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SHORT COMMUNICATION

Circulating extracellular vesicles as potential biomarkers in chronic fatigue syndrome/myalgic encephalomyelitis: an exploratory pilot study

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ABSTRACT

Chronic Fatigue Syndrome (CFS), also known as Myalgic Encephalomyelitis (ME) is an acquired, complex and multisystem condition of unknown etiology, no established diagnostic lab tests and no universally FDA-approved drugs for treatment. CFS/ME is characterised by unexplicable disabling fatigue and is often also associated with numerous core symptoms. A growing body of evidence suggests that extracellular vesicles (EVs) play a role in cell-to-cell communication, and are involved in both physiological and pathological processes. To date, no data on EV biology in CFS/ME are as yet available. The aim of this study was to isolate and characterise blood-derived EVs in CFS/ME. Blood samples were collected from 10 Spanish CFS/ME patients and 5 matched healthy controls (HCs), and EVs were isolated from the serum using a polymer-based method. Their protein cargo, size distribution and concentration were measured by Western blot and nanoparticle tracking analysis. Furthermore, EVs were detected using a lateral flow immunoassay based on biomarkers CD9 and CD63. We found that the amount of EV-enriched fraction was significantly higher in CFS/ME subjects than in HCs ($p = 0.007$) and that EVs were significantly smaller in CFS/ME patients ($p = 0.014$). Circulating EVs could be an emerging tool for biomedical research in CFS/ME. These findings provide preliminary evidence that blood-derived EVs may distinguish CFS/ME patients from HCs. This will allow offer new opportunities and also may open a new door to identifying novel potential biomarkers and therapeutic approaches for the condition.

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Introduction

Chronic Fatigue Syndrome (CFS), also known as Myalgic Encephalomyelitis (ME), is a complex, multisystem and metabolic-neuroinflammatory condition with genetic predisposition of unknown etiology characterised by prolonged and disabling chronic fatigue, which worsens with physical and cognitive exertion. CFS/ME may also be accompanied by other cardinal symptoms that include orthostatic intolerance, unrefreshing sleep, gastrointestinal discomfort, neuropsychological impairment, anxiety/depression, chronic pain and post-exertional malaise lasting more than 24 h. CFS/ME occurs more often in women than in men and is less common among children and adolescents. Currently, no established diagnostic laboratory tests exist, nor are any FDA-approved drugs available for treatment [1].

Over the past decade, many studies have been conducted on Extracellular Vesicles (EVs) in the field of biomedical research. EVs secretion and EV-mediated pathways are known to be important players in a variety of biological processes under physiological and pathological conditions. EVs cargo from donor cells may interact with recipient cells, making them potential intercellular mediators. In fact, several proteins such as heat shock proteins, integrins and tetraspanins can be found in all EVs [2,3]. Moreover, tetraspanins CD9, CD63 and CD81 are commonly used as conventional EV markers since they are enriched in their membranes [3–5].

In addition, EVs may be altered in number and composition in several conditions [6–9], and they have emerging as potential biomarkers of diagnosis and prognosis [10]. To date, there are no published reports of EVs isolation and characterisation in CFS/ME. As the pathomechanisms of CFS/ME described in the literature [11], may promote EVs release, we proposed to characterise circulating EVs in CFS/ME to

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assess their new potential as biomarkers for the condition.

In this proof of concept study, we isolated human serum-derived EVs from CFS/ME subjects and healthy controls (HCs) in order to characterise them in terms of size and concentration. We also analysed their composition using a lateral flow immunoassay (LFIA) system optimised for rapid on-site detection.

Patients and methods

Ethics committee

The study was approved by the local Institutional Review Board and Human Research Ethics Committee of the Vall d'Hebron University Hospital Institute. All participants provided written informed consent prior to participation in the study.

Participants

A total of 10 potentially eligible Caucasian females with CFS/ME (mean age ± SD; 46.3 ± 8.5 years) according to the 1994 CDC/Fukuda definition [12] were recruited from a single outpatient tertiary referral centre (CFS/ME Unit, Vall d'Hebron University Hospital, Barcelona, Spain). Five matched sedentary non-fatigued healthy controls (HCs) (mean age ± SD; 44.2 ± 6.2 years) were also included. Exclusion criteria were comorbid psychiatric illness, endocrino-metabolic, haematological and autoimmune disorders, CVD, pregnancy or breast-feeding, drug abuse, smoking or symptoms of CFS/ME that did not conform to the 1994 CDC/Fukuda definition.

