB analysis while 4 µL of each nuclear and cytoplasmic fraction were loaded for subcellular fractionation analysis. All samples were diluted in Laemmli Loading Buffer (WVR Life Science). WB analysis was performed by incubating the PVDF (polyvinylidene difluoride) membranes (GE Healthcare) for 1h at R.T. or overnight (O.N.) at 4°C in a blocking solution containing 4% dried milk (Serva Electrophoresis GmBH) or BSA (Bovine Serum Albumin, PanReac AppliChem) in TBS (Tris-Buffered Saline, Corning) to which 0.1% Tween-20 was added. After incubation with primary antibody (1.5h at R.T. or O.N. at 4°C), the membranes were extensively washed in TBS and 0.1% Tween-20 and incubated for 1h at R.T. with a specific rabbit or mouse peroxidase-conjugated immunoglobulins (Jackson Immuno Research). The immunoreactivity signals were detected by Super Signal^{TM}West Femto Maximum Sensitivity Substrate (Thermo Scientific) using the following primary antibodies: rabbit polyclonal anti-STX-1a (1:350), rabbit polyclonal anti-ERp57 (1:2000), rabbit polyclonal anti-CD9 (1:500), all from Elabscience; rabbit monoclonal anti-STX-1a (1:750, AbCam); rabbit polyclonal anti-SNAP-25 (1:200, Atlas Antibodies); rabbit polyclonal anti-Lamin A/C (1:200), rabbit monoclonal anti-SV2A (1:200), rabbit monoclonal anti-GFAP (1:1000), rabbit monoclonal anti-PSD95 (1:500), all from Cell Signaling Technology; mouse monoclonal anti-β-actin (ACTB, 1:10000), rabbit polyclonal anti-APO A1 (1:500), rabbit polyclonal anti-NSE (1:400), all from

Biorbyt; mouse monoclonal anti-GM130 (1:200, BD Biosciences); mouse monoclonal anti-CD107a (1:2000, Exbio Antibodies). For WB analysis of serum and plasma, PVDF membranes were subsequently stained for total proteins content with Coomassie-Blue (WVR Life Sciences).

WB densitometric analyses were performed by using Image J software (Meida Cybernetics).

Cells cultures

PBMCs cultures, obtained from an healthy subject, were performed as previously described [38]. Briefly, after their isolation from blood (as stated above), cells have been extensively washed in DMEM (Dulbecco's Modified Eagle medium, Biowest LLC, without FBS, Fetal Bovine Serum, Corning) and resuspended in 1 mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells concentration have been adjusted at 1x10^6 cells/mL and 500 µL of cells suspension have been seeded in a 24 well culture plate and incubated at 37°C in a 5% CO2. As reported, cells adhering to the plate are the monocyte population whereas the floating one are lymphocytes [38]. After 2, 4 and 9 days of culture, cells were harvested, counted and resuspended in Laemmli Loading Buffer to reach 1x10^4 cells/µL, sonicated and 20 µL of each sample (2X10^5cells) has been used for WB analyses.

The SH-SY5Y human glioblastoma cells (ATCC CRL-2266), received from EBRI Rita Levi-Montalcini Foundation, were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin (Aurogene) at 37°C in a 5% CO2 incubator.

Immunofluorescence experiments

For immunostaining of PBMCs, round glass coverslips were coated with poly-L-lysine 0.1 mg/mL solution (Serva Electrophoresis GmBH) for 5' at R.T. and then dried for 2h. Frozen PBMCs were resuspended in 100 µl of PBS and seeded for 1h on coated coverslips at 4°C. Cells were fixed in 1% p-formaldehyde (Thermo Scientific) 15' at 4°C.

PBMC's colocalization analysis between STX-1a and β-actin or STX-1a and GM130 were performed on a subpopulation of 5 HC subjects. In the same way, STX-1a expression levels of PBMC were analyzed, by immunofluorescent experiments, in a subpopulation of 5 IS patients and 5 HC subjects.

SH-SY5Y cells were seeded on round glass coverslips (2.5x10^5 cells each) and incubated for 24h at 37°C and 5% CO2, then washed with PBS (Euroclone) and fixed with 4% p-formaldehyde for 15' at 4°C.

Both PBMCs and SH-SY5Y cells were permeabilized and unspecific binding sites were blocked by incubation at R.T. in PBS supplemented with 0.2% Triton X-100 and 1% BSA. The coverslips were incubated O.N. at 4°C with primary antibodies: rabbit polyclonal anti-STX-1a (1:200), rabbit polyclonal anti-SNAP-25 (1:200), mouse monoclonal anti-β-actin (1:50) and mouse monoclonal anti-GM130 (1:100). Cells were then washed in PBS supplemented with 1% BSA and incubated 1h at R.T., in dark, with anti-rabbit TRITC and anti-mouse FITC (1:400, Jackson Immuno Research). After extensive washing with PBS and PBS supplemented with 0.2% Triton X-100 and 1% BSA, cells were incubated 10' at 4°C, in dark, with

0.1 µg/mL of DAPI (PanReac AppliChem) for nucleus staining. Coverslips with no primary antibodies were analyzed as negative controls.

Fluorescence microscopy and image analysis

Fluorescent images were acquired using NIS-Elements Basic Research software using a fluorescent microscope (Nikon Eclipse Ti2-E). Fluorescence acquisition settings were identical among all images acquired. To avoid, artifacts, the background was subtracted to each image and independent threshold was calculated. Image J software was used to evaluate fluorescent intensity that was calculated by taking in considering at least 15 isolated cells for each subject and the mean was calculated by the analysis of at least 5 subjects for each group. Before calculating colocalization analysis and the STX-1a fluorescent levels in IS patients and HC groups, a median filter was applied to the images to reduce the background noise.

The colocalization parameters (between STX-1a and β-actin or STX-1a and GM130) were estimated by measuring the percentage of colocalized area and Pearson's coefficient. To evaluate the fluorescent areas, for each channel were automatically drawn a color mask using the Color threshold module. In Pearson's coefficient analysis the JACoP plugin was used.

For the quantification of STX-1a levels in IS patients and HC subjects, fluorescence intensity of nuclei (stained with DAPI), cytoplasm (stained with β-actin) and STX-1a were calculated. The quantification of STX-1a expression levels was obtained by dividing the values of its signal by the β-actin value from which the nucleus fluorescence was subtracted. These analyses were made by using an immunofluorescence area mask for DAPI and β-actin channels and the Set Measurement module for STX-1a determination.

Subcellular Fractionation of PBMCs

A cytosolic cellular fraction (containing soluble proteins) and an insoluble nuclear fraction were prepared by differential centrifugation [39, 40]. Briefly, cellular pellets were lysed in hypotonic buffer (10 mM Hepes/NaOH pH 7.5, 250 mM sucrose, 10 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100, protease and phosphatase inhibitor cocktails, from Thermo Scientific) and the cytoplasmic fractions were separated from nuclei by 5' centrifugation at 2000 g at 4°C. Pellets were resuspended in 100 mM Tris-HCl pH 7.4, 1 mM EDTA pH 7.5 and 500 mM NaCl, protease and phosphatase inhibitor cocktails, incubated 10' at 4°C followed by 1:10 dilution and 15' of incubation at 4°C with 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 7.5, 0.5% NP-40, protease and phosphatase inhibitor cocktails and centrifuged 5' at 150 g at 4°C. Both the cytoplasmic and the nuclear fractions were precipitated by the addition of 9 volumes of ethanol, cooled 2h at -80°C and then centrifuged for 30' at 18400 g at 4°C. After discarding the supernatant, pellets (containing cytoplasmic or nuclear proteins) were resuspended in 30 µL of Laemmli Loading Buffer 1X and 4 µL of each sample were loaded for WB analysis.

The analysis of STX-1a expression levels in the cytosolic fractions were performed in a subpopulation of 10 IS patients, 10 HC and 5 Y-HC subjects.

