ORIGINAL ARTICLE

Extracellular vesicles in biological fluids. A biomarker of exposure to cigarette smoke and treatment with chemopreventive drugs

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Keywords

Extracellular vesicles • Cigarette smoke • Celecoxib • Bronchoalveolar lavage fluid • Blood serum • Urines

Summary

Extracellular vesicles (EVs) are released from cells and enter into body fluids thereby providing a toxicological mechanism of cell-cell communication. The present study aimed at assessing (a) the presence of EVs in mouse body fluids under physiological conditions, (b) the effect of exposure of mice to cigarette smoke for 8 weeks, and (c) modulation of smoke-related alterations by the nonsteroidal anti-inflammatory drug celecoxib, a selective cyclooxygenase-2 inhibitor. To this purpose, ICR (CD-1) mice were either unexposed or exposed to cigarette smoke, either treated or untreated with oral celecoxib. EVs, isolated from bronchoalveolar lavage fluid (BALF), blood serum, and urines, were analyzed by nanoparticle tracking analysis and flow cytometry. EVs baseline concentrations in BALF were remarkably high. Larger EVs were detected in urines. Smoking increased EVs concentrations but only in BALF. Celecoxib remarkably increased EVs concentrations in the blood serum of both male and female smoking mice. The concentration of EVs positive for EpCAM, a mediator of cell-cell adhesion in epithelia playing a role in tumorigenesis, was much higher in urines than in BALF, and celecoxib significantly decreased their concentration. Thus, the effects of smoke on EVs concentrations were well detectable in the extracellular environment of the respiratory tract, where they could behave as delivery carriers to target cells. Celecoxib exerted both protective mechanisms in the urinary tract and adverse systemic effects of likely hepatotoxic origin in smoke-exposed mice. Detection of EVs in body fluids may provide an early diagnostic tool and an end-point exploitable for preventive medicine strategies.

Introduction

Extracellular vesicles (EVs) are spherical structures with a lipid bilayer, which include exosomes, having a size of 30-100 nm, microvesicles (MVs), having a size of 100-700 nm, and apoptotic bodies, having a size up to 5,000 nm, which contain cell organelles and nuclear components [1, 2]. The International Society for Extracellular Vesicles proposed a series of criteria, based on current best practice, that represent the minimal characterization of EVs [3]. Exosomes are constitutively generated, stored, and released from the endosomal system, whereas MVs are released by outward budding from the bi-lipid external membrane of cells [4, 5]. Most, if not all, cell types release EVs, which then enter the body fluids [6]. Thus, EVs are endogenous delivery carriers that transport molecules to target cells and, travelling from the cells of origin to target cells, they transfer their contents after having been internalized [7]. In such a way, they provide a mechanism of cell-to-cell communication [8] and of intercellular exchange of cell components, such as nucleic acids, cytokines, lipids and proteins. Therefore, they act as signals both in cell homeostatic processes and in pathological conditions [9], also including inflammation [10] and cancer [11]. EVs can be detected in nearly all biological fluids, such as blood, urine, saliva, cerebrospinal fluid, bronchoalveolar lavage fluid (BALF), amniotic fluid, seminal plasma, and breast milk [2]. Since their content is protected from degradation by extracellular proteases and RNases, they are highly stable in storage conditions.

EVs are of particular interest in the study of lung diseases due to the high blood flow and vascular surface area of the respiratory tract. Surface proteins play a role in EVs pharmacokinetics and in particular in their distribution to the lung [12]. These biomarkers have been investigated in lung diseases, such as pulmonary hypertension [13], chronic obstructive pulmonary diseases (COPD) [14], and lung cancer [15]. Furthermore, it has been reported that a variety of environmental and lifestyle risk factors, such as air pollutants, smoking, alcohol, obesity, nutrition, physical activity, and oxidative stress, can modulate EVs trafficking [2].

The assessment of EVs modulation in biological fluids is of great importance following exposure to cigarette smoke (CS), which is the dominant risk factor for lung cancer and, in addition, has been causally associated with the induction of other cancers affecting the respiratory tract, urinary tract, digestive system, and hematopoietic system [16]. CS is also one of the main risk

factors for other chronic degenerative diseases, such as BPCO [17]. *In vitro* studies have shown the relationships between exposure to CS and release of EVs, for instance by using human macrophages [18], human mononuclear cells, depending on Ca^{2+} mobilization [19], and cultured human bronchial epithelial cells [20]. The last effect could be prevented by the antioxidant thiols glutathione (GSH) and *N*-acetyl-L-acetylcysteine (NAC) [20], which may contribute to understand the benefits of NAC as a chemopreventive agent [21]. In addition, it was shown that circulating endothelial MVs can be assumed as a measure of early lung destruction and emphysema in cigarette smokers [22], and smoking enhanced the levels of MVs in blood cells of healthy volunteers [23]. Furthermore, the analysis of human BALF showed that smoking can alter EVs profiles [24].

