



HAL
open science

Extracellular vesicles of MSCs and cardiomyoblasts are vehicles for lipid mediators

Nathalie Pizzinat, Varravaddheay Ong-Meang, Florence Bourgailh-Tortosa, Muriel Blanzat, Lucie Perquis, Daniel Cussac, Angelo Parini, Verena Poinso

► **To cite this version:**

Nathalie Pizzinat, Varravaddheay Ong-Meang, Florence Bourgailh-Tortosa, Muriel Blanzat, Lucie Perquis, et al.. Extracellular vesicles of MSCs and cardiomyoblasts are vehicles for lipid mediators. *Biochimie*, 2020, 178, pp.69-80. 10.1016/j.biochi.2020.07.013 . hal-03018214

HAL Id: hal-03018214

<https://hal.science/hal-03018214>

Submitted on 30 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Extracellular vesicles of MSCs and cardiomyoblasts are vehicles for lipid mediators.

Pizzinat N.¹, Ong-Meang V.¹, Bourgailh-Tortosa F.¹, Blanzat M.², Perquis L.², Cussac D.¹,
Parini A.¹, Poinso V.^{1*}.

¹I2MC, INSERM/UT3, 1av Jean Poulhès, BP84225, 31432 Toulouse-Cedex

²IMRCP, CNRS/UT3, 118 route de Narbonne, 31062 Toulouse-Cedex

Corresponding author: Verena Poinso, verena.poinso@inserm.fr, I2MC, U1048
INSERM/UT3, 1av Jean Poulhès, BP84225, 31432 Toulouse-Cedex. Phone : +33 05 31 22 41 22

KEYWORDS: Extracellular vesicles, lipid mediator, inflammation, heart, PUFA

1. INTRODUCTION:

In many pathophysiological situations, inflammation is an ubiquitous process which can either be positive, or, on the contrary, represent a deleterious mechanism for the organ or tissue. Indeed, the inflammatory response is a prerequisite to initiate the reparatory processes, but intense and persistent inflammation causes tissue damages. The resolution of inflammation is considered to be an active process, influenced by factors released by the matrix and local cells. Considering the heart, myocardial damages involve acute (myocardial infarction) and/or chronic (heart failure) inflammatory phases which most of the time need to be resolved. Different cell types from the stromal and parenchymal compartments can play a role in these processes through the secretion of paracrine factors that modulate inflammation.

Concerning the stromal compartment, mesenchymal stem cells (also known as mesenchymal stromal cells or mesenchymal progenitor cells) are stromal cells that can be isolated from different adult tissues including bone marrow and adipose tissue. Belonging to the family of progenitor cells, they are implied in the renewal of different cell types (chondrocytes, osteocytes, adipocytes, fibroblasts, smooth muscle cells, endothelial cells). In addition, MSCs secrete a variety of paracrine factors, regulating angiogenesis, cell survival and inflammation. The role of the MSCs secretome in regulation of the immune response is particularly relevant. Indeed, MSC have been shown to regulate the activation and differentiation of many cells of the immune system, including T-cells, B-cells, NK cells, monocytes,

dendritic cells, and neutrophils [1]. The first evidence supporting the role of MSCs in regulation of cardiac homeostasis and repair has been supplied by the studies on cell therapy of heart failure. These studies showed that administration of Bone-Marrow Mesenchymal Stem Cell (BM-MSC) in failing hearts of animal models and humans promoted angiogenesis, decreased fibrosis and improved cardiac function. More recently, several studies showed the existence of cardiac MSC-like cells (cMSCs) playing a critical role in regulation of cardiac function through the cell renewal and the secretion of a variety of paracrine factors [2] and in the chronic low grade inflammation associated with aging [3].

Extracellular vesicles are small vesicles (30 to 150 nm), carried by Multi-Vesicular bodies (MVB) and secreted outside of the cell in the stroma after the membrane fusion of the MVBs [4-6]. Once secreted, these micro-vesicles can be internalized within neighbouring cells and deliver their molecular material. Their envelope composition is specific (eg. enriched in lipid raft and tetraspanins), allowing their addressing and incorporation within the neighboring cells. It enables the parent-cell signals to without direct cell to cell contact [7].