Extracellular vesicles purification from serum samples

Extracellular vesicles were isolated, as previously reported [41], by using 500 µL of frozen sera added to 500 µL of PBS containing three times more than the suggested concentrations of protease and phosphatase inhibitor cocktails and were spun at 4000 g for 20’ at 4° C. Supernatants were gently mixed with 250 µL of ExoQuick (System Biosciences) in a fresh tube, incubated 1h at 4°C and then centrifuged at 1500 g for 20’ at 4° C. The pellets were resuspended in 500 µL of Ultra-Pure Water (Lonza Bioscience Solution) containing three times the suggested concentrations of protease and phosphatase inhibitors and incubated for 2h at R.T. with rotation. Then, 100 µL of samples were stored as total extracellular vesicles proteins (T). NDEs were then immunoprecipitated by using 4 µg of mouse anti-human CD171 (L1 cell adhesion molecule [L1CAM]) biotinylated antibody from eBioscience) in a total volume of 45 µl of 3% BSA in PBS and incubated 1h at 4° C with rotation. Samples were then incubated with 13.5 µl of streptavidin-agarose Ultralink resin (Thermo Scientific) in a total volume of 40 µl of 3% BSA for 30’ at 4° C with rotation. The pH was adjusted to ~ 7.0 and samples were spin-down at 200 g for 10’ at 4° C. Supernatants represent the total extracellular vesicles fraction depleted of NDE (T–N), while pellets were resuspended in 160 µL of 0.1 M Glycine, pH 2.5-3, vortexed 30” and centrifuged at 4500 g, for 5’ at 4° C. Supernatants, which represent NDEs fractions (N), were added of 13.5 µl of 1 M Tris-HCl, pH 8, 22.5 µl of 3% BSA in PBS and 234 µl of RIPA Buffer, in a fresh tube, containing three times the suggested concentrations of protease and phosphatase inhibitors. Samples were subjected to 2 freeze-thaw cycles and sonication, before the determination of the protein concentration and WB analysis.

The analysis of STX-1a expression levels in NDEs and of SNAP-25 in both total extracellular vesicles and NDE fractions were performed in a subpopulation of 10 IS patients and 10 HC subjects.

Transmission electron microscopy

Extracellular vesicles isolated from HC subjects have been used to perform ultrastructural analysis by transmission electron microscopy (TEM), as previously described [42]. Briefly, extracellular vesicles were fixed in 2% paraformaldehyde and adsorbed on formvar-carbon-coated copper grids. The grids were then incubated in 1% glutaraldehyde for 5’, washed with deionized water eight times, and then negatively stained with 2% uranyl oxalate (pH 7) for 5’ and methyl cellulose/uranyl for 10’ at 4°C. Excess of methyl cellulose/uranyl was blotted off, and the grids were air-dried and observed with a TEM (FEI Morgagni268D) at an accelerating voltage of 80 kV. Digital images were taken with Mega View imaging software.

Statistical Analysis

Variation of STX-1a and SNAP-25 levels in the populations analyzed were evaluated by one-way analysis of variance and post-hoc Tukey significance tests by using Kaleidagraph (Synergy Software). Significance was assessed as * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$.

Correlation between NIH-SS, BI and MMSE clinical scales and STX-1a and SNAP-25 levels in the different blood components were determined by Pearson’s correlation coefficient *r* with 95%. As for the strength of

correlation, it has been classified according to British Medical Journal guidelines, which regard significant correlation coefficients of 0-0.19 as very weak, 0.2-0.39 as weak, 0.4-0.59 as moderate, 0.6-0.79 as strong and 0.8-1 as very strong [43].

Results

STX-1a and SNAP-25 are detected in human peripheral blood

In order to confirm the presence of STX-1a and SNAP-25 in human blood fractions, we firstly analyzed, by WB, the different human blood components (i.e. PBMC, serum and plasma) from healthy donors. In PBMC, the band corresponding to STX-1a was found at the predicted molecular weight of 33 kDa and it was comparable to the one observed in the mouse brain cortex lysate loaded as positive control. On the other hand, as expected, no specific signal was detected for SNAP-25 (Fig. 1a). These results have been also confirmed by immunofluorescence analysis in which STX-1a was found expressed around the nuclei (Fig. 1b) similarly to the signal observed in the neuronal-like cells SH-SY5Y, used as positive control (Fig. SI 1a). On the other hand, SNAP-25 specific signal was completely absent in PBMC (Fig. 1b), thus confirming our WB results.

Both STX-1a and SNAP-25 proteins were expressed in serum and plasma, although the immunoreactive band for STX-1a appears to be shifted at higher molecular weight (around 50 kDa) (Fig. 1a), still, further investigations are needed to elucidate the band entity.

The analysis of human blood samples is often complicated by the presence of high concentrations of albumin and IgG, which can count for 70% of total serum proteins [37]. For this reason, serum and plasma samples have been re-probed after the IgG and albumin removal. Indeed, both STX-1a (at the higher molecular weight) and SNAP-25 signals were still observed (Fig. 1c). Flow through samples coming from the IgG/albumin removal were analyzed and they showed very faint bands for both proteins (Fig. SI 1b) confirming the antibodies specificity for the proteins probed.

Subcellular localization of STX-1a in PBMCs from healthy donors

Fluorescence images demonstrated that STX-1a is mostly expressed outside the nucleus (Fig. 1b) with a higher localization in the cytoplasm where strongly colocalizes with the cytoplasmic marker β-actin (Fig. 2a). This interaction is also confirmed by the colocalization areas (60.0 ± 7.7%; Fig. 2b) and Pearson's Coefficient (0.605 ± 0.141; Fig. 2c) analyses. Moreover, we observed a partial colocalization of STX-1a and the specific marker of the Golgi's apparatus (GM130) (Fig. 2a) of which the colocalization area (14.8 ± 4.2%; Fig. 2b) and the Pearson's Coefficient have been calculated (0.380 ± 0.076; Fig. 2c). These results suggest that STX-1a could be synthesized by PBMCs rather than been uptaken from outside.

Then, to confirm the cytosolic localization of STX-1a in PBMCs we performed a subcellular fractionation experiment in which we found that STX-1a was present in the soluble cytoplasmic fraction but not in the nuclear compartment. β-Actin and lamin A/C have been used respectively as markers of cytoplasmic and nuclear compartments to test the quality of the fractionation method (Fig. 2d).

PBMCs chiefly consists of monocytes and lymphocytes, therefore, we finally analyzed whether STX-1a was expressed in both cellular subtypes or one specific sub-population. For this reason, PBMCs have been cultured for 9 days, the longest time point possible before they died, and the expression of STX-1a was monitored at different time points showing that it was expressed in both monocytes and lymphocytes at each time analyzed (Fig. 2e).

STX-1a and SNAP-25 are detected in NDEs purified from human serum of healthy donors

NDEs were purified from the serum of healthy donors in order to confirm the presence of the two SNARE proteins. The fraction prepared are total extracellular vesicles (T), the NDEs (N) and the total fraction depleted of the NDEs (T-N). Firstly, we examined our preparations in order to verify their quality, indeed all extracellular vesicles fractions resulted pure and free from contaminations by other cells organelles such as Golgi apparatus (GM130), endoplasmic reticulum (ERp57), lysosomes (CD107a) and nucleus (lamin A/C) (Fig. 3a). Moreover, extracellular vesicles preparation resulted positive to CD9 antibody, specific for exosomal population, while it was not present in PBMCs, serum and mouse brain cortex (Fig. 3a, b, c). Lipoprotein particles are very abundant in the circulation and are often co-purified with extracellular vesicles fractions [44]. In our preparations, the WB analysis showed that the subsequential purification steps succeeded to obtain NDE fractions (N) with a reduced contamination of apolipoproteins, if compared to the other two extracellular vesicles fractions (T and T-N) (Fig. 3a). The purified NDEs fractions, which are enriched of neuronal proteins, were positively immunoreactive to main neuronal markers NSE and SV-2a (Synaptic Vesicle Glycoprotein 2a), but negative to the astrocyte's marker GFAP and to the post-synaptic marker PSD95 (Post Synaptic Density Protein 95) (Fig. 3b), thus confirming the neuronal origin of these vesicles, potentially from the presynapses. Then, we probed the NDEs for both STX-1a and SNAP-25. Interestingly, STX-1a was present, at the typical molecular weight of 33 kDa, in T extracellular vesicles and also in NDE but not in T-N. In particular, STX-1a appears significantly enriched in NDEs fractions with respect to that of T extracellular vesicles, while it was undetectable in the fractions of T-N. SNAP-25 was present in T and N extracellular vesicles fractions and in less extent also in T-N (Fig. 3c). These results suggest that the presence of both SNARE proteins in human serum may be due to their transport through NDEs.

Finally, the NDE preparation underwent TEM analyses showing the presence of nano-sized, rounded-shaped vesicles with a typical diameter range of 70–100 nm which can definitely correspond to serum isolated NDEs (Fig. 3d).