The present study had various goals. The first one was to evaluate in a preclinical model, under controlled experimental conditions, the physiological background concentration and size of EVs in mouse biological fluids, including BALF, blood serum, and urines, and to determine the proportion of EVs of epithelial origin. The second goal was to assess how exposure to mainstream CS (MCS), which is inhaled by active smokers as an undiluted complex mixture, can affect the concentration of EVs in these biological fluids. These experiments were carried out by using, in part, a subset of mice that had been treated in a study evaluating the release of microRNAs (miRNAs) in the same biological fluids and, additionally, in 10 organs of mice exposed to MCS [25, 26]. A further goal was to explore whether administration of a chemopreventive agent to MCS-exposed mice may be able to further modulate the concentration of EVs in biological fluids. Since chronic inflammation plays a key role at different stages of the carcinogenesis process [27] and is crucial in CS-related carcinogenesis [28, 29], we tested the nonsteroidal anti-inflammatory drug (NSAID) celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene-1-sulfonamide, CAS 169590-42-5). Celecoxib is a selective inhibitor of cyclooxygenase-2 (COX-2), the inducible COX isoform having a pro-inflammatory function, which is expressed in response to certain stimuli such as mitogens, cytokines and growth factors [30]). In contrast, COX-1 is the housekeeping isoform, the prostaglandins derived from COX-1 being involved in the homeostatic maintenance of the gastric mucosa. Therefore, selective COX-2 inhibitors (coxibs) seem to be safer with regard to gastric damage compared to traditional NSAIDs [31]. A rationale for using celecoxib in these experiments is that, at least in synovial fibroblasts, microparticles are able to upregulate the production of prostaglandins by inducing COX-2 [11].

The results obtained showed that: a) there were baseline differences in both size and concentration of total EVs and of EVs of epithelial origin in the 3 examined biological fluids; b) exposure to MCS significantly increased EVs concentrations but only in BALF; and c) treatment of MCS-exposed mice with celecoxib further enhanced EVs concentrations in the urines and especially in the blood serum, which presumably reflects the occurrence of toxic effects related to administration of this NSAID.

Materials and methods

Mice and experimental groups

Two-month old Swiss ICR (CD-1) mice of both genders, purchased from Harlan Laboratories (San Pietro al Natisone, Udine, Italy), were housed in Makrolon™ cages on sawdust bedding and maintained on standard rodent chow (Teklad 9607, Harlan Laboratories) and tap water *ad libitum*. The animal room temperature was 23 ± 2°C, with a relative humidity of 55% and a 12 h day-night cycle.

Sixty mice were randomly assigned to 3 experimental groups, including mice kept in filtered air for 8 weeks (Group 1, sham-exposed mice), mice exposed to MCS for 8 weeks (Group 2, MCS-exposed mice), and MCS-exposed mice treated with celecoxib for the same period of time (Group 3). Each group was composed of 10 males and 10 females.

Housing and treatments of mice were in accordance with NIH, European (2010/63 UE Directive), and institutional guidelines. The issuance of the NIH Office of Laboratory Animal Welfare (OLAW) with the University of Genoa bears the identification number A5899-01 and is effective until February 28, 2021. The IACUC protocol regarding treatment of the same mice for studying modulation of miRNAs was approved by the Fox Chase Cancer Center Committee on April 13, 2015.

Exposure to MCS and treatment with celecoxib

The 40 mice belonging to Groups 2 and 3 were exposed whole-body to the MCS generated by Kentucky 2R4F reference cigarettes (University of Kentucky, Lexington, KY), having a declared content of 9.4 mg tar, 0.73 mg nicotine, and delivering 12 mg CO each. MCS was transferred to the exposure chambers by drawing 15 consecutive puffs, each of 60 ml and lasting 6 s. Each daily session involved 6 consecutive exposures, lasting 10 min each, with 1-min intervals during which a total air change was made in order to avoid excessive accumulation of MCS and toxic effects. The average concentration of total particulate matter measured in the exposure chambers was 784 mg/m^3.

The 20 MCS-exposed mice belonging to Group 3 were treated with celecoxib, starting 3 days before the first exposure to MCS. Celecoxib was supplied by the US National Cancer Institute (NCI) via MRIGlobal (Kansas City, MO). Based on a preliminary subchronic toxicity study in Swiss H mice [32], it was decided to incorporate celecoxib in the mouse diet at the dose of 1,600 g/Kg diet, which corresponded to the 80% of the maximum dose that did not produce any body loss or sufferance or alterations in the behavior of mice after 6 weeks of treatment.

