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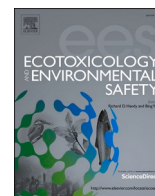
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## Exploration of microRNAs from blood extracellular vesicles as biomarkers of exposure to polycyclic aromatic hydrocarbons

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### ABSTRACT

Exposure to polycyclic aromatic hydrocarbons (PAHs), ubiquitously environmental contaminant, leads to the development of major toxic effects on human health, such as carcinogenic and immunosuppressive alterations reported for the most studied PAH, *i.e.*, benzo(a)pyrene (B(a)P). In order to assess the risk associated with this exposure, it is necessary to have predictive biomarkers. Thus, extracellular vesicles (EVs) and their microRNA (miRNA) contents, have recently been proposed as potentially interesting biomarkers in Toxicology. Our study here explores the use of vesicles secreted and found in blood fluids, and their miRNAs, as biomarkers of exposure to B(a)P alone and within a realistic occupational mixture. We isolated EVs from primary human cultured blood mononuclear cells (PBMCs) and rat plasma after PAH exposure and reported an increased EV production by B(a)P, used either alone or in the mixture, *in vitro* and *in vivo*. We then investigated the association of this EV release with the blood concentration of the 7,8,9,10-hydroxy (tetrol)-B(a)P reactive metabolite, in rats. By performing RNA-sequencing (RNA-seq) of miRNAs in PBMC-derived EVs, we analyzed miRNA profiles and demonstrated the regulation of the expression of miR-342-3p upon B(a)P exposure. We then validated B(a)P-induced changes of miR-342-3p expression *in vivo* in rat plasma-derived EVs. Overall, our study highlights the feasibility of using EVs and their miRNA contents, as biomarkers of PAH exposure and discusses their potential in environmental Toxicology.

### 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a part of a group of ubiquitous environmental contaminants to which humans are routinely exposed. The major source of PAH production is the pyrolysis of organic materials. PAHs constitute a group of several hundred compounds and exposure to these contaminants occurs both at the domestic level, *via* diet and air pollution, and in occupational environments such as those associated with coal-tars and their derived products. Benzo(a)pyrene (B

(a)P) has been the most extensively studied PAH and is listed as a priority toxic substance by international agencies. Indeed, human exposure to B(a)P is associated with the development of various toxic effects, including well-documented carcinogenic effects, immunosuppressive, atherogenic or pro-inflammatory effects (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2016; Holme et al., 2019). Due to these proven impacts on health, human biomonitoring to determine exposure to B(a)P, has received increasing interest in recent years, particularly through the development of biomarkers. In the absence of

**Abbreviations:** B(a)P, benzo(a)pyrene; Ct, threshold Cycle; CTP-I, industrial Coal Tar Pitch; DMSO, dimethylsulfoxide; EVs, extracellular vesicles; GC-MS/MS, gas chromatograph with a triple quadrupole mass spectrometer; HPLC-Fluo, high performance liquid chromatography with fluorescence detection; microRNAs, miRNAs; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp-O-Mefluoromethyl ketone; NTA, Nanoparticle Tracking Analysis; PAHs, Polycyclic Aromatic Hydrocarbons; PBMCs, Peripheral Blood Mononuclear Cells; PBS, phosphate buffer saline; Pyr, pyrene; RNA-seq, RNA-sequencing; Tetrol-B(a)P, 7,8,9,10-hydroxy-B(a)P; Veh, Vehicle.

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robust methods to directly assess B(a)P exposure in humans, indirect processes have been used. We have recently demonstrated the usefulness in measuring urinary tetrol-B(a)P (7,8,9,10-hydroxy-B(a)P), product of the hydrolysis of the reactive metabolite of B(a)P namely 7,8-diol-9,10-epoxide, as biomarkers of B(a)P exposure (El Hajjar et al., 2023).

microRNAs (miRNAs) are a class of small and single-stranded RNAs that do not translate into functional proteins but regulate gene expression by interacting with the 3'-untranslated regions of their target mRNAs to induce their silencing (Ghildiyal and Zamore, 2009). miRNAs thus control many cellular, biological and physiological processes like proliferation, apoptosis, or immune regulation, and also modulate pathological occurrences like inflammation or tumor development (Bueno and Malumbres, 2011; Sati and Parhar, 2021). Interestingly, miRNAs are abundant in extracellular vesicles (EVs), thereby shielding them from degradation (Valadi et al., 2007). EVs are membrane enclosed nanostructures classified into small EVs or exosomes, originating from the endosomal pathway and measuring between 30 and 200 nm, and large EVs or microvesicles, i.e., 200–1000 nm, resulting from budding of the plasma membrane (Colombo et al., 2014). Enriched in different cargoes (lipids, proteins and small RNAs), EVs are released by cells into the extracellular compartment and are found with their contents in biological fluids (blood, urine or saliva...) (Raposo and Stoorvogel, 2013). In recent years, research has demonstrated the potential of EVs and miRNAs as diagnostic and/or prognostic biomarkers in various disorders, including cancer, diabetes, autoimmune and cardiovascular diseases (Schwarzenbach et al., 2014). Currently, a novel role for these EVs and their miRNAs is emerging in environmental toxicology. For example, an association has been described between exposure to air pollutants, such as particulate matter, or cigarette smoke, and the amount of EVs and their miRNA contents (Bollati et al., 2014; Ferrari et al., 2022; Hélot et al., 2017; Pergoli et al., 2017), making them potential new biomarkers of pollutant exposure and effects. Extracellular plasmatic miRNAs were also recently proposed as predictive biomarkers of effect in cardiovascular diseases in a PAH-exposed population (Ruiz-Vera et al., 2019a,b).

Peripheral blood mononuclear cells (PBMCs), constantly circulating in the body and composed primarily of lymphocytes and monocytes, can easily be isolated from blood fluid and cultured *in vitro*. We recently found that primary human cultured PBMCs are highly sensitive to PAHs, with changes seen at the cellular mRNA and miRNA levels (Souki et al., 2023). Human PBMCs also produce EVs, which cargo composition can be altered by exposure to therapeutic irradiation, leading to the discovery of candidate biomarkers for radiation dosimetry (Moertl et al., 2020).

The present study selected individual PAHs such as B(a)P, the most extensively studied associated with major toxic effects and Pyrene (Pyr), another important PAH not carcinogenic, as well as an occupational PAH mixture, extracted from the industrial products coal tar pitch (CTP-I), which mirrors a realistic exposure and then used standard isolation and characterization methods to measure EVs released from primary human cultured PBMCs and from rat plasma, and to evaluate how these PAHs alone and within a realistic occupational mixture, impact EV concentration and size, *in vitro* and *in vivo*. We further determined the blood concentrations of B(a)P and its tetrol metabolites, previously reported as relevant biomarkers for B(a)P exposure (El Hajjar et al., 2023), and analyzed their association with plasmatic EV levels in rats. Finally, we analyzed EV miRNA composition and used RNA-sequencing (RNA-seq) to profile miRNAs in isolated EVs from PBMC-exposed to B(a)P. We next examined whether measurable changes in circulating miRNA levels were also detectable in plasma from rats exposed to B(a)P, alone and within the CTP-I mixture. Through this study, we propose that blood EVs and their associated miRNA contents, are useful environmental toxicology biomarkers.

## 2. Materials and methods

### 2.1. Chemicals

Dimethylsulfoxide (DMSO), B(a)P, Pyr, and N-benzyloxycarbonyl-Val-Ala-Asp(O-Me)fluoromethyl ketone (Z-VAD) were purchased from Merck (Darmstadt, Germany). The PAH mixture (CTP-I) was directly collected from metallurgical companies. Its PAH profile and its preparation adjusted to the specified B(a)P concentration were described elsewhere (Bourgart et al., 2019; Souki et al., 2023).