Demographic and clinical characteristics of the human subjects selected for the study

A population of 30 IS patients (mean age 70.0 ± 11.5; 50% females) and 30 age-and-sex matched healthy controls (HC, mean age 72.1 ± 7.3; 50% females) has been selected to study the expression levels of STX-1a and SNAP-25 in serum, PBMCs and NDEs isolated from serum. Being the SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) migration of the serum STX-1a at a molecular weight higher than normal, we decided to analyze the expression levels of STX-1a only in PBMCs and NDEs, while being SNAP-25 absent in PBMCs, we analyzed it in serum and NDEs. Moreover, 15 healthy young subjects (Y-HC, mean age 32.6 ± 4.0; 60% females) have been enrolled in the study to assess whether the expression levels of STX-1a and SNAP-25 varied during aging. Table 1 presents the demographic and clinical features of the population studied.

Age or gender distribution was homogeneous between the IS and the HC population groups. Patients suffering from transient ischemic attack (TIA) and hemorrhagic stroke have been excluded from the study. Blood samples of all IS patients were obtained between 14 to 51 days (mean 30.3 ± 12.1 days) from the ischemic event, which is considered the beginning of the post-acute phase of IS, and the majority of the stroke events had origin in the anterior circulation (46.7%). The average of the specific stroke clinical scales, i.e. NIH-SS and the modified BI were 4.9 ± 2.8 and 59.4 ± 20.0 respectively, while MMSE is 25.6 ± 8.8 vs 29.1 ± 1.4 of the HC population.

Peripheral levels of STX-1a and SNAP-25 were significantly increased in IS patients compared to HC subjects and STX-1a correlates with IS clinical scales

First of all, the expression levels of STX-1a in PBMCs and of SNAP-25 in serum have been analyzed by WB analysis and subsequent densitometric measurement in IS patients, HC and Y-HC subjects. Our analyses revealed that both SNARE proteins significantly increased in IS patients compared to either HC or Y-HC (Fig. 4). We also confirm the “altered” migration of STX-1a in sera of IS patients (around 50 kDa, Fig. SI 2a) and the concomitant absence of SNAP-25 in their PBMCs (Fig. SI 2b).

Interestingly, the densitometric analyses showed an increased expression level of STX-1a in PBMCs of IS patients compared to both HC and Y-HC subjects (1.5 ± 0.5 vs 0.97 ± 0.3 and 0.94 ± 0.2 for HC and Y-HC, respectively, p=0.0002; Fig. 4a, b). We then analyzed a possible correlation between protein expression level in PBMCs and the NIH-SS, BI and MMSE clinical scales. We found a moderate negative correlation with NIH-SS scale (-0.407) (Fig. 4c) and a moderate positive correlation with BI score (0.400) (Fig. 4d). Both these correlations are statistically significant (p=0.03). On the other hand, no significant correlation has been found between STX-1a concentration measured in IS PBMCs and MMSE (Fig. SI 2c).

Moreover, the expression levels of SNAP-25 have been found significantly increased in serum of IS subjects with respect to both HC and Y-HC subjects (0.47 ± 0.07 vs 0.28 ± 0.12 and 0.09 ± 0.02 for HC and Y-HC, respectively, p=0.00004; Fig. 4e, f). Of note, in this case, SNAP-25 is significantly more abundant in the HC population in comparison with the Y-HC subjects. Nevertheless, the Pearson’s analysis between these data and the three aforementioned clinical scales did not lead to any statistically significant results (Fig. SI 2d, e, f).

Finally, we demonstrate that the increased SNAP-25 expression levels in IS sera were not affected by the IgG and albumin content since SNAP-25 expression has been observed in serum of the three analyzed population (Fig. 4g), while no signals have been observed in the flow through samples (Fig. SI 2g).

STX-1a increased in PBMC samples of a subpopulation of IS patients

Immunofluorescence analysis of PBMCs of a subpopulation of IS patients showed a higher presence of STX-1a signal in this group compared to the HC subjects (Fig. 5a). Indeed, the fluorescent analysis of STX-1a expression confirms that STX-1a was significantly increased in IS patients (107 ± 15.4 vs 73.1 ± 12.9, p=0.005; Fig. 5b).

Later we performed subcellular fractionation of PBMCs of a subpopulation of IS patients. The WB confirmed the presence of STX-1a in the cytoplasm of PBMCs in the population analyzed (Fig. 5c). Moreover, STX-1a expression levels have been found significantly higher in the cytoplasm of PBMCs of IS patients with respect to HC subjects (1.6 ± 0.6 vs 0.59 ± 0.1, p=0.0002; Fig. 5d) and Y-HC (0.77±0.3, p=0.0027; Fig. 5d). No significant differences were observed between the two control groups, HC and Y-HC (p=0.70) (Fig. 5d).

STX-1a and SNAP-25 expression levels are significantly increased in NDEs purified from serum of a subpopulation of IS patients

STX-1a and SNAP-25 proteins content have been analyzed in the extracellular vesicles' fractions isolated from the serum of a subpopulation of IS patients and HC subjects. All extracellular vesicle fractions have been tested for the exosomal marker CD9 and the neuronal marker NSE (Fig. 6). The STX-1a signal was barely visible but not quantifiable in the total extracellular vesicles' fractions (Fig. 6a), and absolutely absent in the total extracellular vesicles fractions depleted from NDEs (T-N) (Fig. 6f), but it was significantly increased in NDEs derived from IS patients with respect to HC (1.8 ± 0.3 vs 1.1 ± 0.3, p=0.00005; Fig. 6c, d). On the other hand, SNAP-25 resulted strongly in the total extracellular vesicles fractions compared with STX-1a, but differences of SNAP-25 expression were not significant between the two above-mentioned populations (p=0.91) (Fig. 6a, b). However, SNAP-25 expression level was statistically higher in NDEs isolated from IS patients than HC subjects (4.1 ± 0.9 vs 2.9 ± 0.9, p=0.02; Fig. 6c, e).

Finally, SNARE protein contents in NDEs have been correlated with the clinical scales through a Pearson's correlation analysis and only STX-1a showed significant results. In particular, a moderate positive correlation between STX-1a and NIH-SS scale (0.521) (Fig. 6g) and a moderate negative correlation with BI scores (-0.431) (Fig. 6h) were found. Both correlations are statistically significant (p=0.003 and p=0.02, for NIH-SS and BI scales, respectively). On the other hand, no correlation has been observed between STX-1a and MMSE (Fig. SI 3a) and between SNAP-25 NDE's concentration and the clinical scales (Fig. SI 3b, c, d). Finally, even the levels of the two SNARE proteins in the NDEs are not statistically correlated with each other (Fig. SI 3e).

Discussion

The identification of IS blood-based biomarkers would represent a fundamental step for unsure a better prediction of the outcomes of the ischemic event and for the identification of a personalized treatment for each patient with the advantage to be very little invasive and relative at low-cost. Emerging evidences demonstrate that exosomes regulate brain intercellular communication after IS [45], for this reason, the development of techniques to isolate NDEs from peripheral blood has opened interesting opportunities in the field of the identification of IS biomarkers. In addition, PBMC, being present at the peripheral level, can represent another source of IS biomarkers. These cells selectively migrate and infiltrate the ischemic brain tissue [46] and an alteration of their gene expression profile has been observed in IS patients [47]. Indeed, the destruction of the blood-brain barrier allows proteins to leak into the blood flow and their serological detection become therefore possible. Interestingly, it has been demonstrated that some cerebral proteins are released and detected into blood samples of IS patients [19].

In this work, we investigated the IS peripheral expression levels of two proteins member of the SNARE complex, STX-1a and SNAP-25, that play a fundamental role in the release of neurotransmitters from the pre-synaptic side [48, 49] and which concentration is altered in the brain tissue of IS animal models [16-18]. Recently, both proteins have been detected in NDEs isolated from blood serum and the reduction of their expression levels have been associated with Alzheimer and Parkinson diseases [30, 32]. Moreover, both proteins have yet been described in blood cells like neutrophils [28, 50, 51] and platelets [52, 53], but only the mRNA of STX-1a (but not that of SNAP-25) was detected in PBMC [29].