Collection of body fluids

After 8 weeks, all mice were euthanized. The 2013 AVMA guidelines on euthanasia were followed using slow

introduction of CO_2 for asphyxiation of mice. Death was confirmed by absence of respiration and/or heartbeat. Cell-free biological fluids were obtained as follows. Immediately after sacrifice, BAL was performed by intubating the trachea and lavaging with 5 ml of phosphate buffered saline (PBS) per mouse. The BAL samples were centrifuged and the supernatant fluids (BALF) were pooled within each experimental group, separately for males and females. The blood was collected by heart puncture and used for preparing serum, which was pooled within each experimental group separately for males and females. The urine was collected for 8 h and pooled from the male mice belonging to each experimental group by using metabolic cages during the day preceding euthanasia of mice. The urine was centrifuged in order to remove the sediment.

Isolation of EVs from body fluids

Differential ultracentrifugation methods were used to isolate EVs from BAL, plasma and urine samples, which remain the most widely used primary isolation procedure [33]. In particular, EVs were isolated by ultracentrifugation as described previously [1]. Briefly, blood serum and BALF samples from male and female mice were centrifuged first at 1,000 x *g* for 5 min to pellet the intact cells and then at 2,000 x *g* for10 min to discard the dead cells. The supernatants were further centrifuged at 10,000 x *g* for 30 min in order to remove cell debris. EVs were isolated from the final supernatant by ultracentrifugation at 100,000 x *g* for 1 h. The EVs pellets were resuspended in a final volume of PBS corresponding to 1:100 of the original volume. Urine samples pooled from male mice were collected and centrifuged first at 1,000 x *g* for 5 min to pellet the intact cells and then at 3,000 x *g* for 10 min at 4°C to remove cell debris. The supernatants were further centrifuged at 10,000 x *g* at 4°C for 30 min to remove large membrane fragments and other debris. Finally, the supernatants were ultracentrifuged at 110,000 x *g* for 75 min at 4°C [34]. The EVs pellets were resuspended in 400 µl PBS filtered 3 times through 0.10 µm pore size membranes (EMD Millipore, Billerica, MA, USA).

Nanoparticle Tracking Analysis (NTA)

Concentrations and size of EVs were assessed by nanoparticle tracking analysis (NTA), a technique that measures the Brownian motion of vesicles suspended in fluids and displays them in real time through a charge-coupled device (CCD) camera with high sensitivity. Using a NanoSight LM10-HS system (NanoSight Ltd., Amesbury, UK), EVs were visualized by laser light scattering. Five 30-s recordings were made for each sample. The collected data were analyzed with NTA software, which provided high-resolution particle-size distribution profiles and concentration measurements of EVs.

EVs characterization

EVs were characterized by MACSQuant analyzer flow cytometer (Miltenyi Biotec, Calderara di Reno, Bologna, Italy) according to the customer protocol. 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) was used to discriminate the integrity of the vesicles before the specific antibody staining. CFSE is a cell permeant non-fluorescent dye. Intracellular esterases in EVs cleave the acetate groups which results in the green fluorescent molecule carboxyfluorescein that is membrane impermeant. In particular, 60 µl sample aliquots were stained with 0.02 µM CFSE at 37°C for 20 min in the dark. The CFSE-stained sample was incubated with 6 µl monoclonal antibody CD326 (EpCAM)-APC (clone: caa7-9G8) in the dark for 20 min at 4°C. The double staining with CFSE and EPCAM antibody discriminates EVs from other contaminants, such as cell membrane fragments, and allowed us to quantify the EVs from epithelial cells. Thirty µl of double stained sample were acquired on the MACSQuant Analyzer. Due to the very low amounts of blood serum remaining after Nanosight analysis, it was possible to execute the EVs characterization analysis only in urine and BALF.

Statistical analysis

Quantitative data were expressed as the mean ± SD of 5 replicate recordings. Continuous variables were tested for normality and linearity. The comparison between the EVs concentration/size curves was made by calculating the subtended areas by adding each other histogram column values recorder for each interval size. The statistical significance of the differences between groups was evaluated by ANOVA followed by Student's *t* test for unpaired data. *P* values lower than 0.05 were regarded as statistically significant. All statistical analyses were performed by using the statistical software Statview software (Abacus Concept Inc., Berkeley, CA, USA).