### 2.2. Cell isolation and exposure

Human PBMCs were isolated from the buffy coat of blood donors (convention AC-2019–3853, French Blood Establishment, Rennes) and  $100 \times 10^6$  cells were cultivated *per condition* as previously reported (Souki et al., 2023). Thereafter PBMCs were exposed to 10 nM and 2  $\mu$ M of B(a)P, Pyr or CTP-I mixture, diluted in DMSO, as presented in Fig. S1. Control cultures were exposed to the same dilution of DMSO as treated cultures (0.2 %).

### 2.3. Animal and experimental design

#### 2.3.1. Animal housing

Animal procedures were carried out in agreement with the Directorate of Veterinary Services, of the French Ministry of Agriculture (Directive 2010/63/EU of the Council of the European Communities and health monitoring recommendations of the Federation from European Laboratory Animal Science Associations, reviewed by the local ethics committee). The protocol was conducted in an Animal High Technology Platform (Agreement C38–516100006, Université Grenoble Alpes) as previously reported (El Hajjar et al., 2023).

#### 2.3.2. Animal treatment

Male Wistar rats aged 11 weeks (310–435 g) (Charles River, L'Arbresle, France) were randomly divided into 5 groups of 9 rats before oral exposure by gavage with PAHs for 4 days. A first group was exposed to a dose of  $200 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  and a second at a dose of  $2000 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of B(a)P. The third and the fourth groups were exposed to the CTP-I mixture adjusted to  $200 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of B(a)P and  $2000 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of B(a)P. A control group received vegetable oil (Veh) ( $4 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ). As previously (El Hajjar et al., 2023), the quantity of PAH given was adjusted to the weight of the rats. After the last exposure, rats were fasted for 4 h and then euthanized with carbon dioxide inhalation and the blood sample was collected from the aorta in EDTA tubes. No major abnormalities were reported during exposure to PAHs (data not shown).

### 2.4. Analysis of blood concentrations of B(a)P and tetrol-B(a)P

For blood B(a)P analysis, 100  $\mu$ L sample was diluted in 2 mL of acetate buffer before a liquid-liquid extraction with 5 mL of dichloromethane. After dry evaporation of the organic solvent, the residue was dissolved in 250  $\mu$ L of acetonitrile. Extraction yields ranged from 80 % to 100 %. B(a)P was analyzed using an high performance liquid chromatography with fluorescence detection (HPLC-Fluo) with a Waters® PAH C18 column (250  $\times$  3 mm, 5 $\mu$ m). The limit of quantification was  $0.3 \text{ ng} \cdot \text{mL}^{-1}$  for B(a)P.

Blood tetrol-B(a)P analysis in urines was performed using a gas chromatograph with a triple quadrupole mass spectrometer (GC–MSMS) as described previously (Barbeau et al., 2018). The limit of quantification was  $2 \text{ ng} \cdot \text{L}^{-1}$  for tetrol-B(a)P.

### 2.5. Isolation of EVs from PBMCs and plasma

For PBMC-derived EVs, culture media were harvested at the end of the PAH treatment. The media was centrifuged at 800 g for 5 min to

eliminate cells, and at 3700 g for 10 min to remove apoptotic bodies and cell debris. Total EVs were recovered by ultracentrifugation of supernatants at 110,000 g for 2 h at 4 °C in a SW32 Ti rotor (Beckman Coulter, Villepinte, France) and then washed with phosphate-buffered saline (PBS) at 110,000 g for 2 h at 4 °C, before a suspension in 25 µL PBS.

For rat plasma-derived EVs, 3 mL of blood freshly sampled was centrifuged at 1 300 g for 10 min at 4 °C to separate blood phases. The supernatant was collected and centrifuged at 3000 g for 10 min at 4 °C to eliminate cell debris and apoptotic bodies. Thereafter, EVs were pelleted by ultracentrifugation and washed with PBS, as described for PBMCs.

## 2.6. EV analysis by Nanoparticle Tracking Analysis (NTA)

A Nanosight NS300 equipped with a syringe pump, a sCMOS camera and a Blue488 laser (Malvern Panalytical, Palaiseau, France) was used to quantify size distribution and concentration of EVs, diluted at a concentration of  $10^7$  to  $10^8$  particles/mL in PBS. Results were retrieved from 3 × 60 s videos using NanoSight NTA 3.4.4 software (Malvern).

## 2.7. RNA isolation

Isolation of vesicular small RNAs from PBMCs and plasma was performed using the total exosome RNA isolation kit (Thermo Fisher Scientific) with minor changes. Briefly, one volume of each sample was combined with one volume of 2X denaturing solution before incubation on ice for 5 min. The spike-in cel-miR-39 (*Caenorhabditis elegans* miRNA-39) was added in each sample before RNA extraction. Small RNAs were eluted in 50 µL nuclease-free water.

## 2.8. RNA-seq of small RNAs

### 2.8.1. Library preparation

Libraries were constructed from 12 independent cultures of PBMCs, each in the presence of vehicle (DMSO) or 2 µM B(a)P as in our previous study (Souki et al., 2023), except that the conditioned media were combined into 3 equal pools of 4 different cultures to isolate EVs. Thereafter, small RNAs were extracted and a volume of 5 µL was used to construct libraries using the Qiaseq miRNA library preparation kit (Qiagen, Hilden, Germany). The sequences are indicated in GenBank (GSE256027). After removing defective reads, a total of  $3,958,065 \pm 426,495$  and  $4,587,812 \pm 299,676$  miRNA reads were identified for DMSO and B(a)P-treated PBMCs, respectively (Table S1).

### 2.8.2. RNA-seq data mapping

The Qiagen pipeline was used to align Fastq files (QIaseq® miRNA Primary Quantification) and the DESeq2 package determined the number of differentially expressed miRNAs as previously reported (Souki et al., 2023).

## 2.9. RT-qPCR assays

miRNAs from purified small RNAs (2 µL) were reverse-transcribed using the TaqMan Advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific) with preamplification procedures according to the manufacturer's protocols. qPCR was performed using TaqMan Fast Advanced Master Mix and predesigned TaqMan Advanced miRNA Assays (Thermo Fisher Scientific) with a 1:10 dilution of cDNA template and thermocycling conditions of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. qPCR assays were conducted using cel-miR-39 as an external standard to calibrate the assay (Souki et al., 2023). The coefficient of variation for this exogenous miRNA was used as a normalization measure to remove experimental bias as already reported (Mestdagh et al., 2009).

## 2.10. Statistical analysis

GraphPad Prism 7 (GraphPad Software, INC., La Jolla, CA, USA) was used for statistical analysis. Variable distribution was tested using the Shapiro–Wilk normality test. Statistical significance was assessed by paired Wilcoxon or Student's *t* test, or by one-way parametric or non-parametric analysis of variance (repeated measures or not) followed by tests for multiple comparisons. Associations between variables were evaluated using Pearson's correlations done on log-transformed data to satisfy normality. We considered a *p*-value  $\leq 0.05$  as significant.