The parent-cell information, packed into extracellular vesicles in the form of lipids, proteins and nucleic acids, is called "cargo". Extracellular vesicles derived from MSCs (MSC-Ex) have been used in regenerative medicine. MSC-Ex function is often explained by the constant transfer of miRNAs and cytokines[8]. The anti-inflammatory signals as well as the angiogenic signals carried by the extracellular vesicles remain to be fully identified.

Long chain polyunsaturated fatty acids (PUFAs) derivatives, known as lipid mediators or oxilipins, are among the most efficient factors promoting or resolving inflammation. Released from membrane phospholipids or supplied by diet (α -linolenic acid), PUFAs are modified (cyclized, oxygenated) by different classes of enzymes. Cyclooxygenases (COX) initiated the transformation of arachidonic acid AA (C₂₀ ω -6) into prostaglandins (PG) D, E, F or I or into thromboxane (TX) and Lipoxygenases transform them into leukotrienes (LT) or lipoxins (LX) [9-11]. Elongases lengthen their chain to 22 carbon atoms, then the lipoxigenase oxidize them to other compounds such as resolvins (Rv) and maresins (Ms) [12,13] thus limiting the synthesis of pro-inflammatory compounds such as PGs or LT. The switch from pro-inflammatory lipid mediators (LT, PG, TX) to pro-resolvers (LX, Rv, Ms) is regulated by the very long chain fatty acids oxidizing enzymes. The LX produced leads to the arrest of the inflammatory response and the recruitment of resolutive macrophages [14]. Finally, the n-3 protectants (PD) derived from endogenous ω -3 docosapentaenoic (ω -3 DPA) initiated by the 15-LOXs play a role in the macrophage polarization [15-17] whose stromal cells are orchestrators [18-21]. These molecules could contribute to the beneficial effects of MSCs as the increase in the production of prostacyclin by MSCs has been shown to be more effective for facilitating perfusion recovery and to promote host-tissue regeneration than MSCs alone [22]. Beside, inhibition of the COX-2 mediated prostaglandin E₂ synthesis enhanced cardiac reprogramming and subsequent silencing of inflammation and fibroblast program [23].

Recent studies supported the role of lipid-mediators in the heart failure [eg. 23-26]. Obesity induced heart failure starts with the accumulation of arachidonic acid (C20:4 ω -6 or AA) [27] and promotes progression of heart failure [28]. Thus partial substitution of ω 6 PUFA by ω 3 PUFA (C18:3 ω -3 ALA), (C20:5 ω -3 EPA) and (C22:6 ω -3 DHA) in the diet reduces the proportion of AA in cardiac phospholipids [29] and protect the heart against several diseases [30-32].

To date, the presence of such lipid cargo in the extracellular vesicles and their role in organs/tissues function is almost unknown. This is probably due to the difficulty to analyse these extremely well-regulated signals within the tenuous extracellular vesicles. One single work has been reported very recently concerning the analysis of Resolvins and prostaglandins in MSC-extracellular vesicles [33].

Combined together, it appeared us interesting to know if extracellular vesicles of BM-MSCs and the cardiomyoblast cell line H9c2 are putative vehicles for the inflammation lipid mediator's signalization. H9c2, are particularly interesting as they present characteristics of proliferative neonatal cardiomyocytes [34]. In addition, these cells seem to be involved in the generation of the inflammation through the release of inflammatory cytokines (interleukin 1 β and tumor necrosis factor α) in pro-inflammatory model [35]. These cells may also modulate inflammation by releasing microRNAs (such as miR-146a) in extracellular vesicles which could attenuate inflammation through an inhibition of the expression of Il-1 and TNF- α receptors [36]. Therefore, we studied and compared the lipid-mediators content of the extracellular vesicles produced by BM-MSCs and H9c2 cells supplemented with ω 3-PUFA to investigate if the positive effect of fish oil diet on the heart function can be explained by such extracellular vectorization of lipid mediators.

2. MATERIAL AND METHODS:

2.1. Biological material

The experiments were carried out on three cell types: A commercial H9c2 cardiomyoblast line (ATCCR CRL-1446 TM, Sigma Aldrich), mesenchymal stem cells from adult Lewis rat bone marrow (BM-MSC) previously characterized by our group [37] and human BM-MSCs obtained from donors (Etablissement Français du sang, EFS).