Sample collection and processing

Fresh blood samples were collected at the local site between 8 and 9 a.m. after fasting for at least 12 h. Blood samples were collected from antecubital vein by venipuncture with a 21-gauge needle in a 10 ml BD Vacutainer® Plus serum tube after discarding the first millilitre. All tubes were kept upright and allowed to clot for 30 min. Serum was separated after centrifugation at 1,500 *x g* for 15 min at RT within the first hour (Heraeus Megafuge 16R centrifuge with a 75,003,629 rotor, Thermo Scientific, Madrid, Spain). All 1.5 ml aliquots were snap-frozen in liquid nitrogen, and then stored at −80ºC until further analysis. Frozen serum aliquots in dry-ice from all participants were sent to University of Oviedo (Asturias, Spain) for further isolation and characterisation of EVs.

Isolation of EVs

EVs were purified using the ExoQuick™ precipitation solution (System Biosciences) according to the manufacturer's instructions. Briefly, 250 µl of serum from all subjects were centrifuged at 3,200 *x g* for 15 min at RT to remove platelets and cell debris and mixed with ExoQuick Precipitation Solution. EVs were precipitated by low speed centrifugation (1,500 *x g*) and resuspended in the appropriate buffer following the manufacturer's instructions.

EVs characterisation

Protein quantification and western blot analysis

Protein concentration of the EV fractions was measured by bicinchoninic acid (BCA) assay kit (Thermo Scientific, Waltham, MA, USA). The EV fractions were homogenised with 1× RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% Na-Deoxycholate, 0.1% SDS) containing proteases and phosphatases inhibitors. Equal volumes of each sample were mixed with reducing 1x Laemmli-buffer, loaded and run on 12% SDS-PAGE gel under non-reducing conditions. Proteins were transferred to PVDF membrane (Amersham; GE Healthcare, Munich, Germany). Membranes were blocked in 5% non-fat dry milk using TBS containing 0.1% Tween-20 (TBS-T) and then probed with polyclonal anti-CD63 diluted 1:500 (Santa Cruz Biotech; Santa Cruz, CA, USA) overnight at +4ºC with gently rocking. Membranes were washed with TBS-T for 3 times and 10 min each, and finally HRP-conjugated secondary antibodies diluted 1:2000 (Dako, Glostrup, Denmark) were added for 1 h at room temperature. Blots were developed using the ECL detection system (GE Healthcare, Madrid, Spain).

Nanoparticle tracking analysis

Concentration and size distribution of the isolated EVs were determined by NanoSight LM10 instrument (Malvern, Worcestershire, UK) and nanoparticle tracking analysis (NTA) 3.1 software at NanoVex Biotechnologies S.L (Madrid, Spain). Each EV fraction was diluted between 1:1000 and 1:200,000 in 10 mM HEPES pH 7.4 to achieve a particle concentration ranging from 10^6 to 10^9 particles/ml. Three recordings were performed at 25ºC for each sample. Zetasizer software version 7.03 was used for data processing and analysis.

Lateral flow immunoassay

Detection of purified EVs from CFS/ME patients and HCs by single-targeted LFIA was performed as

previously described [13], using the following monoclonal antibodies: anti-CD9 (clone VJ1/20) and anti-CD63 (clone Tea3/18) (both from Immunostep, Madrid, Spain). Briefly, EV samples were homogenised with the detection antibody conjugated to gold nanoparticles (AuNP-anti-CD63). Anti-CD9 immobilised on the strip was used as capture antibody. Then the immunostrip was added and the samples were allowed to run for 15 min. Unbound AuNP-conjugated migrated further to be captured with an anti-IgG antibody, and this was used as system functional verification. The signal intensities were measured with the ESEQuant LR3 lateral flow strip reader (Qiagen, Madrid, Spain). For more details regarding the nanoparticle-antibody conjugation and the preparation of the immunostrip, see the studies previously published by Oliveira-Rodríguez et al. [14,15].