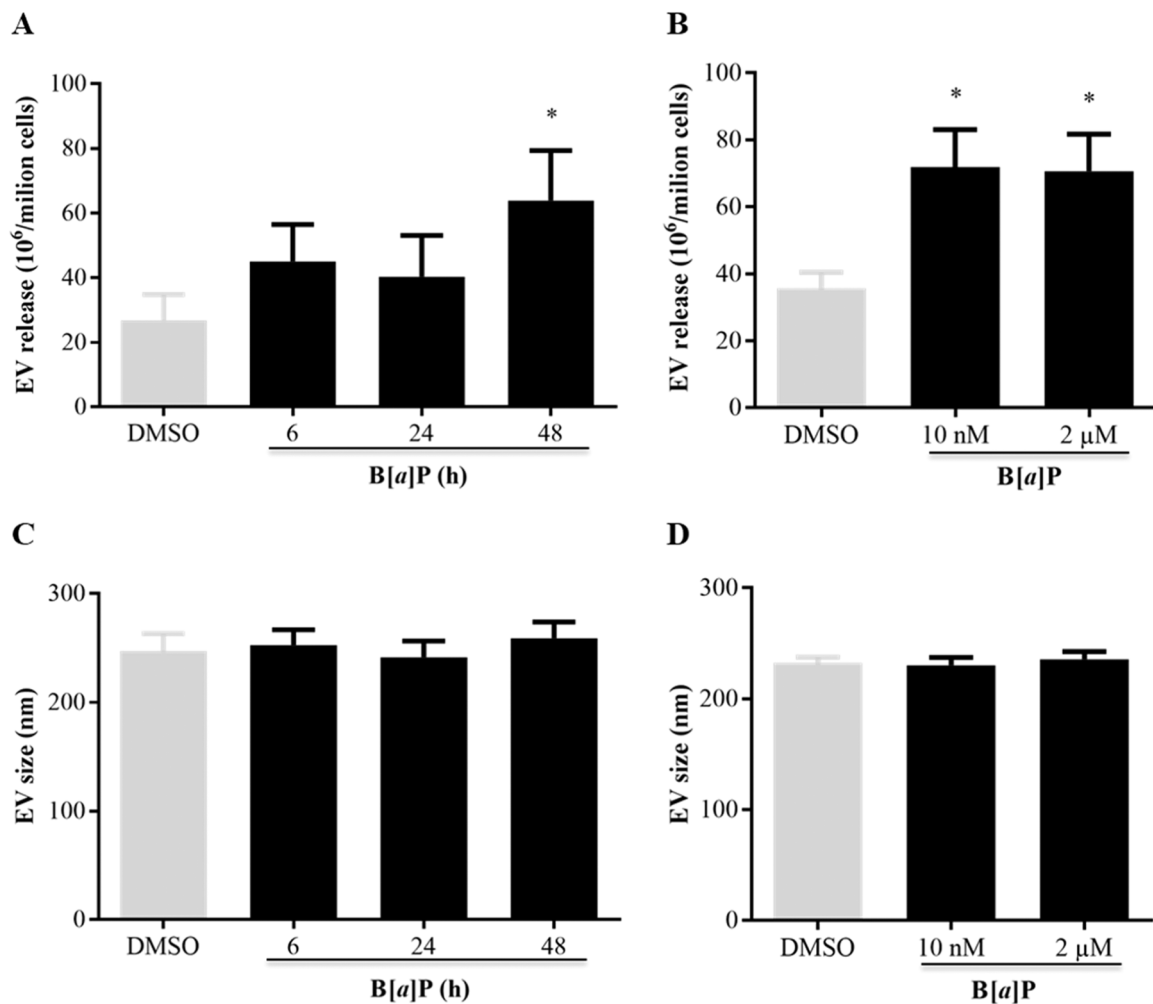
## 3. Results

### 3.1. EV production is increased by an *in vitro* and *in vivo* exposure to B(a)P

We firstly performed a comprehensive characterization of EVs released by primary human cultured PBMCs. The EV isolation procedure consisting of differential centrifugations allowed the preparation of total EVs including small and large EVs, as confirmed by NTA (Fig. S2A), TEM (Fig. S2B), and immunoblotting of marker proteins (Fig. S2C). As indicated in the representative NTA graph, most EVs released by PBMCs had a diameter  $> 200$  nm (Fig. S2A). EV morphology, examined by TEM, showed the presence of small EVs, *i.e.*, exosomes, with cup-shaped structures (Fig. S2B, top left panel), and large EVs, *i.e.*, microvesicles, typically exhibiting a diameter from 200 to 1000 nm (Fig. S2B, bottom left panel). As formulated by the international society for EVs, we also looked for the presence of typical protein markers by western blotting (Lötvald et al., 2014). As shown in Fig. S2C, detection of the tetraspanin CD63 and the lipid-raft protein FLOTILIN indicated the presence of exosome particles. The mitochondrial protein (COX IV) and CALNEXIN, an endoplasmic reticulum membrane protein, were easily detected in whole cells but scarcely, if not, in EVs, indicating the purity of the EV preparations (Fig. S2C).

NTA was used to quantify EVs released by PBMCs in response to increased exposure to B(a)P. A time-course of EVs released by PBMCs exposed to 2 µM B(a)P reported a significant increase after 48 h (Fig. 1A). At 48 h, a lower B(a)P concentration of 10 nM also significantly enhanced the release of EVs by PBMCs (Fig. 1B). Moreover, NTA was used to measure the effect of B(a)P exposure on the size of EVs. We did not observe an effect of the length of B(a)P exposure or concentration on EV size (Fig. 1C and D). Due to our previous observation that, 2 µM of B(a)P for a period of 48 h, induces apoptosis in PBMCs (Souki et al., 2023), we next examined whether the release of EVs was a consequence of apoptotic stress signals. As shown in Fig. 2A, a pre-treatment of PBMCs by Z-VAD, an inhibitor of caspase-dependent apoptosis (Souki et al., 2023; van Meteren et al., 2019), did not modify the B(a)P-induced release of EVs (Fig. 2A). Furthermore, the concentration of 10 nM of B(a)P, not associated with significant apoptosis (Souki et al., 2023), led to a significant increase in EV production at 48 h (Fig. 1B), which is therefore independent of this cell death. We thereafter analyzed the effects of other PAHs on the production of EVs by PBMCs. Whereas Pyr, at concentrations of 10 nM and 2 µM for 48 h, did not increase the production of EVs (Fig. 2B), a realistic industrial mixture of PAHs (CTP-I) collected directly from metallurgical production sites and, adjusted at 10 nM B(a)P (Bourgart et al., 2019; Souki et al., 2023), significantly increased EVs released by PBMCs (Fig. 2C). As indicated for the B(a)P treatment, the size of PBMC-derived EVs was not significantly altered by exposure to Pyr or to the CTP-I mixture (Fig. S3A and S3B).

Since blood cells secrete a large proportion of circulating EVs (Alberro et al., 2021), we conducted *in vivo* experiments to study plasmatic EVs in rats exposed during 4 days to 200 and 2000 µg.kg<sup>-1</sup>.d<sup>-1</sup> B(a)P, alone and with other PAHs in the CTP-I mixture. Using NTA to measure the concentration of EVs in the rat plasma (Fig. S3C), we found that the exposure to PAHs increased EV concentration, with significant



**Fig. 1.** B(a)P increases EV release *in vitro* by primary human PBMCs. PBMCs were cultured and exposed to DMSO (control) or to 2  $\mu$ M B(a)P for 6, 24 and 48 h (A, C), and to 10 nM and 2  $\mu$ M B(a)P for 48 h (B, D). EVs were isolated by ultracentrifugation and NTA determined their concentration (A, B) and size (C, D). Data are expressed as the mean  $\pm$  SEM (n=8 (A, C) or 9 (B, D) independent cultures). \*,  $p \leq 0.05$  compared with control (Friedman's analysis of variance followed by the Dunn's multirange test (A), analysis of variance for repeated measures followed by the Dunnett's multiple comparison test (B)).