2.2. Cell cultures

The cell cultures were carried out in a CO₂ incubator thermostatically controlled at 37 °C with 5% CO₂. Cardiomyoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCCR) culture

medium and MSCs in Alpha Modified Eagle's Medium (MEM- α no nucleosides). All media were supplemented with 10% foetal calf serum (FCS) and a mixture of antibiotics (100 U / ml of streptomycin and 100 U / ml of penicillin).

The number of apoptotic and necrotic BM-MSCs and H9C2 present in cell cultures after serum starvation and before ultracentrifugation was determined by flow cytometry with Annexin V labelling Kit (BD pharmingen). The number of apoptotic or necrotic cells was always below 5% (Supplementary figure 1). For collecting the vesicular fraction, the dishes were rinsed with 10 mL of PBS and the medium replaced with the same medium but deprived at 1% of ultra-centrifuged foetal serum out of 24 hours. The supernatant is collected over an additional 24 hours period after renewal of the medium.

2.3 Dynamic Light Scattering

The extracellular vesicles' size distributions were determined by Dynamic Light Scattering (DLS) using a Malvern Instruments Nano ZS, with a He-Ne laser (633 nm), at a scattering angle of 173 °. The analyses were performed at $25.0 \pm 0.1^\circ\text{C}$. The deconvolution of the measured intensity autocorrelation function of the sample was realized with the CONTIN algorithm on Malvern Instruments software to obtain the distribution of diffusion coefficients (D). The apparent equivalent hydrodynamic diameter (d_h) was then obtained using the Stokes-Einstein equation. Hydrodynamic diameter values were obtained from three different runs.

2.4 Cryo-SEM

Extracellular vesicles were observed through Scanning Electron Microscopy with freeze-fracture (CryoSEM) using an ESEM Quanta FEG250 (FEI), operating at 5 kV and about 10^{-4} Pa. The samples were mounted on pin specimen holders and frozen in liquid nitrogen. They were fractured in a vacuum chamber at -140°C , then a 10 min sublimation at -90°C was performed. It was followed by a platinum coating using an Ion-sputter JFC-1100 (JEOL). Finally, the specimen holder was transferred to the observation chamber kept at -140°C under a flow of nitrogen gas.

2.5 RNA extraction and RT-PCR

The RNAs were extracted from H9C2 and rat BM-MSC cells using the RNA extraction kit (PromegaRelia prep RNA Cell Miniprep System) and quantified by spectrophotometry (NanoDrop2000). They were then reverse transcribed using the Reverse Transcription Kit (Applied Biosystems). A volume of RNA containing 1 μg of total RNA and 1 volume of RT Master Mix (RT buffer, dNTP (100mM), RT random primers, RNase inhibitor and MultiScribe RT were mixed and then quickly incubated on an appropriate

thermal cycler according to manufacturer. Gene mRNA expression was normalized with respect to the housekeeping gene Rplp0.

Table 1

2.6. Sample preparation

The supernatants of the cell culture are centrifuged for 20 minutes at 10,000 g in order to remove the apoptotic bodies. The supernatants were then subjected to precipitation by ultracentrifugation (100,000g; 1h30, 4°C.) (Beckman and Coulter).

Cells were collected using a scraper in 1 mL PBS and transferred to haemolysis tubes. An aliquot of 50 µL was taken in order to carry out an assay of the cellular proteins.