First of all, we confirmed the presence of both proteins at peripheral levels and we found that SNAP-25 was present in serum and in both total extracellular vesicles and NDEs lysates, but not in PBMC, as already reported in the literature [29, 31]. On the other hand, STX-1a expression was observed in NDEs, while in total extracellular vesicles was almost undetectable by WB analyses, and surprisingly we found it expressed in PBMC. Although, it was reported that STX1a mRNA is present in PBMC [29], its protein expression in these cells is here described for the first time. Still, there are no available data about the role of STX1a in PBMC, although it is known that these cells are able to release cytokines [54], so potentially syntaxins protein family could be useful for the release machinery in these cells.

Regarding the cellular distribution of STX-1a, we observed, by mean of immunofluorescence and subcellular fractionations experiments, that it was expressed mainly in the cytosol. Interestingly, the partial colocalization of STX-1a with the GM130-Golgi apparatus marker led us to assume that STX-1a could be processed by the Golgi organelle during its in-situ syntheses. Moreover, another hypothesis on the cause of the presence of STX-1a in the PBMC could be that it is up-taken by the cells from the quote of the protein freely moving in the blood flow. Interestingly, we demonstrated that both monocytes and lymphocytes, the two main cellular sub-population of PBMC, express STX-1a. In line with our results, syntaxin proteins, but not STX-1a, were reported to play an important role in the cellular release of cholesterol and choline-phospholipids to apolipoprotein A-I (apoA-I) in monocytes by binding ATP-binding

cassette transporter A1 (ABCA1) [55], and in addition, STX-11 was found to regulate lymphocyte-mediated secretion and cytotoxicity [56].

We analyzed the peripheral expression of STX-1a and SNAP-25 in a cohort of IS patients that has been characterized by the classical IS clinical scales NIH-SS and BI as well as by MMSE [36] and CIRS [35] in order to also evaluate their cognitive function as well as to select patients with low comorbidity levels. Our data indicated that in all blood components analyzed, the levels of both SNARE proteins were significantly higher in IS patients compared to the two control populations (HC and Y-HC). In particular, we demonstrated that the level of STX-1a was increased in PBMCs of IS patients and no significant difference was observed between HC and Y-HC subjects leading us to speculate that the expression protein level fluctuation is related to the ischemic event rather than to the aging process. These results are in line with reported data showing that other brain pathologies stimulate up-regulation of proteins in PBMCs as for example happens for α-synuclein in Parkinson's disease patients [57] or the increase of mRNAs like IL-1 (Interleukin) beta, IL-8, and IL-17 mRNA in PBMC during brain ischemia [58]. Likewise, SNAP-25 levels were significantly increased in sera of IS patients compared to the age-matched healthy subjects and, interestingly, its expression level seems to be also influenced by aging processes, since HC subjects were characterized by significant higher SNAP-25 serum levels with respect to the younger population. The role of SNAP-25 in aging is so far not explored, however our findings suggest that its peripheral levels could reflect some physiological modification of brain synapses which occurs during the aging.

In addition, both STX-1a and SNAP-25 levels were significantly enriched in NDEs purified from sera of IS patients. The release of neuronal proteins in extracellular vesicles has been reported to show a potential use of their quantification as a biomarker for different pathologies like Alzheimer's in the case of SNAP-25 [30] and Parkinson's, for the presynaptic SNARE protein complex (i.e. STX-1a, SNAP-25 and VAMP-2) [32]. Moreover it has been demonstrated that STX-1a plays an important role in the regulation of the release of exosomes from the central nervous system [59] and, interestingly, other syntaxin isoforms were found to have a role mainly in exosomal secretion from different cell types [60, 61].

The Pearson's correlation analysis of STX-1a levels, in both PBMCs and NDEs, lead us to hypothesize that the presence of STX-1a is correlated with the IS pathology since it is associated with NIH-SS clinical scale, which is among the most frequent clinical and demographical scale associate with stroke mortality [33] and with BI, that measures the performance in activities of daily living [34]. However, we did not observe any correlation with the MMSE ,which is not a specific clinical scale for the IS, but an assessment of cognitive functions [36]. These results demonstrate that STX-1a not only is linked to the pathology onset but also correlated with its severity, being higher in patients with a more severe score in clinical scales. On the other hand, the levels of SNAP-25 never showed any statistical difference when compared with the clinical scales, probably the presence of these proteins is not strictly related to the severity of the IS (indeed, we also observed its blood accumulation in aging), but its peripheral levels increase is anyway associated with brain ischemia pathology.

All these findings prompted us to propose that the observed biological modifications of STX-1a and SNAP-25 could potentially represent the extent of brain damage and, consequently, they could be studied as potential prognostic biomarkers for IS. A deeper knowledge on the role of these two synaptic molecules in the peripheral blood samples could be used to support the clinical investigations on the severity of the brain damage occurring in IS and, after following studies, as a molecular predictor of the functional recovery of individuals affected by IS.

Conclusion

Our study demonstrates the SNARE proteins STX-1a and SNAP-25 are present in human blood fractions and to the best of our knowledge, this is the first time that the neuronal STX-1a protein is observed in PBMCs. The analysis of their expression levels showed that both the two SNARE proteins analyzed are augmented in the blood fractions of a cohort of IS patients, HC and Y-HC subjects. Interestingly, STX-1a increase, in both PBMCs and NDEs, always correlates with the severity of the pathology assessed by using the IS clinical scales NIH-SS and BI. Indeed, these findings might set the basis to study the synaptic plasticity changes, following an ischemic attack, at peripheral level and STX-1a and SNAP-25 could be considered as potential prognostic biomarkers for understanding the effects of rehabilitation interventions on the population affected by IS.

Abbreviations

ABCA1
ATP-Binding Cassette transporter A1
ApoA-I
Apolipoprotein A-I
ATP
Adenosine Tri-Phospahte
BI
Bartel Index
BSA
Bovine Serum Albumin
CCPP
Casa di Cura Privata del Policlinico
CIRS
Cumulative Illness Rating Scale
DMEM
Dulbecco’s Modified Eagle medium
FBS
Fetal Bovine Serum
GFAP

Glial Fibrillary Acid Protein

HC

Healthy Controls

HSP70

Heat Shock Protein 70

IgG

Immunoglobulin G

IL

Interlerukin

IS

Ischemic Stroke

K2EDTA

ethylenediaminetetraaceticacid-K2

L1CAM

L1 Cell Adhesion Molecule

Lp-PLA 2

Lipoprotein-associated Phospolipase A2

MMP-9

Matrix Metalloproteinase 9

mRNA

Messenger Ribonucleic Acid

MMSE

Mini-Mental State Examination

NDE

Neuronal Derived Extracellular Vesicles

NIH-SS

National Institutes of Health Stroke Scale

NSE

Neuron Specific Enolase

O.N.

Over Night

PBMC

Peripheral Blood Mononuclear Cells

PBS

Phosphate Buffered Saline

PSD95

Post Synaptic Density Protein 95

PVDF

polyvinylidene difluoride

RIPA

Radioimmunoprecipitation Assay Buffer

RPMI

Roswell Park Memorial Institute culture medium

R.T.

Room Temperature

S100β

S100 calcium-binding protein B

SDS-PAGE

sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SNAP-25

Synaptosomal Associated Protein, 25 kDa

SNARE

Soluble N-Ethylmaleimide-Sensitive Fusion Protein Attachment Protein Receptor

STX

Syntaxin

SV-2a

Synaptic vesicle Glycoprotein 2a

TIA

Transient Ischemic Attack

TBS

Tris-Buffered Saline

TEM

Transmission Electron Microscopy

WB

Western Blot

Y-HC

Young Healthy Controls

Declarations

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Author's contributions: Conceptualization was performed by [Marco Feligioni], data curation and methodology were performed by [Pamela Cappelletti and Marco Feligioni], formal analysis, investigation and writing-original draft- were performed by [Pamela Cappelletti], funding acquisition, project administration, supervision and writing-review and editing- were performed by [Massimo Corbo and

Marco Feligioni], TEM investigation was performed by [Laura Masuelli and Roberto Bei], blood sample resources was performed by [Melania Filareti], writing-review and editing- was performed by [Kambiz Hassanzadeh].

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethic approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Milan: n ° 285_2019 bis for sample collection and n° 729-2021 for the experimental study.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent to publish: Patients signed informed consent regarding publishing their data.