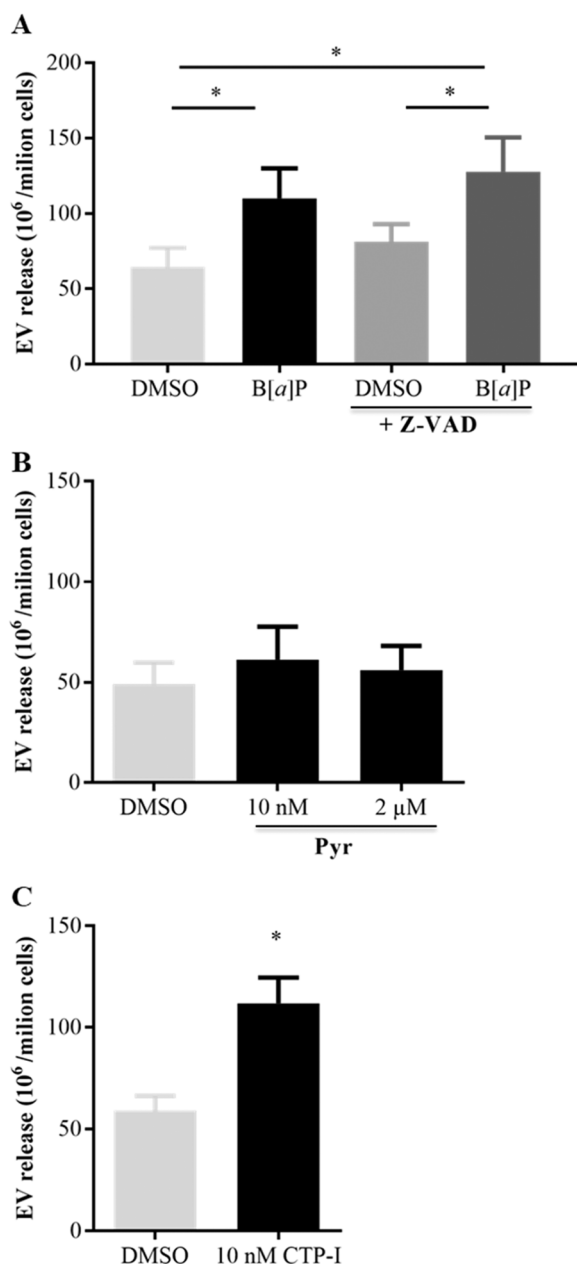
effects caused by 200  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> B(a)P or 2000  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> CTP-I (Fig. 3A). A similar increase was caused by exposure to 2000  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> B(a)P although it did not present statistical significance ( $p = 0.0949$ ) (Fig. 3A), confirming the lack of a dose-response effect in the increase of EVs caused by B(a)P exposure. As seen *in vitro*, PAH exposures did not modify the size of total EVs secreted *in vivo* (Fig. S3D). Our previous study showed that urinary levels of tetrol-B(a)P, produced from the hydrolysis of the reactive metabolite of B(a)P, are good biomarkers of exposure and metabolic activation of PAHs (El Hajjar et al., 2023). Here, we analyzed blood concentrations of tetrol-B(a)P using GC-MSMS. As presented in Fig. 3B, blood concentrations of tetrol-B(a)P were comparable between rats exposed to B(a)P alone and within the CTP-I mixture, and considerably higher after exposure to 2000  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> compared to 200  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> (6 and 9 times higher for B(a)P and the CTP-I mixture, respectively). In parallel, higher blood B(a)P concentrations were observed after exposure to the dose of 2000  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> compared to that of 200  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup>; the increase was particularly important with the CTP-I mixture (32-fold increase compared to a 4-fold increase with B(a)P alone, Fig. 3C). Finally, for the dose of 2000  $\mu$ g.kg<sup>-1</sup>.d<sup>-1</sup> of B(a)P alone and within the CTP-I mixture, associated with significantly increased levels of tetrol-B(a)P, we investigated the association between tetrol-B(a)P levels and EV concentrations. As indicated in Fig. 3D and E, a significant correlation between EV and tetrol-B(a)P concentrations was found for treatment with B(a)P alone whereas no association was

seen for exposure to the CTP-I mixture.

Altogether, these data describe that the increase in EV production is a biological marker of *in vitro* and *in vivo* exposure to B(a)P.

### 3.2. Increased expression of EV-derived miRNAs as signatures of B(a)P exposure *in vitro* in human PBMCs and *in vivo* in rat plasma

Since circulating miRNAs largely derived from blood cells (Pritchard et al., 2012), we performed deep sequencing of the miRNome of EVs isolated from conditioned media of primary human cultured PBMCs exposed, or not, to B(a)P in culture. In addition to their ability to produce EVs (Moertl et al., 2020), we previously showed that these PBMCs possess the components of the miRNA machinery, not altered by B(a)P exposure (Souki et al., 2023). A pilot sequencing experiment was first conducted to establish a standardized procedure and a workflow for characterizing the EV-miRNome by RNA-seq (data not shown). We then sequenced miRNAs, which are abundant among small RNAs in EVs produced by PBMCs (~76 %, Fig. S2D), from pooled PBMC-derived EVs after exposure to 2  $\mu$ M B(a)P or to DMSO for 48 h. As shown in Fig. 4A, the percentage of miRNA reads did not appear significantly altered after B(a)P exposure. In total, we detected 1745 known miRNAs expressed on the criteria of base Mean number of reads > 10 in at least one of the 2 conditions (Tables S2 and S3), with 1332 (76 %) shared by DMSO and B(a)P (data not shown). After correction with an adjusted  $p$ -value  $\leq 0.05$ ,



**Fig. 2.** B(a)P-induced EV release *in vitro* is linked to PAH exposure and not a consequence of apoptotic stress in primary human PBMCs. PBMCs were cultured and exposed for 48 h to DMSO (control) or to 10 nM and 2 μM Pyr (B), to the CTP-I adjusted to 10 nM of B(a)P (C) and, pre-exposed or not to 10 μM Z-VAD for 1 h before a B(a)P exposure to 2 μM (A). EVs were isolated and NTA determined their concentration. Data are expressed as the mean ± SEM (n=6 (A), 10 (B) or 11 (C) independent cultures). \*,  $p \leq 0.05$  for multiple comparisons (analysis of variance for repeated measures followed by the Tukey's multiple comparison test (A) or compared with control (paired Student's test (C)).

the miR-132-3p was the only one significantly up-regulated in EVs from B(a)P-exposed PBMCs compared to DMSO ( $\log_2FC=1.3$  and adjusted  $p$ -value=0.02). By filtering miRNAs using the individual  $p$ -value ( $\leq 0.05$ ), we selected 18 miRNAs, showing differential expression ( $|\log_2$ -FoldChange  $> 0.5$ ) after B(a)P treatment; 11 miRNAs were up-regulated (132-3p, 361-3p, 339-5p, 155-5p, 181a-2-3p, 342-3p, 29b-3p, 29a-3p, 146a-5p, 181b-5p and 320c), and 7 miRNAs were down-regulated (615-5p, 3195, 7847-3p, 597-5p, 6838-3p, 3960 and 451a) (Fig. 4B, Tables S2 and S3). A selection of abundant miRNAs (baseMean number

of reads  $> 2000$ ) retained 8 over-expressed miRNAs (155-5p, 361-3p, 342-3p, 339-5p, 146a-5p, 29a-3p, 29b-3p and 181b-5p) and 1 down-regulated miRNA (451a) (Fig. 4C). Fig. 4C indicates their differential expression based on the number of reads in each condition; unsupervised hierarchical clustering shows the distinct expression between DMSO and B(a)P exposures (heatmap inset, Fig. 4C).

We then realized RT-qPCR assays on the 8 most expressed and differentially up-regulated miRNAs (342-3p, 146a-5p, 29a-3p, 155-5p, 29b-3p, 181b-5p, 339-5p, and 361-3p), using EVs from separate cultures of PBMCs exposed or not to PAHs for 48 h. As shown in Fig. 5, RT-qPCR assays confirmed significant increases in miRNA levels identified by RNA-seq upon exposure to 2 μM of B(a)P. Interestingly, a lower concentration of B(a)P, *i.e.*, 10 nM, shown to induce EV release from PBMCs (Fig. 1B), also significantly increased the expression of all the miRNAs tested (Fig. 5A-H). Pyr, at a concentration of 10 nM, which did not affect EV release at 48 h (Fig. 2B), did not significantly modify the levels of these miRNAs (Fig. 5A-H). Additionally, all the miRNAs tested were significantly increased, after exposure to 10 nM of the CTP-I mixture (Fig. 5A-H), which also increased EV production (Fig. 2C). By contrast, B(a)P did not induce any changes of miR-93-5p and miR-24-3p levels (Fig. S4), highly expressed and not regulated in our RNA-seq experiments (Tables S2 and S3). This data excludes a generalized increase in the levels of miRNAs that could result from the increase in the production of EVs. The panel of vesicular miRNAs made up of miR-342-3p, -146a-5p, -29a-3p, -155-5p, -29b-3p, -181b-5p, -339-5p and -361-3p, is selectively up-regulated in PBMCs after exposure to B(a)P alone and within a mixture.