2.7. Protein assay and immunodetection

Protein extraction of the samples was carried out using the RIPA lysis buffer containing phosphatase and protease inhibitors (composition: TrisHCl 50 mM; NaCl 150 mM; NP40 1%; Desoxycholic Acid 1%; SDS 0, 1%; EDTA 5 mM; NaF 20 mM). The lysis was carried out in ice, 5 µL of buffer were added to the samples, shaken with a vortex and finally incubated for 30 minutes at 4°C. The determination of total proteins was carried out by the BCA method (BiCinchoninic acid Assay) in a 96-well plate. A standard range was produced from a protein solution of BSA (bovine serum albumin) at 1 mg / ml (0; 62.5; 125; 250; 500; 1000 µg / ml). The samples were diluted 1/10 in PBS and then 10 µL were deposited in the wells in the presence of 200 µL of the BCA reagent. After incubation at 37 ° C for 30 minutes, the absorbance was measured at 570 nm using a spectrophotometer (TECAN). Immunodetection was realized using JESS Separation Capillary Cartridge 12-230 kDa with 1.2 µg of purified extracellular vesicles fraction. Alix protein detected with the rabbit anti Alix antibody (cell signalling 1/50) according to the manufacturer and total protein content was evaluated by protein normalization buffer. The signals generated were detected and analyzed by Compass software (Protein Simple).

2.8. Free-lipid extraction

Deuterium-labeled eicosanoids (LxA4-d5, LTB4-d4 and 5-HETE-d8) were mixed at a concentration of 400 ng/mL in MeOH and used as internal standard (IS) solution. The 1 mL cell and extracellular vesicles suspensions were placed in the presence of 10 µL of IS and the lipid extraction was carried out in glass haemolysis tubes with twice 500 µL of CHCl₃. The samples were vortexed and then left to settle. The organic phases were collected, mixed and evaporated to dryness under nitrogen flow at 45 ° C and the

samples stored in pill boxes with glass inserts in 20 μ L of MeOH at -80° C, then subjected to spectrometric analysis by LC / MS-MS.

2.9. LC / MS-MS analysis

Mass spectrometric analyzes were performed by Metatoul facilities, according to LeFaouder et al. [38]. Briefly, High performance liquid chromatography was performed using an Agilent 1290 Infinity. The analytical column was a ZorBAX SB-C18 column (2.1 mm, 50 mm, 1.8 μ m) (Agilent Technologies) maintained at 40° C. The mobile phases consisted of water, ACN and FA (75:25:0.1; v/v/v) (A) and ACN, FA (100:0.1, v/v) (B). The linear gradient was as follows: 0% B at 0 min, 85% B at 8.5 min, 100% B at 9.5 min, 100% B at 10.5 min and 0% B at 12 min. The flow rate was 0.35 mL/min the injection volume was 5 μ L. The HPLC system was coupled on-line to an Agilent 6460 triple quadrupole MS (Agilent Technologies). Electrospray ionization (ESI) was performed in negative ion mode. Analyses were performed in Selected Reaction Monitoring detection mode (SRM). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) by correlation with the IS response.

3. RESULTS

3.1. Characterization of secreted micro- and nano-vesicles.

The two cell types (BM-MSK and H9c2) were deprived each over 24h to induce the same stress than the ones described in the literature. Then, the supernatants were collected for 24 hours more. The apoptotic bodies (AB) were removed by a 10 000g centrifugation. The extracellular vesicles were collected after ultra-centrifugation at 100000g (1:30 hour), suspended in PBS and submitted to differential light scattering analyzes (DLS).

Figure 1 :

The two cell-supernatants produced very similar DLS signals, therefore we chose to present only the H9c2 results in figure 1 and 2 to gain in clarity. DLS measurements indicated the presence of three populations (figure 1). The first one around 500-1000 nm corresponds to non-spherical objects, probably residual ABs. The second one close to 100 nm correspond the signal of the targeted extracellular vesicles. Finally, particles around 30 nm could be a second population of smaller

extracellular vesicles or aggregates of residual proteins. Without information on the refraction index of the nano-vesicles, the DLS-signal is given in intensity, not in number of particles. It is well established that Exosomes are impossible to isolate in pure grade, even if the purification and characterization guidelines are in constant amelioration [39, 40]

In order to confirm that the signal around 100 nm could be extracellular vesicles, and to discriminate between both hypotheses for the smallest population, Cryo-SEM experiments were performed. Again three populations of particles could be detected around 1000 nm, 100-200 nm and a smaller one around 50-70 nm. The biggest population (figure 2Aa), which corresponds to non-spherical objects, is most likely residual ABs which confirms our hypothesis. On the contrary, both smaller populations are composed of spherical vesicles, ranging for the biggest one from 100 to 300 nm (figure 2Ab), which could be attributed to micro-vesicles and the smallest population of nano-vesicles (figure 2Ac), around 60 nm, are most probably the expected extracellular vesicles.