Results

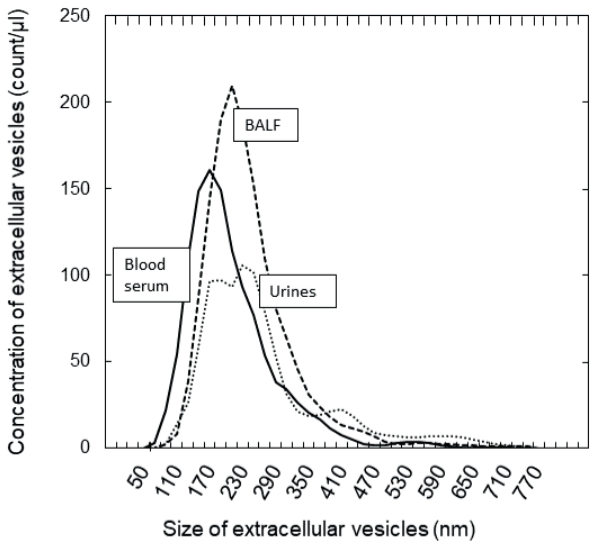
Survival and body weights

All 60 mice survived throughout duration of the experiment (8 weeks). At the beginning of the study, before starting the treatments, the body weights (means ± SE) were 38.3 ± 0.83 g in the 30 males and 28.8 ± 0.82 g in the 30 females. After 8 weeks, the body weights in males and females were 42.3 ± 1.09 g and 37.5 ± 1.16 g in Group 1 mice (sham-exposed mice), 39.4 ± 0.70 g and 31.7 ± 1.22 g in Group 2 mice (MCS-exposed mice), and 34.6 ± 1.55 g and 26.7 ± 1.16 g in Group 3 mice (MCS-exposed mice treated with celecoxib). The slight body weight loss recorded in MCS-exposed mice was statistically significant in both males ($P < 0.05$) and females ($P < 0.01$), and it was not further significantly affected by administration of dietary celecoxib.

Physiological spread of EVs into body fluids

We first evaluated comparatively the shedding of EVs into mouse BALF, blood serum, and urines under baseline conditions. To this purpose, we used sham-exposed male mice, for which all three biological fluids were available. As summarized in Figure 1, the EVs differed in the body fluids both in size and in concentration. In

Fig. 1. Concentration of EVs according to their size in the BALF (dashed line), blood serum (continuous line), and urines (dotted line) of sham-exposed male mice.



fact, the EVs curves in blood serum and BALF were unimodal, with maximum concentration peaks at a diameter of about 170 nm and 230 nm, respectively. In both cases, the curves fit a quasi-Gaussian distribution ranging between 70 and 530 nm, with a queue of larger EVs spanning until about 730 nm. Conversely, the EVs curve in the urines was multimodal, with two major peaks at 170 nm and 230 nm, and a minor peak at 430 nm, with a more abundant presence of larger EVs. As assessed by calculating the areas under the curves, the concentrations of EVs were 41,198.4/µl BALF, 23,436/µl blood serum, and 19.462.0/µl urines. The differences between blood serum and urines were not statistically significant, whereas EVs concentrations in BALF were significantly higher than those measured in each one of the other two biological fluids ($P < 0.05$ in both cases).

Concentration of EVs in body fluids as related to exposure to MCS and treatment with Celecoxib

Figure 2 shows the curves relating the size of EVs to their concentrations in the BALF and blood serum of mice of both genders and in the urines of male mice as related to exposure to MCS and treatment with celecoxib. Exposure to MCS did neither significantly affect the EVs size distribution nor their concentrations in blood serum and urines, whereas it significantly increased their concentrations in BALF, as compared with sham-exposed mice ($P < 0.001$). The oral administration of celecoxib did not further affect EVs concentrations in the BALF of MCS-exposed mice of both genders. In contrast, treatment of MCS-exposed mice with celecoxib remarkably and significantly ($P < 0.001$) increased the concentrations of EVs in the blood serum of both male and female mice as compared to either sham-exposed or MCS-exposed mice. In the urine of male MCS-exposed mice, treatment with celecoxib caused a slight but significant ($P < 0.05$) increase in the concentrations of EVs in the 150-200 nm range as compared with both sham-exposed mice and MCS-exposed mice in the absence of the COX-2 inhibitor.

Evaluation of EpCAM-positive EVs

EpCAM was characterized by flow cytometry in the BALF of both male and female mice and in the urines of male mice, as related to treatment of mice. The resulting fluorescence-activated cell sorting (FACS) scatter plots are shown in Figure 3, which relates the concentration of EpCAM-positive EVs to SSC (Side Scatter) intensity. In sham-exposed male mice, the concentration of EpCAM-positive EVs in urines was much higher than in BALF (1,249.2 *vs.* 55.1 EVs/µl, $P < 0.001$).

Following exposure to MCS, the concentration of EpCAM-positive EVs in BALF was significantly increased as compared with sham-exposed mice, both males (76.5 *vs.* 55.1 EVs/µl, $P < 0.05$) and females (102.7 *vs.* 55.4 EVs/µl, $P < 0.001$), whereas it was significantly decreased in urines (725.3 *vs.* 1,249.2 EVs/µl, $P < 0.05$).