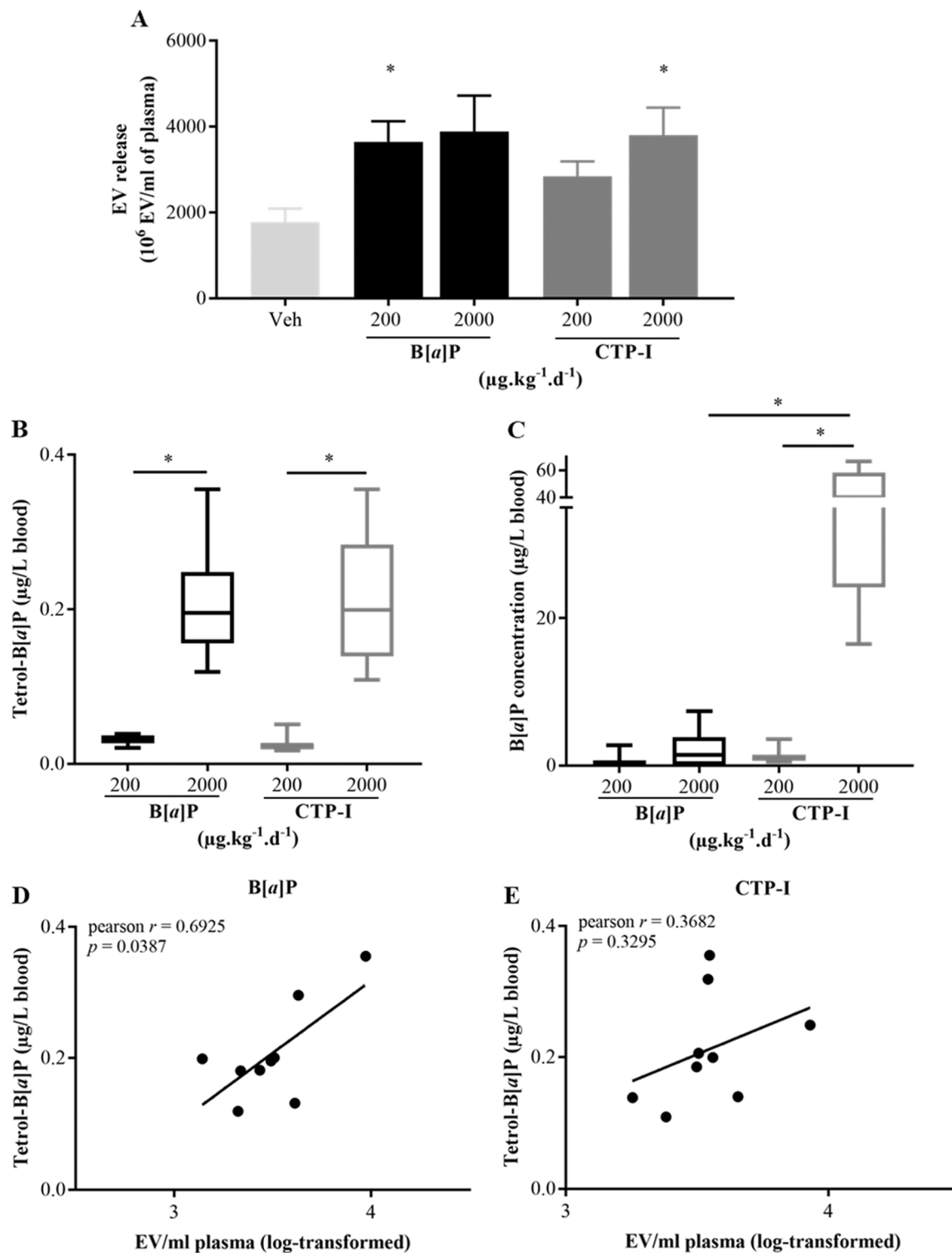
Finally, we examined the expression and regulation by PAHs of these miRNAs *in vivo* in EVs from rat plasma using RT-qPCR. As it has been reported that miRNAs are generally conserved across species (Li et al., 2010), we aligned sequences between human and rat for miR-342-3p, -146a-5p, -29a-3p, -155-5p, -29b-3p, -181b-5p, -339-5p, and -361-3p. For miR-342-3p, -29a-3p, -29b-3p, 181b-5p, and -339-5p, the sequences were 100 % conserved unlike those of miR-146a-5p, -155-5p and -361-3p (Fig. S4). Thereafter, using RT-qPCR, we measured levels of miR-342-3p, -29a-3p, -29b-3p, -181b-5p and -339-5p and their regulation in plasma of rats treated with 200 and 2000  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  of B(a)P or CTP-I. The level of miR-339-5p was very low in rat plasma (threshold cycle (Ct)  $> 30$ ) and could not be measured. The level of expression of miR-29a-3p, -29b-3p, -181b-5p and -342-3p, were sufficient for analysis (Ct values ranging from 24 to 29) and displayed a large variability in their levels after exposure to B(a)P, alone and within the CTP-I mixture. Nevertheless, we were able to observe a statistically significant up-regulation of miR-342-3p after exposure to 2000  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  B(a)P (Fig. 6A).

Altogether, these data identify the circulating miR-342-3p as a biomarker of *in vitro* and *in vivo* exposure to B(a)P; its potential to be informative and its functional implications will need further exploration.