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Figures

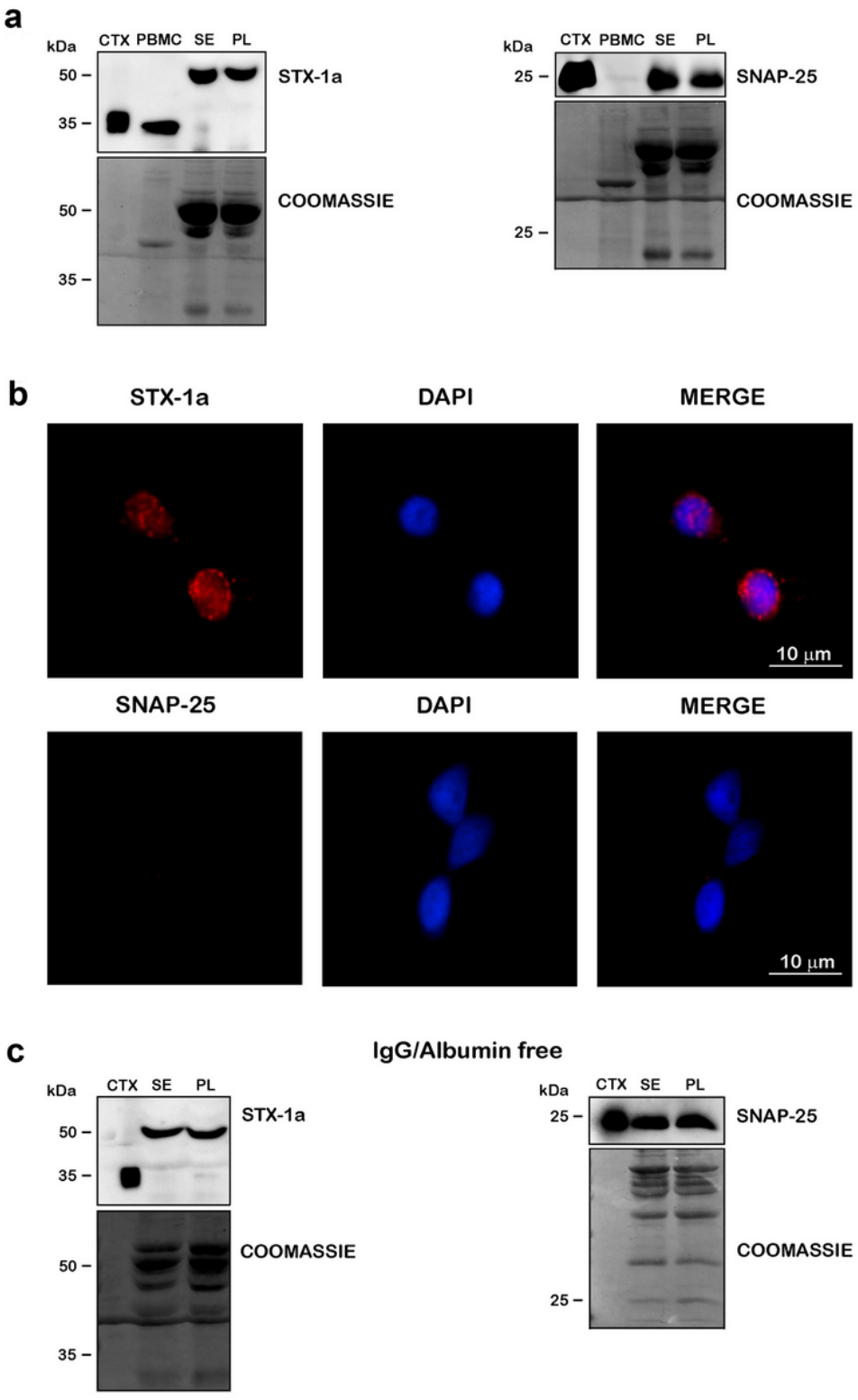

Figure 1

Detection of STX-1a and SNAP-25 in peripheral blood of healthy donors. a) WB analysis of the SNARE proteins in blood components. PBMCs, serum (SE), plasma (PL) and mouse brain cortex lysate (CTX, loaded as positive control) have been immunostained with STX-1a and SNAP-25 antibodies. STX-1a was

detected in all blood components, but only in PBMCs at the predicted M.W., while SNAP-25 was observed in serum and plasma but not in blood cells. b) Immunofluorescence analysis of STX-1a and SNAP-25 in PBMCs. Fluorescence analysis of PBMCs confirms the presence of STX-1a but not that of SNAP-25 (red channels). Nuclei have been labeled with the marker DAPI (blue channels). Original magnification: 60X. Bars correspond to 10 μm. c) WB analysis of STX-1a and SNAP-25 in serum and plasma depleted from IgG and albumin. After the stripping of IgG and albumin from serum and plasma (as shown by Coomassie Blue staining) both STX-1a (at the shifted M.W) and SNAP-25 immune-recognition signals persist. For WB analysis 100 μg of blood components, 0.5 μg of mouse brain cortex lysate and 10 μL of flow through have been loaded in each lane

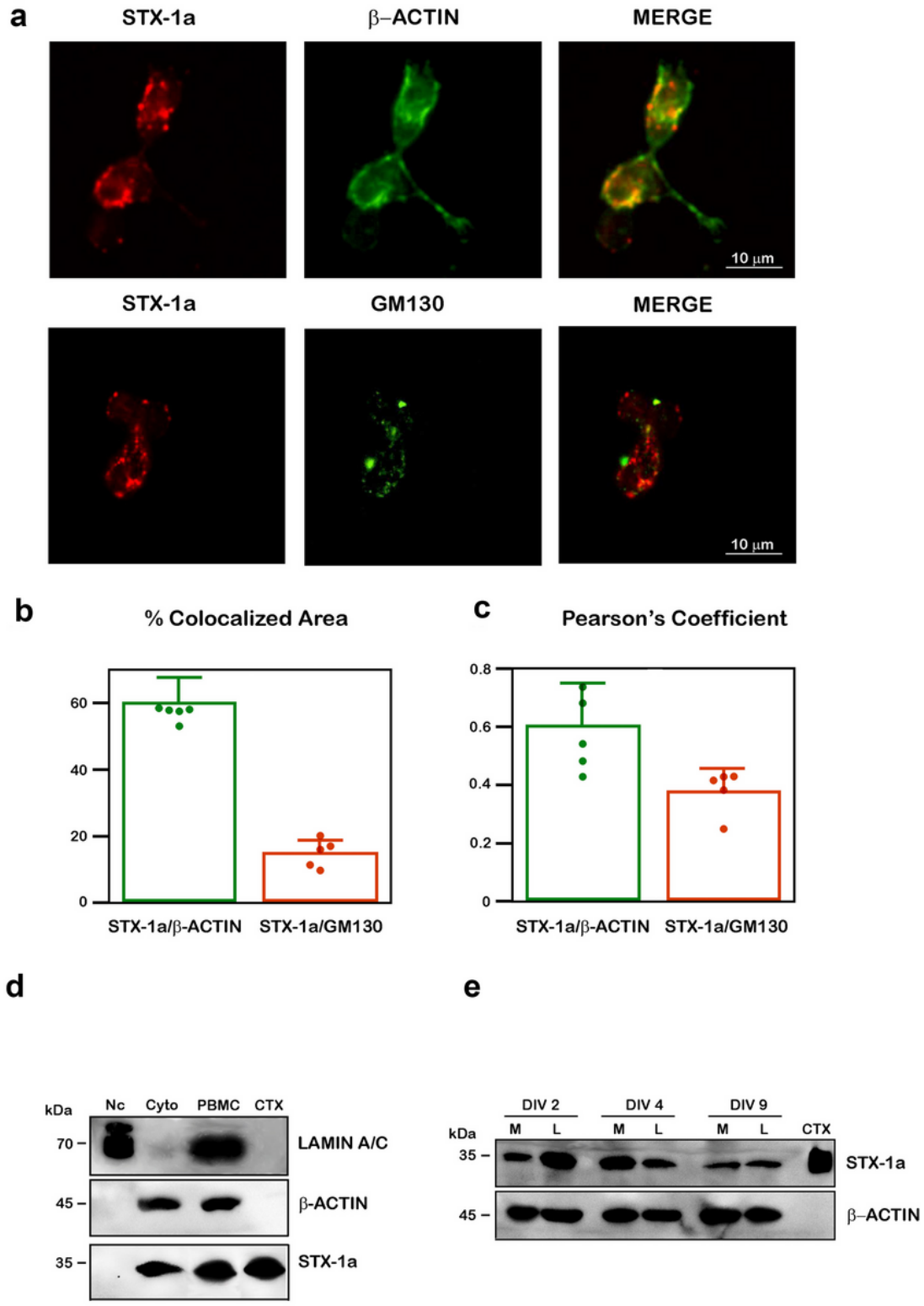

Figure 2

Analysis of the subcellular and cellular localization of STX-1a in PBMCs of healthy donors. a) IF analysis of the cellular distribution of STX-1a in PBMCs. PBMCs have been immunostained with the cytoplasmic marker β-actin (upper panel) or the Golgi apparatus marker GM130 (lower panel) (green channels) and STX-1a (red channels). In the merge images, a clear colocalization of STX-1a with β-actin is evident thus indicating a cytoplasmic localization of the SNARE protein in PBMCs. A partial colocalization of STX-1a