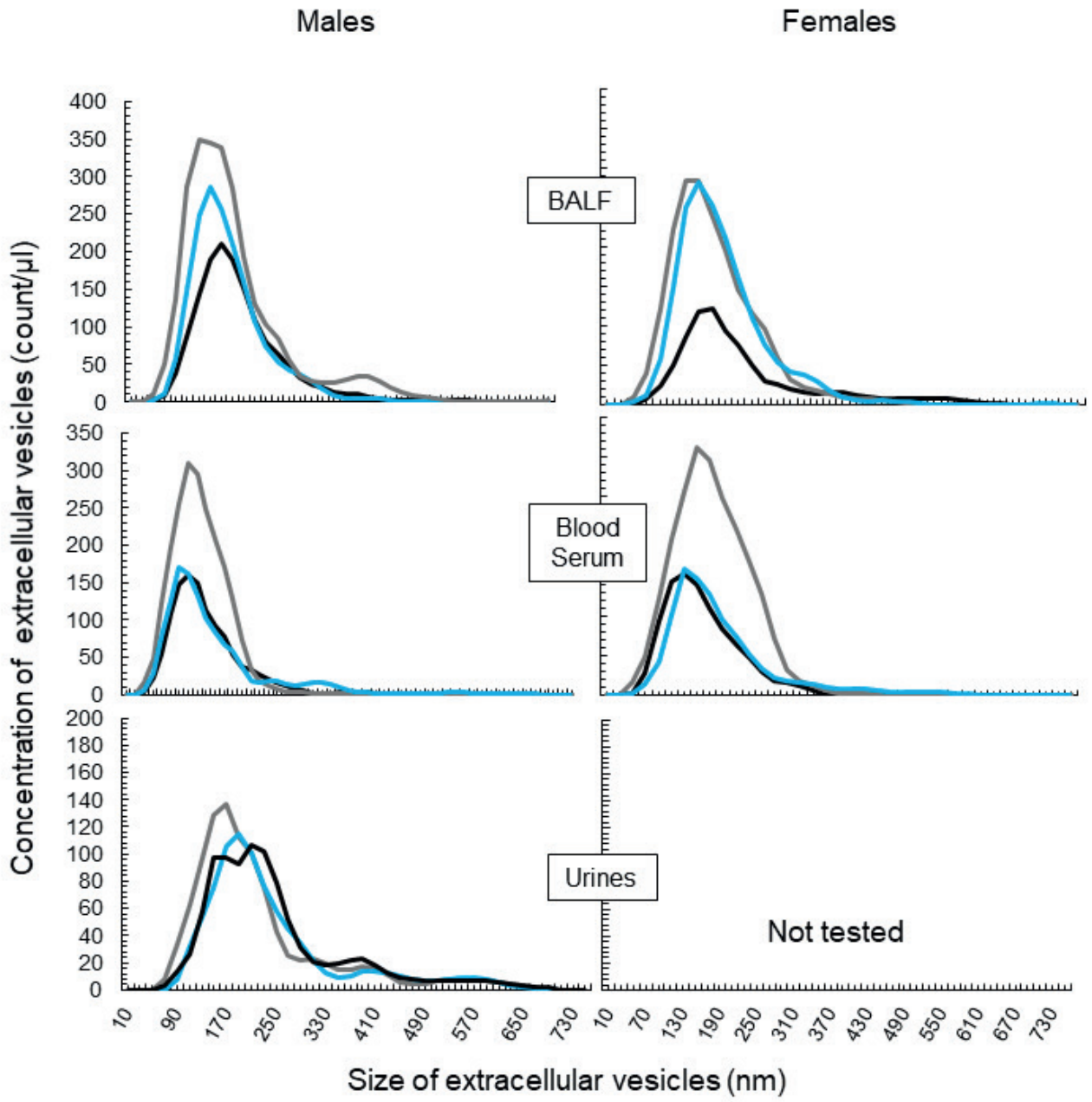
Administration of celecoxib to MCS-exposed mice did not affect the concentration of EpCAM-positive EVs in the BALF of female mice (115.2 EVs/µl) and slightly but significantly increased it in the BALF of male mice (108.3 EVs/µl, $P < 0.05$). Conversely, celecoxib significantly decreased the concentration of these EVs in the urines of male mice (478.1 EVs/µl, $P < 0.05$).