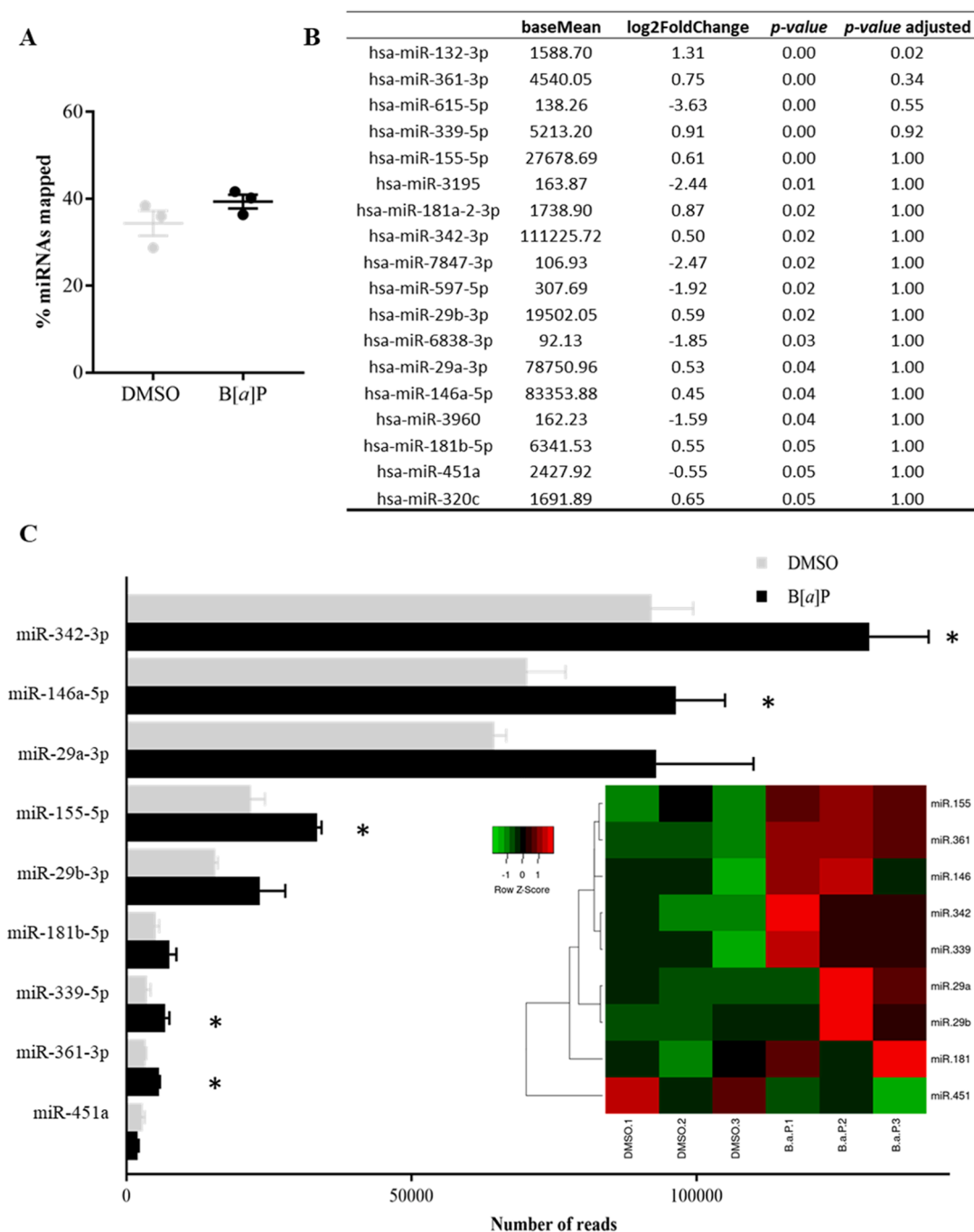
#### 4. Discussion

Among ubiquitous environmental contaminants, PAHs, in particular B(a)P, exert various effects on human health such as carcinogenicity, immunosuppression, atherogenesis or inflammation (Holme et al., 2019; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2016; Stading et al., 2021). Due to these effects, the development of biomarkers of PAH exposure is necessary to assess health risks. Recently, circulating EVs and their biological contents have emerged as new players in environmental toxicology (Carberry et al., 2023). Thus, EV-derived miRNAs have been suggested as biomarkers of exposure and/or effect to environmental stresses like radiations (Moertl et al., 2020; Wagner et al., 2018). The objective of this work was to explore blood EVs and their miRNA contents as novel biomarkers of exposure to PAHs.

PBMCs are cells present at the entry routes of environmental



**Fig. 3.** B(a)P increases EV release *in vivo* in rat plasma. Rats were orally exposed during 4 days to Vehicle (Veh), to 200 and 2000  $\mu\text{g.kg}^{-1}.\text{d}^{-1}$  of B(a)P, or to the CTP-I mixture adjusted to 200 and 2000  $\mu\text{g.kg}^{-1}.\text{d}^{-1}$  of B(a)P. Blood was collected 4 h after the last exposure. (A) EVs were isolated and NTA determined their concentration. (B, C) Blood concentrations of tetrol-B(a)P and B(a)P were determined using GC-MSMS and HPLC-Fluo, respectively. (D, E) Relationships were analyzed between concentrations of EVs (log-transformed) and tetrol-B(a)P for 2000  $\mu\text{g.kg}^{-1}.\text{d}^{-1}$  B(a)P, and the CTP-I adjusted 2000  $\mu\text{g.kg}^{-1}.\text{d}^{-1}$  of B(a)P, the line indicates the linear trend; Pearson's  $r$  correlation coefficient and  $p$ -value are shown. Data are expressed as the mean  $\pm$  SEM (A), or as the median with min/max values, upper and lower quartiles (B, C) ( $n=9$  rats *per* group (A-E)). \*,  $p \leq 0.05$  compared with Veh (A) or for multiple comparisons (B, C) (Kruskal-Wallis' analysis of variance followed by the Dunn's multirange test).

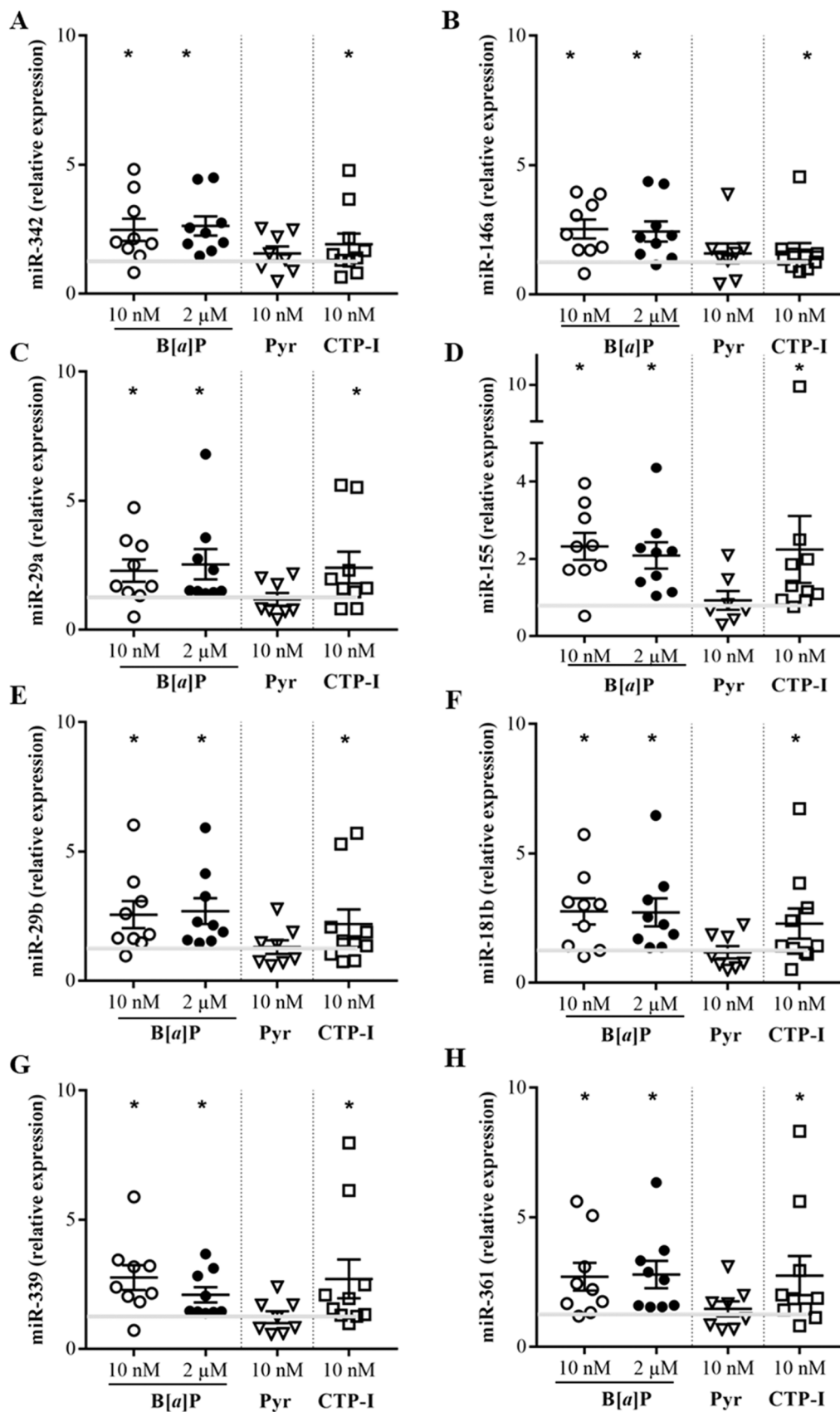


**Fig. 4.** RNA-seq identifies B(a)P-regulated miRNAs in EVs from primary human cultured PBMCs. PBMCs were exposed to DMSO (control) or to 2  $\mu$ M B(a)P for 48 h. EVs were isolated, small RNAs were extracted and miRNA expression was measured by RNA-seq. (A) Percentages of miRNAs mapped. (B) Differentially expressed miRNAs after B(a)P exposure filtered using the individual *p*-value from RNA-seq experiments ( $\leq 0.05$ ). (C) miRNA abundance ranked by the number of reads associated with each miRNA after DMSO and B(a)P exposure for the top 9 expressed miRNAs; an unsupervised hierarchical clustering using Pearson correlation on normalized reads is displayed (<http://www.heatmapper.ca/expression>). Data are expressed as the mean  $\pm$  SEM ( $n=3$  equal pools with 4 independent cultures *per* pool). \*,  $p \leq 0.05$  compared with control (paired Student's *t* test (C)).