and GM130 is also shown. Original magnification: 60X. Bars correspond to 10 μm. b) Colocalization analysis. Histogram showing the percentage of colocalized area compared to the total fluorescent area. c) Pearson's Coefficient. Histogram showing Pearson's Coefficient. Green points and histograms represents the % of colocalization area (b) and the Pearson's Coefficient (c) of STX-1a compared to β-actin, orange points and histograms represents the % of colocalization area (b) and the Pearson's Coefficient (c) of STX-1a compared to GM130. d) PBMC's subcellular fractions analysis. WB analysis confirmed the cytoplasmic localization of STX-1a in PBMCs. The successful separation of the nuclear (Nc) and the cytoplasmic fractions (Cyto) of PBMCs is demonstrated by the presence of the nuclear marker lamin A/C only in the nuclear fraction and that of β-actin only in the cytoplasmic one. e) STX-1a is expressed in both major PBMCs subtypes in cultures. WB analysis reveals that STX-1a is expressed in both monocytes (M) and lymphocytes (L) and that its expression is maintained over time in cultured cells. For IF analysis at least 15 cells for each subject (n=5) have been analyzed. For WB analysis 4 μL of PBMCs subcellular fractions (i.e. Nc and Cyto), 100 μg of whole PBMCs, 0.5 μg of mouse brain cortex lysate and 2X10^5 of cultured PBMCs have been loaded in each lane

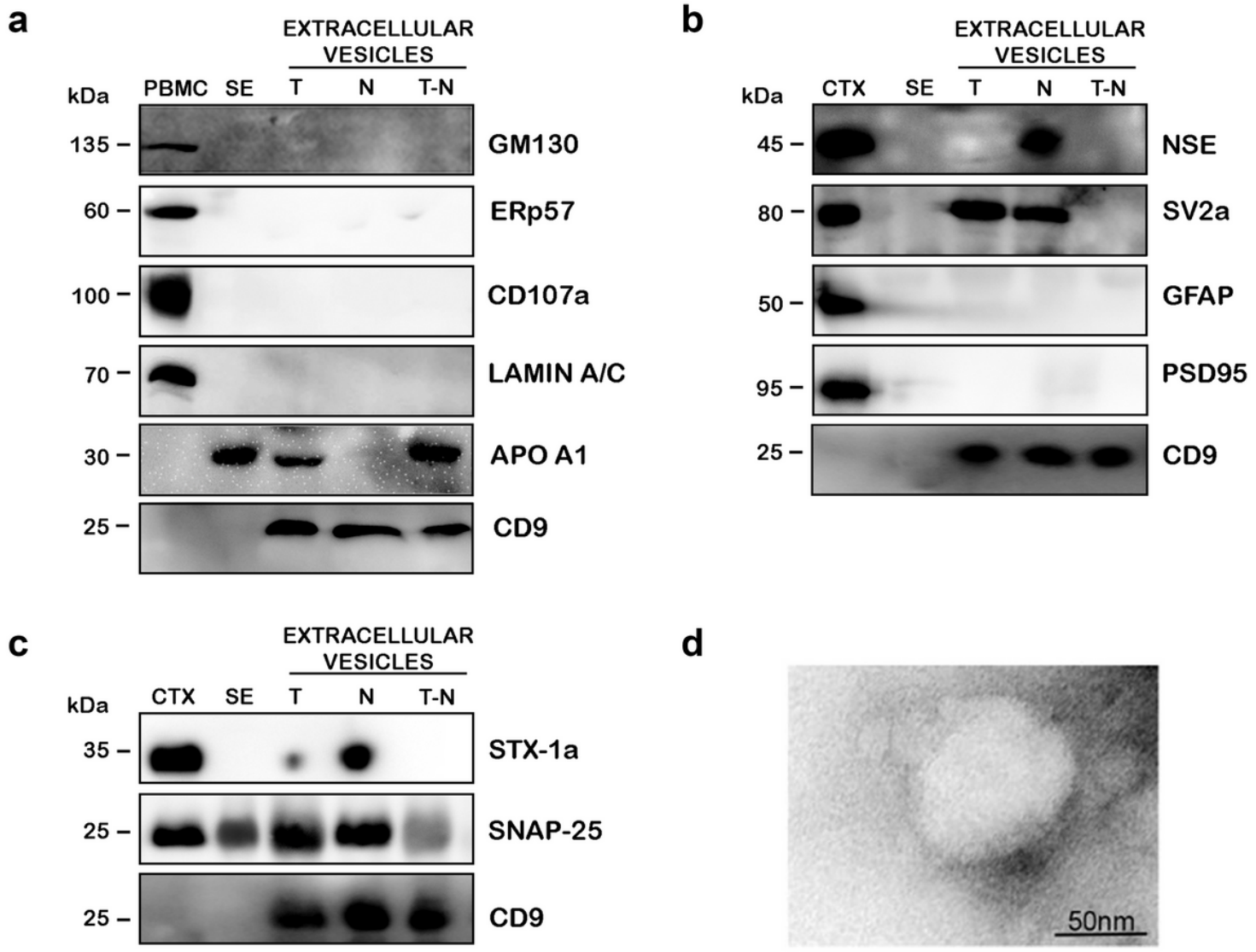

Figure 3

WB analysis of a representative NDE purification from the serum of healthy donors. a) Quality analysis of extracellular vesicle preparation. Extracellular vesicles samples are not contaminated by Golgi apparatus (GM130), endoplasmic reticulum (Erp57), lysosomes (CD107) and nucleus (lamin A/C). The presence of the HDL marker APO A1 is detectable in both total extracellular vesicles (T) and total extracellular vesicles depleted to NDE (T-N), but disappear in NDEs fractions (N). CD9 is a common exosome marker. b) NDE characterization. WB analysis show an enrichment of neuronal markers (NSE and SV-2a) in NDEs with respect to the other two extracellular vesicles fractions. Moreover, NDE samples are negative to the astrocytes and the post synaptic markers GFAP and PSD95, respectively. c) STX-1a and SNAP-25 are enriched in NDE fractions. The levels of both SNARE proteins are increased in NDE with respect to the two other extracellular vesicles fractions. d) Representative EM analysis of NDE. The ultrastructural analysis of NDE shows the presence of rounded-shaped vesicles with a diameter range of 70–100 nm. Bars correspond to 50nm. For WB analysis 100 µg of whole PBMCs, 50 µg of serum (SE), extracellular vesicles fractions (T, N, T-N) and 0.5 µg of mouse brain cortex lysate have been loaded in each lane