Discussion

The results obtained show that, under physiological conditions, the concentrations of EVs in the extracellular environment of the 3 examined mouse body fluids is of a similar order of magnitude, ranging between almost 20,000 particles/µl in urines and more than 40,000 particles/µl in BALF. There were some differences regarding the size of EVs, which in BALF and blood serum were mainly MVs but also contained some exosomes, whereas in urines almost all EVs were MVs and included larger vesicles having a multimodal distribution. These differences correlated with the variable proportion of EVs of epithelial origin among total EVs, as assessed by evaluating the proportion of EVs positive for EpCAM, which was much higher in the case of urines. The low proportion of EpCAM-positive EVs in BALF correlates with the finding that epithelial cells range from 0.05% to 1.5% of the total number of cells recovered in human BAL samples [35]. It is noteworthy that, besides mediating cell-cell adhesion in epithelia [36], EpCAM plays a role in tumorigenesis and metastasis of carcinomas, being expressed in most neoplastic epithelial cells [37]. In fact, normal epithelia express this cell surface glycoprotein at a variable but generally lower level than carcinomas [38].

The data concerning the baseline concentrations of EVs in the examined body fluids should be related to the volumes of the same fluids. An adult mouse excretes daily

Fig. 2. Size distribution and concentration of EVs in the BALF and blood serum of mice of both genders and in the urines of male mice, either sham-exposed (black lines) or exposed to MCS (blue lines) or exposed to MCS and treated with celecoxib (gray lines).



around 0.5-1.0 ml urine and has a total blood volume of approximately 1.5 ml (https://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling). More difficult are the estimates for BALF. This fluid was obtained by lavaging the lungs of each mouse with 5 ml of physiological saline and therefore it is likely that the EVs present in terminal airways were considerably diluted in the examined samples. BAL recovers the pulmonary epithelial lining fluid (ELF), which includes the surfactant and bronchial-bronchiolar secretions and, in mammals, has a relatively constant composition of about 90% lipids and 10% proteins. Surfactant lipids, which are produced by alveolar type II pneumocytes and to a lesser extent by bronchiolar non-ciliated epithelial cells, are synthesized in preformed intracytoplasmic lamellar bodies that are secreted into the aqueous subphase of ELF [39]. We have no information about the volume of ELF in mice, but in humans its volume is pretty high, having been reported to be between 37.5 and 75 ml [40]. It should be also taken into account that the EVs found in different body fluids undergo a different fate. In fact, the EVs present in urines get in contact with the mucosae of the urinary tract and thereafter are excreted from the body. Those present in the bloodstream are either eliminated through emunctory organs or transmitted to distant organs. Those found in BALF may trigger cell-to-cell communication mechanisms in bronchoalveolar cells before being removed from the lower respiratory spaces via the