Statistical analysis

All statistical data analyses were performed with the freely available software R.3.1 (www.r-project.org). Data were analysed by Mann–Whitney U test (non-parametric test), and $p < 0.05$ was considered as statistically significant.

Results and discussion

Determination of protein amount and detection of CD63 in EV fractions

EVs were isolated from 250 µl of serum of 10 CFS/ME patients and five matched-HCs. The amount of protein in the EV fractions was determined (Figure 1(a)). We found significantly higher protein content in CFS/ME patients than in controls (mean ± SD, 65.49 ± 10.75 mg/ml vs. 43.62 ± 5.10 mg/ml; $p = 0.001$, respectively). The Bradford assay is commonly used to determine the protein content of isolated EVs and to estimate the amount of circulating EVs [13]. Moreover, analysis of the EV protein content may differentiate between CFS/ME individuals and controls. The content of EVs has been reported to be higher in certain CNS disorders [16], which may serve as a biomarker in the development of new diagnostic tools for early stages of CFS/ME. In our study, a significant abundance of blood-derived EVs in CFS/ME subjects was shown by a 1.5-fold increase on average compared to HCs.

We also determined the presence of the commonly used marker CD63 in the purified EV fractions of CFS/ME patients and healthy controls. In addition, since our LFIA system for the detection of EVs uses anti-CD63 as detection antibody, we checked that the levels of this tetraspanin in our CFS/ME cohort were

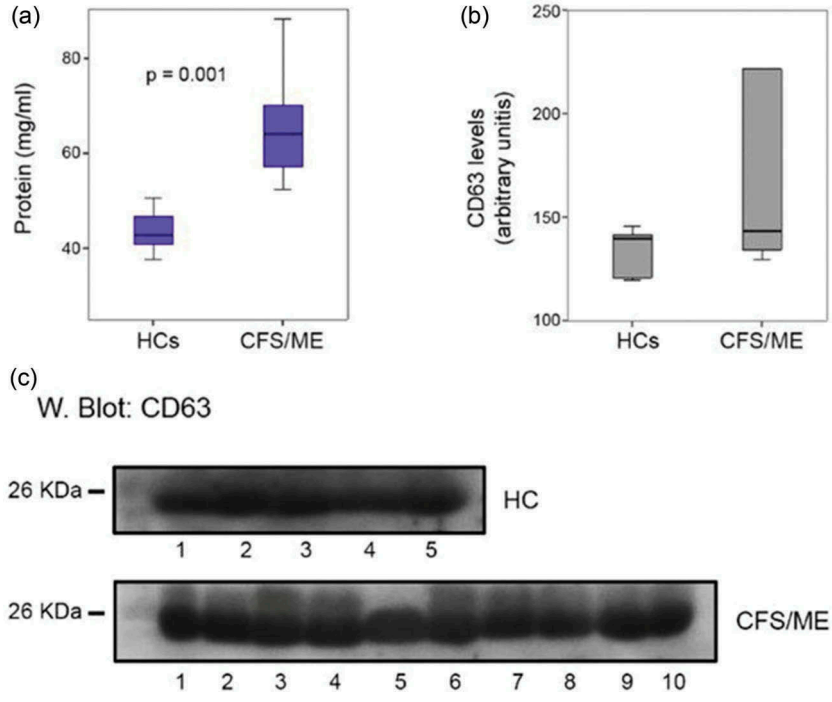

Figure 1. (a) Box plot for the protein concentration of the EV fractions isolated in 10 CFS/ME patients and 5 healthy controls (HCs). (b) Box plot for the measurement of CD63 levels by optical density (O.D.) in CFS/ME individuals ($n = 10$) and HCs ($n = 5$). (c) Detection of CD63 (25 kDa) in EV fractions from CFS/ME patients and HCs by western blotting (left lane shows 26 kDa band corresponding to the molecular weight marker).

sufficient to use this approach. Equal volumes of each sample were analysed by Western blot (Figure 1(b)). Levels of CD63 were also higher in CFS/ME patients than in the control group (Figure 1(c)), although the difference did not reach statistical significance.