contaminants, which can alter their phenotype, as we and other have previously reported for PAH exposure (Souki et al., 2023; Vayssier-Taussat et al., 2001). In addition, PBMCs have been shown to produce EVs (Wagner et al., 2018), which can be subsequently found in plasma, and reflect exposure to environmental pollutants and cellular responses. However, the potential and functional utility of EVs as biomarkers require further study. Here, we report an increased production of EVs in conditioned media from PBMCs after exposure to B(a)P, alone and within a mixture, which was not accompanied by variations in the

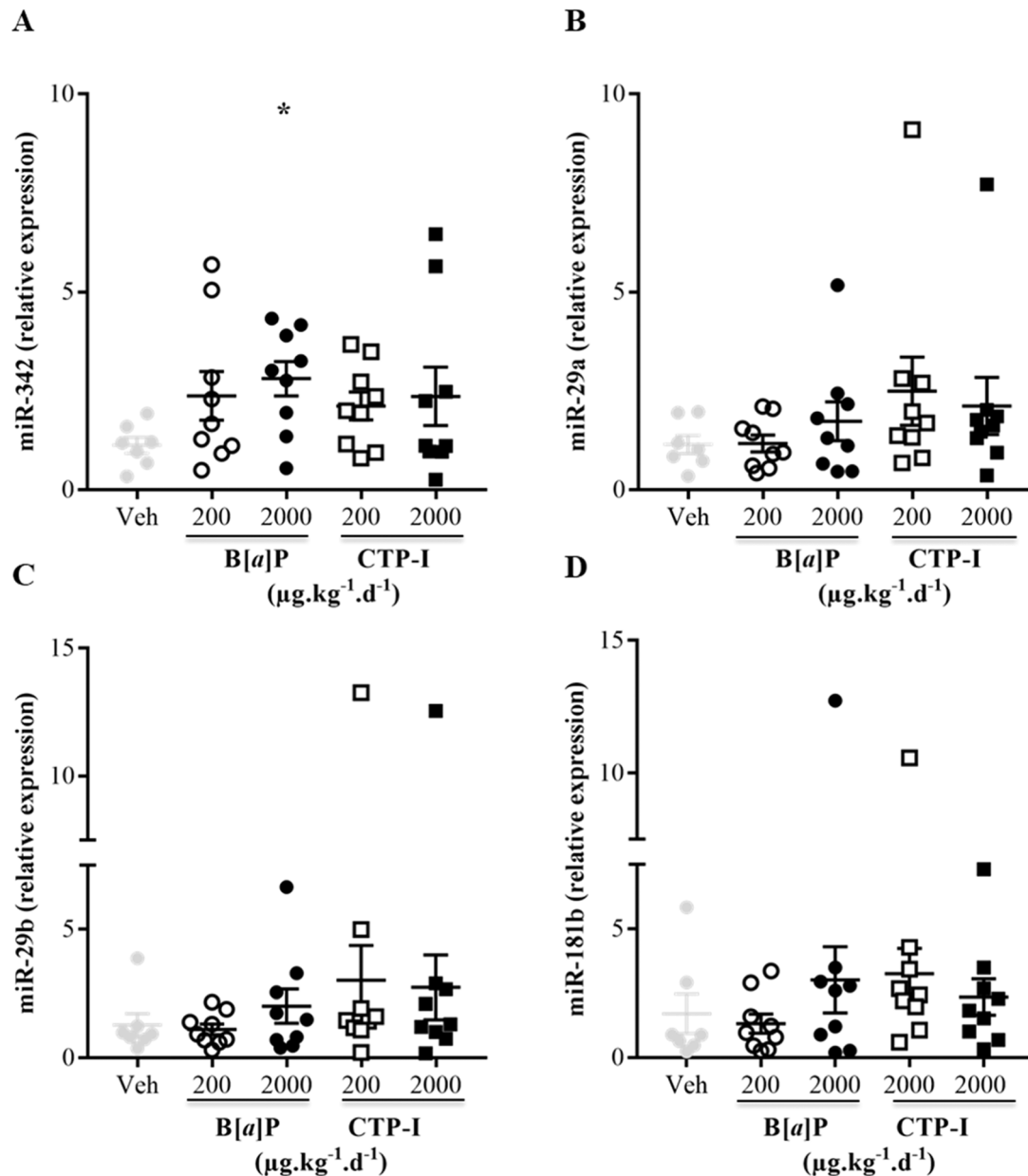
size of EVs. Interestingly, the increased release of EVs by PBMCs observed *in vitro* also occurred *in vivo* in rat plasma. As seen for PBMCs, the size of EVs was not modified after *in vivo* exposure to B(a)P. So far, only a few studies have focused on the effects of PAHs on EV production. Our team reported that PAHs, in particular B(a)P, increased EV production in both endothelial and hepatic cells *in vitro* (Le Goff et al., 2019; van Meteren et al., 2019). We also reported increased EVs in urine of rats treated with a mixture of PAHs (Le Goff et al., 2019). Altogether, our data suggest that the whole organism responds to PAH exposure by





(caption on next page)

**Fig. 5.** RT-qPCR validates selected vesicular miRNAs identified by RNA-seq in primary human cultured PBMCs exposed to PAHs. PBMCs were exposed to DMSO (control), to 10 nM or 2 μM B(a)P, to 10 nM Pyr, or to the CTP-I mixture adjusted to 10 nM of B(a)P for 48 h. EVs were isolated, small RNAs were extracted and miRNA expression was measured using RT-qPCR. Data are normalized relatively to miRNA expression levels in control-treated conditions (set at 1-unit (gray line)), and are shown as the mean ± SEM (n=9 (B(a)P), 8 (Pyr) or 10 (CTP-I) independent cultures with triplicata *per* experiment, different for each treatment conditions (dotted lines)). \*,  $p \leq 0.05$  compared with control (Friedman's analysis of variance followed by the Dunn's multirange test (A-H, B(a)P), paired Wilcoxon's test (A-H, CTP-I)).



**Fig. 6.** RT-qPCR validates miR-342-3p regulation in rat plasma after B(a)P exposure. Rats were orally exposed during 4 days to Vehicle (Veh), to 200 and 2000 μg.kg<sup>-1</sup>.d<sup>-1</sup> of B(a)P, or to the CTP-I mixture adjusted to 200 and 2000 μg.kg<sup>-1</sup>.d<sup>-1</sup> of B(a)P, during 4 days. Blood was collected 4 h after the last exposure. EVs were isolated, small RNAs were extracted and miRNA expression was measured using RT-qPCR. Data are normalized relatively to miRNA expression levels in Veh-treated conditions and are shown as the mean ± SEM (n=7 (Veh) or 9 rats *per* group). \*,  $p \leq 0.05$  compared with Veh (Kruskal-Wallis' analysis of variance followed by the Dunn's multirange test (A)).

increasing plasma EV concentration from PBMCs but also probably from heterogenous cellular origins as reported (Alberro et al., 2021). By enabling the preparation of total EVs, both small and large EVs as indicated by NTA and TEM experiments, our isolation procedure reflects the complexity of the body. Indeed, many studies on EVs released upon environmental stresses only referred to a selected exosome population, as reported by Moertl et al. (2020), but the potential to use total EV

levels as markers of pollutant susceptibility has already been reported by Rota et al. (2020) in the context of exposure to particulate matter. By highlighting the potential of EVs as predictive biomarkers of exposure/effect to B(a)P and given the recently described role of EVs in the increased risk of diseases after exposure to environmental pollutants (Eckhardt et al., 2022), circulating levels of EVs could be proposed as interesting indicators of both exposure and systemic response to

environmental pollutants, like PAHs.