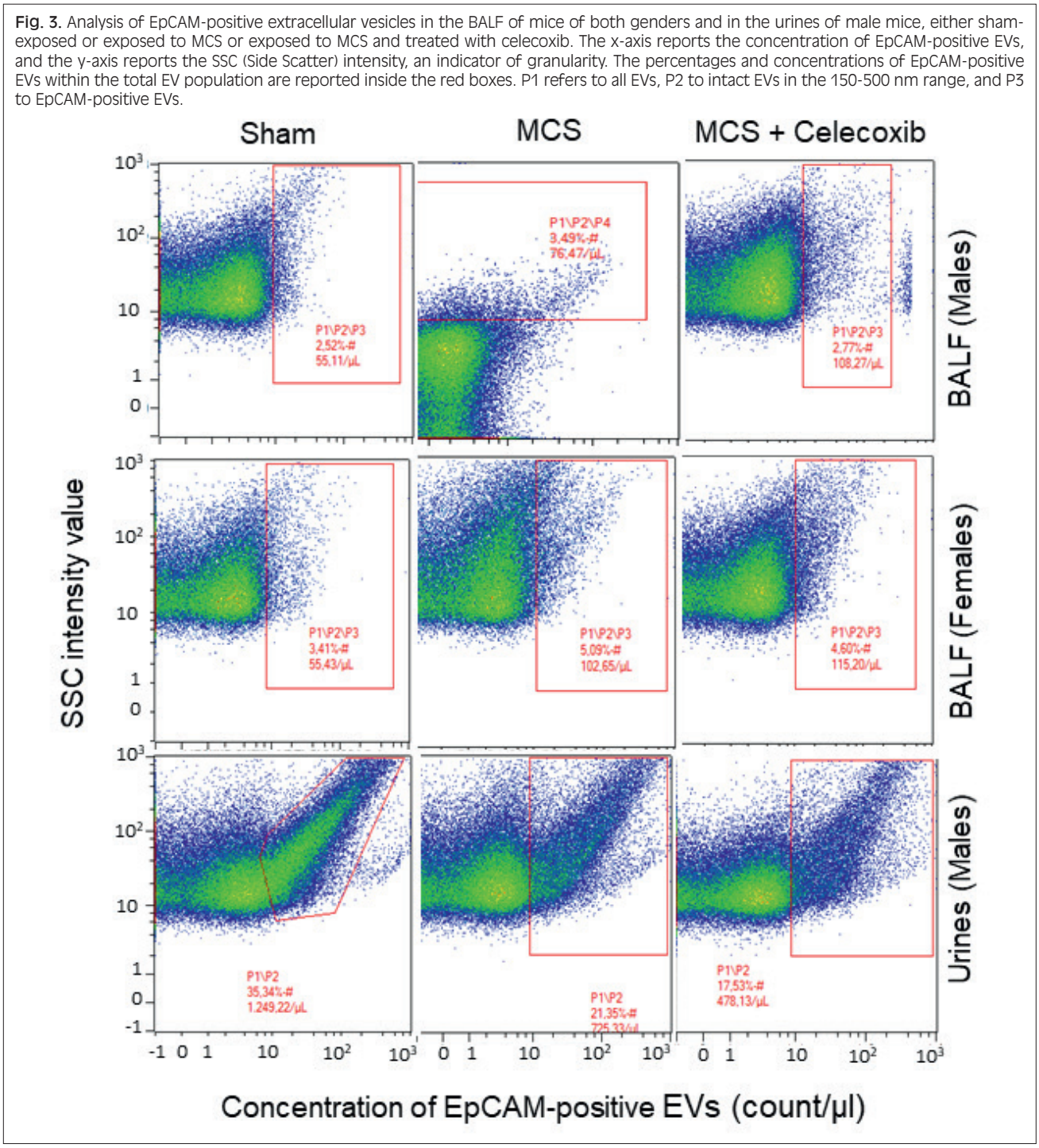


Fig. 3. Analysis of EpCAM-positive extracellular vesicles in the BALF of mice of both genders and in the urines of male mice, either sham-exposed or exposed to MCS or exposed to MCS and treated with celecoxib. The x-axis reports the concentration of EpCAM-positive EVs, and the y-axis reports the SSC (Side Scatter) intensity, an indicator of granularity. The percentages and concentrations of EpCAM-positive EVs within the total EV population are reported inside the red boxes. P1 refers to all EVs, P2 to intact EVs in the 150-500 nm range, and P3 to EpCAM-positive EVs.

mucociliary escalator and being either eliminated from the body by expectoration or swallowing. Swallowed EVs are expected to get in contact with the mucosae of the GI tract, thus being either transported to the liver or ultimately eliminated with feces.

Exposure of mice to MCS resulted in a significant increase of EVs in the BALF of both male and female mice, whereas the baseline concentrations of EVs in blood serum and urines were not affected following exposure to MCS. Similar findings were observed by evaluating the miRNA profiles in 10 organs and 3 biological fluids of the same mice used in the present study [25, 26]. In fact, the miRNAs detectable in the BALF were mainly of pulmonary origin, whereas the skeletal muscle gave a striking contribution to the presence of MCS-dysregulated miRNAs in the blood serum, and the kidney was the main source of miRNAs detectable in urines. Among the examined organs, dysregulation of miRNA expression was by far most prevalent in the lung, which is consistent with the observed upregulation by MCS of proteins that defend the respiratory tract by triggering a variety of protective mechanisms, such

as antioxidant pathways, detoxification of carcinogens, DNA repair, anti-inflammatory pathways, and apoptosis. At the same time, however, MCS activates toxic and carcinogenic mechanisms, such as modulation of oncogenes and oncosuppressor genes, cell proliferation, recruitment of undifferentiated stem cells, inflammation, inhibition of intercellular communication, angiogenesis, invasion, and metastasis [41]. In addition, exposure of mice to MCS in the medium term (7.5-9 months) induces a significant increase in preneoplastic and neoplastic lesions and in other histopathological alterations, also including malignant tumors in the lung [42].

The distribution patterns of both EVs (present study) and miRNAs [25, 26] clearly reflect pharmacokinetic mechanisms. Inhaled MCS undergoes a multiorgan distribution, contains systemic toxicants, and causes cancers in about 15 human tissues [16]. Indeed, blood and urines can be used to detect some smoking-related alterations, such as increased carboxyhemoglobin levels in the blood [43] and mutagenicity of urines [44]. However, these body fluids do not appear to be suitable substrates to detect alterations of other smoking-related biomarkers, such as miRNA dysregulation [26] and shedding of EVs (present study). This is not exclusive of experimental data, but also in humans miRNA signatures in plasma do not correspond with miRNA signatures in BAL samples of lung cancer patients [45], and studies in humans have suggested that smoking alters lung EVs profiles in BALF that are expected to influence the surrounding bronchial epithelial cells [24].

Therefore, in spite of the fact that the collection of BALF is semi-invasive, the analysis of this fluid appears to be more appropriate to detect smoking-related biomarker alterations than analysis of blood serum and urine, where the alterations of pulmonary origin are confounded by contributions from other organs. In fact, BAL has widely been used in preclinical and clinical studies because this fluid contains both biochemical and cytological indicators of cellular responses to infection, cancer, or inhaled drugs or toxicants [46, 47].