Characterization of EVs from CFS/ME patients and healthy controls

Isolated EV fractions were further characterised for the analysis of size distribution and concentration by NTA (Figure 2(a)). The mean diameter measured in both groups is shown in Figure 2(b). The population of particles was quite homogeneous in terms of size of circulating EVs from CFS/ME patients (range 103–183 nm), but EVs isolated from HCs were more heterogeneous, with diameter sizes ranging from 140 to 271 nm. In fact, EVs from the control group were significantly larger than those from CFS/ME subjects ($p = 0.014$). Regarding the concentration of the isolated EV fractions, we found larger number of particles in CFS/ME patients than in controls (10^{15} particles/ml vs. $4.88x10^{12}$ particles/ml; $p = 0.007$, respectively) (Figure 2(c)).

It is currently accepted that the size of EVs of endocytic origin (commonly known as exosomes) is typically in the range of 30–150 nm [5,17,18]. Our results indicate that EV fractions from CFS/ME patients may be rich in exosomes in comparison with HCs, in whom we found larger diameters that may correspond mostly to microvesicles. EVs are emerging targets for biomedical research and these findings show the first evidence that the number of circulating EVs may distinguish between CFS/ME patients and healthy controls.

Detection of EVs expressing tetraspanin CD9 by LFIA

In the study of EV biology, their isolation is as challenging as their detection. In this respect, LFIA provides a powerful tool for low-cost on-site detection. In this study, subsequent LFIA analysis was performed to allow rapid detection of circulating EVs upon isolation [19]. Anti-CD63 was used as detection antibody and anti-CD9 as the capture antibody printed in the test line, anti-IgG was used for the control line [14]. Figure 3(a) shows a representative example of the results obtained with EV fractions isolated from a CFS/ME patient and a healthy control subject. EV-depleted serum was used as a negative control. Unbound anti-CD63-AuNPs captured with anti-IgG were used for system functional verification.

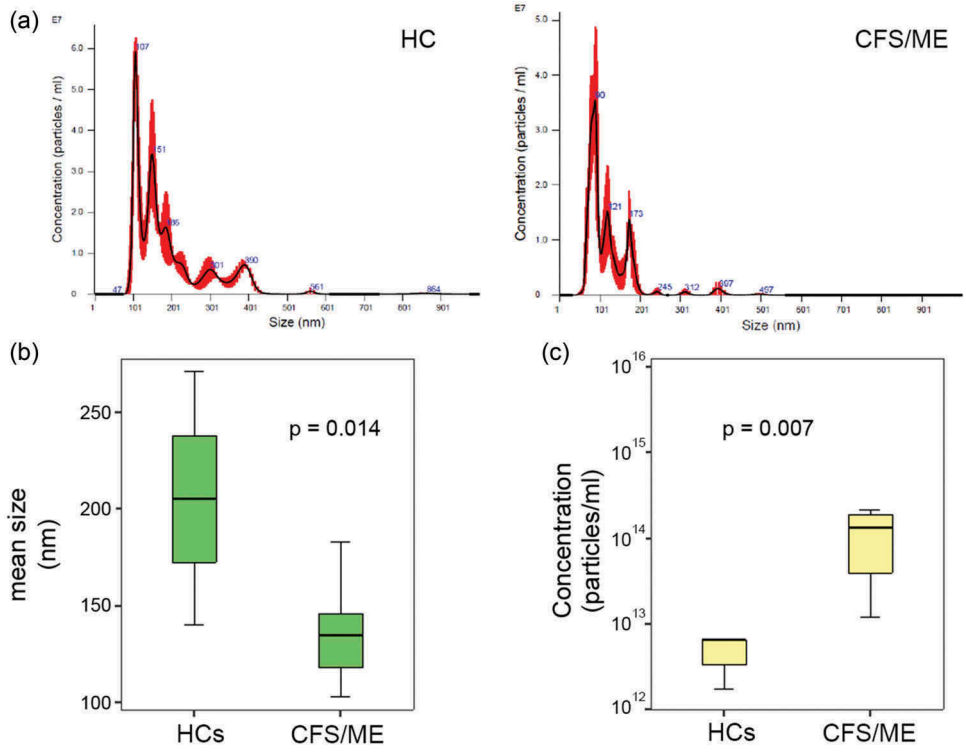

Figure 2. Characterization of isolated EVs by NTA. (a) Representative graph depicting the hydrodynamic size distribution profiles of isolated EVs from a CFS/ME subject and an HC, measured by NTA. (b) Box plots for the diameter sizes and (c) particle concentration of the EV fractions of study participants.