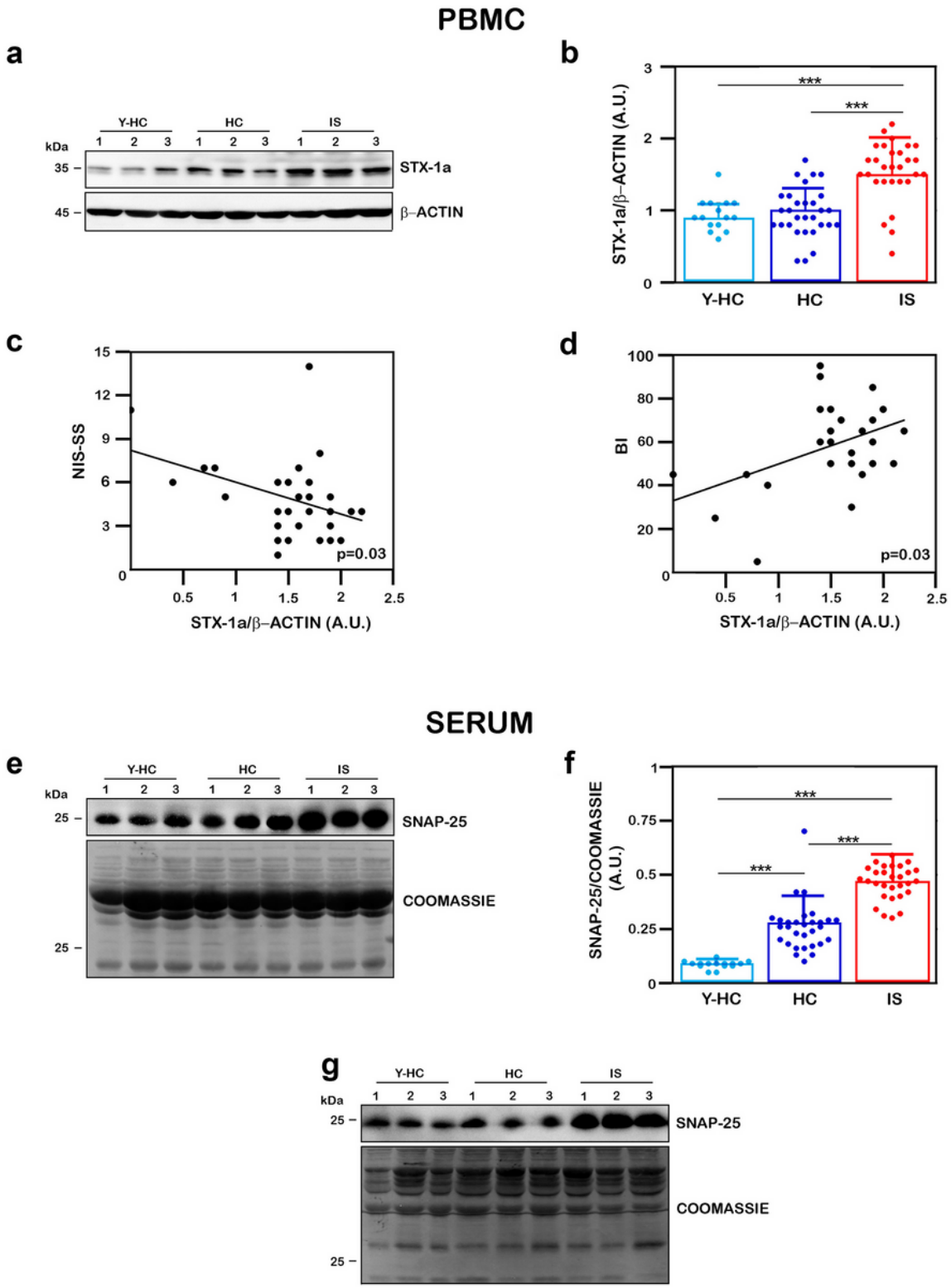

Figure 4

Quantitative analysis of STX-1a in PBMCs and of SNAP-25 in sera in all population analyzed. a, e) Representative WB analysis of STX-1a in PBMCs (a) and of SNAP-25 in serum (e) in IS and HC subjects. PBMCs are immunostained for STX-1a (a) and serum for SNAP-25 (e). For each sample, the STX-1a densitometric value is normalized to that of β-actin, while the SNAP-25 densitometric values is normalized to that of total blood proteins content revealed by Coomassie Blue staining. b) Densitometric

analysis STX-1a expression levels in PBMCs. STX-1a expression levels have been analyzed by using One-way ANOVA with post-hoc Tukey test: STX-1a significantly increase in PBMCs of IS patients with respect to HC and Y-HC subjects ($p \leq 0.0001$), while no differences are observed between control groups ($p=0.97$). c, d) Pearson's correlation analysis between expression of STX-1a in IS PBMC and the clinical stroke scales. STX-1a levels in PBMCs of IS patients present a moderate negative correlation with NIH-SS ($p=0.03$) (c) and a moderate positive correlation with BI (d) ($p=0.03$). f) Densitometric analysis SNAP-25 expression levels in sera. SNAP-25 expression levels have been analyzed by using One-way ANOVA with post-hoc Tukey test: SNAP-25 levels are significantly increased in serum of IS patients with respect to HC and Y-HC subjects ($p \leq 0.0001$). A significant increase of SNAP-25 expression is also observed in HC with respect to Y-HC subjects ($p \leq 0.0001$). g) WB analysis of SNAP-25 in sera depleted of IgG and albumin. The increased SNAP-25 levels in IS patient are not influenced by the presence of serum proteins. For WB analysis 100 µg of whole PBMCs and of serum (SE) have been loaded in each lane. In quantitative graphs (panels b and f) each point in a frame depicts the value for a single subject (n=30 IS patients, 30 HC subjects and 15 young HC subjects) while bars represent the median value ± SD. ***$p \leq 0.001$. In panels b and f light blue points and histograms correspond to Y-HC subjects, blue points and histograms correspond to HC subjects, red points and histograms correspond to IS patients