After determining the impact of PAHs on EV concentration and size, we examined the composition of EV miRNAs. We used RNA-seq to determine the relative distribution and quantification of miRNAs from EVs. We previously characterized cellular miRNAs regulated after a B(a)P exposure in primary human cultured PBMCs (Souki et al., 2023). To date, no studies have been conducted to identify the unique expression profiles of EV-derived miRNAs in PAH-exposed PBMCs. Firstly, using the same PBMC cultures exposed to B(a)P as in our previous paper, we observed a higher and distinct number of vesicular miRNAs, as compared to cellular one's (Souki et al., 2023). These data are consistent with the proposed role of EVs as a distinct compartment, that is, not simply a copy of the contents of their original cells (Chung et al., 2023). One could then postulate that cell-derived EVs represent a more improved fraction compared to the cells themselves in detecting miRNA changes. However, miRNAs appeared to be less significantly regulated in EVs than in cells (Souki et al., 2023). We also noted high variability as well as low miRNA copy numbers in EVs, suggesting a higher diagnostic potential of PBMC miRNomes compared to those of EVs, as previously reported (Almenar-Pérez et al., 2020). Indeed, in our RNA-seq study, adopting the stringent adjusted *p*-value led to the identification of only miR-132-3p as significantly up-regulated miRNA. Such a low number of altered miRNAs using the adjusted *p*-value has already been described (Sisto et al., 2019) and is probably due to the human variability of responses (Liamin et al., 2018; Souki et al., 2023; Sparfel et al., 2010). Because of its low and undetected expression in EVs derived from human PBMCs and rat plasma, respectively, we did not focus our attention on miR-132-3p, but selected other highly expressed miRNAs such as miR-342-3p, -146a-5p, -29a-3p, -155-5p, -29b-3p, -181b-5p, -339-5p, and -361-3p. Among them, polymorphisms in miR-146a were associated to PAH-related effects in coke oven workers (Deng et al., 2016), and circulating miR-155 levels were increased in women exposed to biomass smoke (Ruiz-Vera et al., 2019b). These miR-146a and miR-155 are not 100 % conserved between human and rat. In animal studies, miR-29a has been examined upon PAH exposure and suggested as non-relevant in the animal model (Tomesz et al., 2020), whereas miR-29b expression was significantly up-regulated (1.48 times) in mice orally exposed to 150 mg.kg<sup>-1</sup> B(a)P (Halappanavar et al., 2011). In the present study, only miR-342-3p was significantly regulated in rat plasma upon exposure to 2 000 mg.kg<sup>-1</sup>.d<sup>-1</sup> B(a)P. To our knowledge, this is the first description of an association between circulating miR-342-3p levels and B(a)P exposure. In benzene-exposed mice, altered expression of miR-342-3p has been reported in hematopoietic progenitor cells (Wei et al., 2015). The expression and role of miR-342-3p have mainly been studied in many types of cancer. For example, miR-342 acts as a tumor suppressor by regulating the proliferation and apoptosis of cancer cells by targeting the oncogene *myc* and the anti-apoptotic gene *Bcl2* (Chen et al., 2021; Komoll et al., 2021). An increased expression of miR-342-3p has also been associated with a less glycolytic phenotype (Romero-Cordoba et al., 2018). Decreased serum miR-342-3p levels were correlated with poor prognosis and it was proposed as a promising biomarker in some tumors (Qin et al., 2018). Consistent with these data and since miR-342-3p is not altered in cells (Souki et al., 2023), we can hypothesize that B(a)P causes a selective release of miR-342-3p into EVs to produce a protective response on adjacent cells and limit the damage induced by the HAP, such as the antitumor effect previously suggested (Han et al., 2019). Additional studies are necessary to determine whether genes such as *Bcl2* or the glycolytic pathway, previously shown to be affected by B(a)P (Hardonnière et al., 2017; Souki et al., 2023), are involved in this response.

Finally, our study identified the blood release of EVs and the EV miR-342-3p as biomarkers of exposure to B(a)P in an environmentally relevant context, while Pyr alone, another PAH, did not modify them. Interestingly, the concentration of 10 nM of B(a)P is in the same order as that of human exposure and below those classically used in the literature

(Bukowska et al., 2022). Additionally, we used a PAH mixture, the CTP-I, adjusted at a concentration of 10 nM B(a)P, that is not synthetic as already reported (Bouchard et al., 2002, 1998; Grova et al., 2017). It is an element of furnace electrodes from the metal production industry, closely recapitulating worker exposure to PAHs (El Hajjar et al., 2023). With regards to environmental exposure assessments, growing data support the utility of EVs as biomarkers of exposure in humans: due to their inherent stability and efficiency in transferring cargoes, EVs serve as biomarkers of harmful environmental exposures and diseases (Eckhardt et al., 2022). Few studies have explored the role of vesicular miRNAs as novel biomarkers of PAH exposure (Deng et al., 2016; Ruiz-Vera et al., 2019a,b). Currently, exposure to PAHs can be measured by determining levels of their metabolites in biological fluids (Barbeau et al., 2014, 2018). However, the relevance of many metabolites as biomarkers of PAH exposure has been questioned (Valière et al., 2022). We and others proposed the urinary levels of tetrol-B(a)P, derived from the B(a)P-7,8-diol-9,10-epoxide, as the most relevant biomarker for the assessment of B(a)P exposure (Barbeau et al., 2018; Hilton et al., 2017; Zhong et al., 2011). Our present data also indicate that blood tetrol-B(a)P are useful to evaluate the internal exposure to B(a)P with an association with blood EV concentrations for exposure to 2000 µg.kg<sup>-1</sup>.d<sup>-1</sup> B(a)P. However, we noted a lack of a dose-response in either EV production or miRNA expression after *in vitro* and *in vivo* exposure to B(a)P. Some studies have suggested and highlighted dose-response relationships in the release of EVs after exposure to toxicants (Le Goff et al., 2019; Moertl et al., 2020) but a lack of effect depending on the concentration has also been described for bisphenol A (Rodosthenous et al., 2019). In addition, likely by following the increase in EVs, extracellular miRNAs could be proposed as indicators of an adaptative biological response, probably by acting as housekeepers of homeostasis in metabolic diseases as reported (Mori et al., 2019).

## 5. Conclusion

Altogether, this study associates B(a)P exposure with increased EV levels, thereby increasing the amount of miR-342-3p. It also positions EVs as informative markers of the cell response and highlights the importance of finding circulating miRNAs specific of exposures that, once validated, will contribute to improve the biomonitoring of exposure to B(a)P.

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## CRedit authorship contribution statement

**Alexis Février:** Writing – review & editing, Validation, Methodology, Investigation. **Valentine Genêt:** Writing – review & editing, Validation, Methodology, Investigation. **Benjamin Saintpierre:** Writing – review & editing, Visualization, Software, Resources, Formal analysis, Data curation. **Morgane Le Gall:** Writing – review & editing, Software, Resources, Formal analysis. **Rima Souki:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Christine Demeilliers:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jérémy Amossé:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Christine Demeilliers:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Marie**

**Marques:** Writing – review & editing, Validation, Methodology, Investigation. **Maguy El Hajjar:** Writing – review & editing, Validation, Methodology, Investigation. **Lydie Sparfel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Eric Le Ferrec:** Writing – review & editing, Investigation. **Franck Letourneur:** Writing – review & editing, Validation, Software, Methodology, Investigation. **Dominique Lagadic-Gossmann:** Writing – review & editing, Methodology, Investigation.

## Declaration of Generative AI and AI-assisted technologies in the writing process

Authors did not use generative AI tools in writing the manuscript.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lydie SPARFEL reports financial support was provided by National Agency for Food Environmental and Occupational Health and Safety. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2024.117065](https://doi.org/10.1016/j.ecoenv.2024.117065).

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