A further goal of the present study was to evaluate how a putative chemopreventive agent can modulate the release of MCS-related EVs into biological fluids, which can be assumed as an indicator either of protective effects or of adverse effects. Irrespective of gender, the oral administration of celecoxib did not further affect the increase in EVs concentration in BALF caused by exposure of mice to MCS and had poor effects on the concentration of EpCAM-positive EVs in this biological fluid. This means that this selective COX-2 inhibitor failed to modulate the MCS-related release of EVs from respiratory tract cells.

On the other hand, celecoxib considerably increased the concentrations of EVs in the blood serum of mice of both genders exposed to MCS. A similar but less pronounced effect occurred in the urines of MCS-exposed male mice, which however was accompanied by a loss of EpCAM-positive EVs. Such an effect may correlate with the observed protective effects of celecoxib towards induction by MCS of preneoplastic alterations in the urinary tract of mice [32]. Moreover, celecoxib has been shown to prevent *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced bladder carcinomas in rats [48]. It is noteworthy that urinary EpCAM is overexpressed in bladder tumors to such an extent that it could act as a biomarker of bladder cancer detection [49]. Thus, our results suggest that, even at an early stage, the decrease of EpCAM produced by celecoxib in urines may predict the protective effects of this drug towards MCS-induced neoplastic alterations in the urinary tract.

The increased systemic concentrations of EVs in MCS-exposed mice treated with celecoxib are likely to be related to their release from organs other than the lung, such as skeletal muscle and liver. In this light, there seems to be a relationship between upregulation of circulating EVs by celecoxib and its hepatotoxicity. In fact, the dose of celecoxib used in the present study (1,600 mg/kg diet) did not produce any apparent toxic effect in a preliminary 6-week test in smoke-free mice. However, in the long-term, this drug became toxic to MCS-exposed mice, as inferred from the decrease in survival and body weight gain as well as from some histopathological signs of hepatotoxicity in mice treated with celecoxib at the same dose [32]. It should be noted that such a dose is rather high but is comparable to the pharmacological dose in humans. In fact, a dose of 1,000 mg/kg diet of celecoxib administered to mice resulted in a plasma concentration of 1.6 μg/ml, which approximates the reported therapeutic plasma concentration of celecoxib in humans [50]. The hepatotoxicity of celecoxib observed in MCS-exposed mice is consistent with the detection of hepatocellular alterations produced by this drug in rats [51, 52]. From a mechanistic point of view, it should be taken into account that celecoxib is metabolized primarily by cytochrome P450 2C9 (CYP2C9) [53], which at the same time is involved in the metabolism of polycyclic aromatic hydrocarbons contained in CS [54]. Mediators derived from COX-2 have an important hepatoprotective function and accordingly the risk of drug-induced liver injury may be increased by COX-2 inhibition [55]. In fact, an increased clinical vigilance is required during the co-administration of celecoxib and other substrates or inhibitors of CYP2C9 [50], as it could be the case with smoking. Since drug-induced liver damage increases the number of circulating EVs [56], it may be assumed that the hepatotoxicity caused by celecoxib-induced COX-2 inhibition in MCS-exposed mice was responsible for the observed increase of EVs in blood and urines.

In conclusion, the findings of the present study shed light on the role of EVs as biomarkers of exposure to MCS, the dominant risk factor for lung cancer, other cancers, and other diseases of toxicological relevance. It has been postulated that there is immense potential for the use of EVs for biomarker detection in clinical settings [57]. Our data provide evidence that the effects of MCS on that end-point are well detectable in the extracellular environment of the lower respiratory tract, where they could behave as endogenous delivery carriers of a variety of molecules to target cells. On the other hand, the effects of MCS on EVs release into body

fluids were not discernible at a systemic level, presumably due to confounding factors such as the contribution from organs other than the lungs. The assay of a putative chemopreventive agent, the selective COX-2 inhibitor celecoxib, modulated EVs spread at a systemic level by suggesting the occurrence of both protective mechanisms in the urinary tract and adverse effects of likely hepatotoxic origin in MCS-exposed mice. It should be emphasized that these changes were detected after only 8 weeks of treatment of mice, whereas the development of MCS-related tumors and other histopathological alterations requires longer periods of time [32]. Therefore, the data regarding the spread of EVs in biological fluids may be translated to the clinical practice and taken as an early diagnostic tool and as an end-point exploitable for toxicological studies and preventive medicine strategies.

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Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

AP, AI and SDF conceived the experiments, LP, AC, AP and VB conducted the experiments, S LM and RTM collected body fluids, all authors were involved in analyzing the results, AP and SDF wrote and all authors reviewed the manuscript.

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