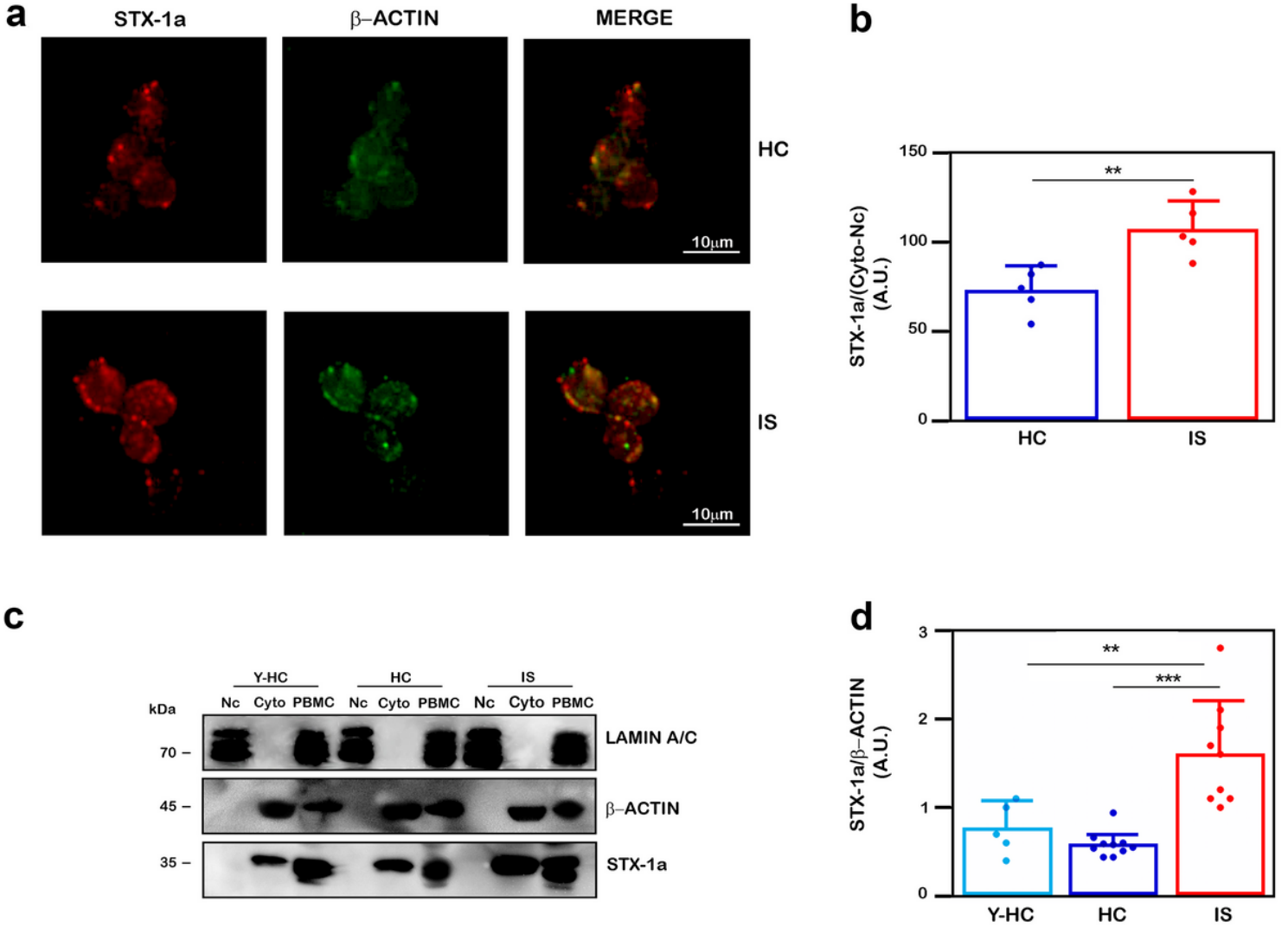

Figure 5

Confirmation of increased STX-1a levels in PBMCs of IS patients with respect to HC subjects. a) Representative Immunofluorescence analysis of STX-1a in PBMCs of IS patients and HC subjects. PBMCs have been labeled with β-actin (green) and STX-1a (red). The STX-1a fluorescence intensity has been normalized by dividing the measure of its intensity by that of the β-actin subtracted from that measured for the nucleus (DAPI immunostaining). b) Quantitative analysis STX-1a levels in PBMCs. The normalized STX-1a expression levels have been analyzed by using One-way ANOVA with post-hoc Tukey test: STX-1a levels are significantly increased in PBMCs of IS patients with respect to HC subjects ($p=0.005$). Original magnification: 60X. Bars correspond to 10 μm. c) Representative WB of STX-1a expression levels in PBMC cytoplasmic fractions. Representative WB analysis of cytoplasmic and nuclear fractions of PBMCs. For each sample, the STX-1a densitometric values have been normalized to that of β-actin. The successful separation of the nuclear (Nc) and the cytoplasmic fractions (Cyto) of PBMCs is demonstrated by the presence of the nuclear marker lamin A/C in the nuclear fraction and that of β-actin in the cytoplasmic one. d) Quantitative analysis of cytoplasmic STX-1a. STX-1a expression levels have been analyzed by using One-way ANOVA with post-hoc Tukey test: STX-1a significantly increase in PBMCs of IS patients with respect to HC ($p\leq0.0001$) and Y-HC ($p\leq0.003$) subjects, while no difference is observed between the two control groups ($p=0.7$). For IF analysis at least 15 cells for each subject have been analyzed. For WB analysis 4 μL of each fraction (Nc and Cyto) and 100 μg of whole PBMCs have been loaded in each lane. In quantitative graphs (panels b and d) each point in a frame depicts the value for a single subject (n=5-10 IS patients, 5-10 HC subjects and 0-5 young HC subjects, respectively in b and d) while bars represent the median value ± SD. **$p\leq0.005$; ***$p\leq0.001$. In panels b and d light blue points and histograms correspond to Y-HC subjects, blue points and histograms correspond to HC subjects, red points and histograms corresponds to IS patients

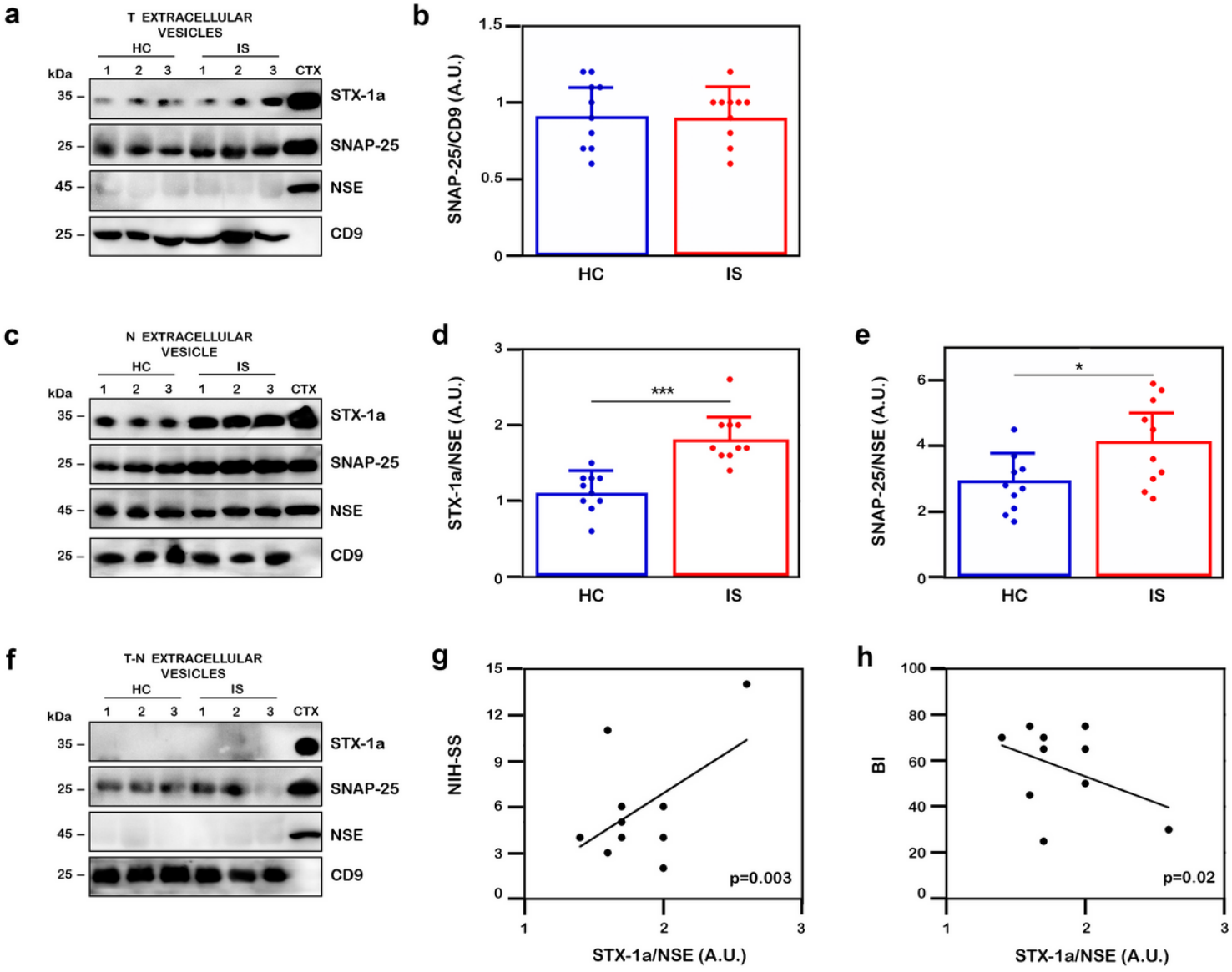

Figure 6

Analysis of SNARE in extracellular vesicles of IS and HC subjects. a, c, f) Representative WB analysis of the expression levels of STX-1a and of SNAP-25 in extracellular vesicles derived from sera. Representative WB analysis of total (T) extracellular vesicles (a), NDE (N) (c) and total extracellular vesicles depleted of NDE (T-N) (f) fractions. Each sample has been tested against CD9 (total exosomes marker), NSE (NDE marker), STX-1a and SNAP-25. The SNAP-25 densitometric values in total extracellular vesicles fraction have been normalized to that of CD9 (a), while the STX-1a and SNAP-25 densitometric values in NDE have been normalized to that of NSE (c). b, d, e) Densitometric analysis of STX-1a and SNAP-25 in extracellular vesicles The normalized SNAP-25 levels in T extracellular vesicles (b) and those of both STX-1a (d) and SNAP-25 (e) in NDE have been analyzed by using One-way ANOVA with post-hoc Tukey test. Both STX-1a and SNAP-25 expression levels are significantly increased in NDE of IS patients with respect to HC subjects ($p \leq 0.0001$ and $p \leq 0.02$ for STX-1a (d) and SNAP-25 (e) respectively), while no differences is observed for SNAP-25 in the T fraction ($p=0.91$) (b). g, h) Pearson's correlation analysis between expression of STX-1a in IS NDEs and the clinical stroke. STX-1a levels in

NDE of IS patients present a moderate positive correlation with NIH-SS (p=0.003) (g) and a moderate negative correlation with BI (p=0.02) (h). For WB analysis 50 µg of each extracellular vesicles fraction and 0.5 µg of mouse brain cortex lysate have been loaded in each lane. In quantitative graphs (panels b, d and e) each point in a frame depicts the value for a single subject (n=10 IS patients and 10 HC subjects) while bars represent the median value ± SD. *p≤0.05; ***p≤0.001. In panels b, d and e blue points and histograms correspond to HC subjects, red points and histograms correspond to IS patients

Supplementary Files

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