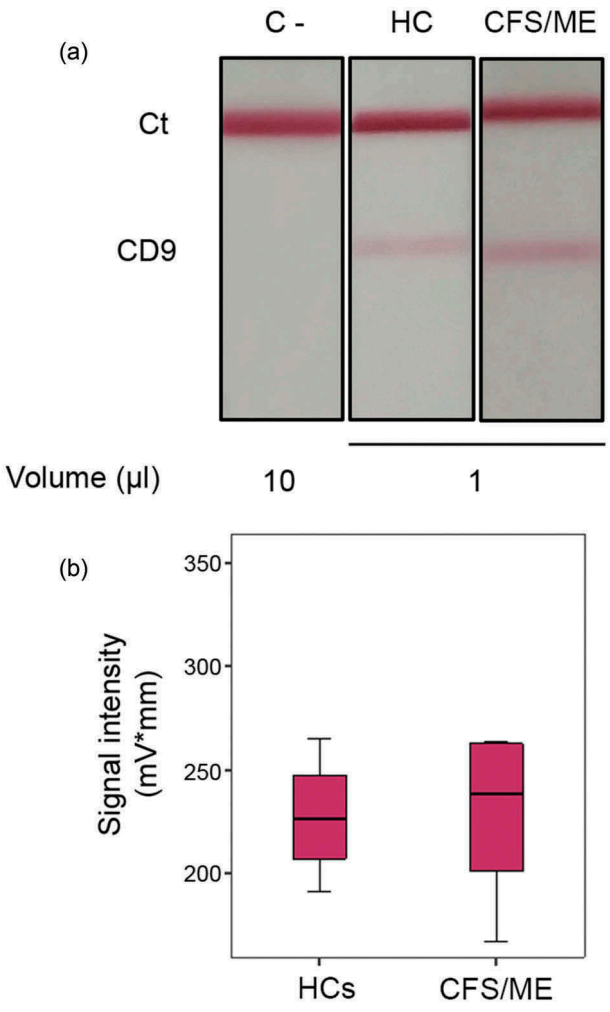

Figure 3. Detection of serum isolated-EVs using anti-CD9 as capture antibody and AuNP-anti-CD63 as detection probe. (a) Representative immunostrip test obtained of HCs and CFS/ME individuals. EV-depleted serum was used as negative control (C-). Control line (Ct). Test line (CD9). (b) Box plots for the signal intensities measured with the ESEQuant LR3 lateral flow strip reader of study participants.

Optical signal intensities were evaluated by reflectance measurements. It is worth noting that only 1 µl of the fractions was required to obtain a visible signal (red line) and therefore to detect EVs. We found no significant differences regarding the intensity of the CD9 signal on EV-enriched fractions between CFS/ME patients and HCs as shown in Figure 3(b). This implies questioning the use of CD9 as a standard exosomal marker on CFS/ME EVs; since the expression level of this tetraspanin was not significantly differ between both groups.

The LFIA platform was used as an initial approach and it is suitable for rapid detection of EVs, as previously reported [13,16]; in addition, our LFIA system can be further optimised or adapted to detect other biomarkers of interest. Therefore, we consider it to be a good starting point. Nevertheless, further studies with more CFS/ME patients and healthy controls are needed to explore possible differences in circulating EVs regarding the presence of other tetraspanins or other EV-encapsulated markers such as DNA, RNA species, lipids, proteins, cytokines and metabolites among others.

Conclusions

This report is the first show that numbers of blood-derived EVs are elevated in patients suffering from CFS/ME, indicating their potential involvement in disease pathogenesis. This promising finding may not only provide insights into the mechanisms involved in the disease but also shows that EVs may be useful for early diagnosis of illness. Moreover, isolation of circulating EVs coupled to our prototype for their detection by LFIA may constitute a powerful diagnostic tool, which can be performed in a single step and in minutes. We concluded that EVs may play a critical role in CFS/ME. Studies with larger sample size, outcome measures and different study designs (i.e. cross-sectional vs. longitudinal cohorts) are now urgently needed. These studies should stratify subgroups according to illness onset and progression, and assess patients at baseline and following induction of post-exertional malaise (PEM), using the 2-day cardiopulmonary exercise test (CPET).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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