To confirm the significant presence of exosomes within these nanometric spherical objects, Immunodetection of Alix, an exosome specific marker, was performed on these purified extracellular vesicles fractions (human and rat BM MSCs or H9C2 (Figure 2B).

Put together, the 100 000 g precipitated fraction is enriched exosomes, even if important residual proteins are clearly visible.

Figure 2:

3.2. BM-MSC and H9c2 cells grown under standard culture conditions secrete a wide oxilipins profile

To evaluate the implication of lipid mediators as paracrine signals, we first analyzed the lipid content of BM-MSC and H9c2. The two cell types were cultivated, scraped after 1 to 3 passages, and the lipids were extracted using Chloroform. We tested several solvents (hexane, ethyl acetate, methyl formate, dichloromethane), but chloroform was the most reproducible and the more efficient over the whole profile (Supplementary figure 2). The free-lipids were analyzed using SRM LC-MS/MS. The relative quantification was calculated by comparison with internal perdeuterated standards.

Bone marrow-MSC and cardiomyoblasts exhibit very different oxilipin profiles. The BM-MSCs produce high amount of prostaglandins derived from arachidonic acid indicating a strong COX activity. 13- and 9-HODE as well as 15-HETE were also detected as major compounds. In contrast, only a minor prostaglandins distribution has been observed in H9c2 cells. The oxilipins produced by them under normal culture conditions are almost HETEs and HDoHE. 5-HETE is the precursor of LTB4, but this

strong pro-inflammatory compound could not be detected. Resolvins and maresins are reported as the most efficient pro-resolving mediators, but they were not detectable in the cells lipid extracts.

As the biological variation is important under the used culture conditions, around 10 repeats were necessary to obtain acceptable variations around the mean values. The global production of oxilipins was in the 4.5 ng/mg of cell protein range for the BM-MSC and close to 3ng/mg cell protein range for the H9c2 cells.

Figure 3

3.3. Extracellular vesicles are oxilipins transporters.

To explore the participation of lipid mediators in extracellular vesicles mediated cell communications, we measured their cargo. The oxilipin profiles of the vesicles deriving from the two types of parent cells are different from those of the cells. For example, even if the prostaglandin $\text{PGF2}\alpha$ was abundantly present in BM-MSCs, it was not detected in their extracellular vesicles. In contrast LXB4, weakly observable in cells, was largely represented in extracellular vesicles. Actually, LXB4 and 9- and 13- hydroxy-octadecadienoic acid (9-/13-HODE) principally derived from the dietary linoleic acid were the major products of BM-MSCs and H9C2 extracellular vesicles. In addition, we also analysed the presence of eicosanoids in the culture media depleted from extracellular vesicles. The oxilipin quantified in the medium were at least 100 times less abundant than in the vesicular-fractions. This points out that the analysed lipid mediators are specifically included in the extracellular vesicles.

Figure 4

Figure 5:

3.4. The cells express genes encoding for lipoxygenase and CYP450

The principal routes of synthesis of lipoxins involve the action of a series of enzymes on arachidonic acid which then gets converted into 15-HpETE. Therefore, we examined the expression of lipoxygenase. Both cell types expressed Alox15, whereas Alox5 was not detected. Alox12 expression could be found only in BM-MSCs.

Figure 6

3.5. Lipid profiles of human BM-MSC cells resemble the rat BM-MSC profiles

We next investigated the lipid mediator content of human BM-MSC, cells extensively used for therapy purpose. Actually, the human BM-MSC had a comparable profile with rat BM-MSC, they also exhibited large cellular content of prostaglandins suggesting that tissue origin of cells determined the lipid profile independently of the animal species. Even if the extracellular vesicles derived from the human cells carry important amounts of PGF, again, a LXB4 enrichment is observed in the human cell-derived EVs.

Figure 7

3.6. Modifications of the oxilipin profiles in the presence of PUFA.

ω -3 fatty acids are increasingly being used in the management of cardiovascular disease. In order to understand the effect of fish oil on cell communications, we analyzed the effect of EPA and DHA (in comparable concentrations to those measured in serum) on the oxilipins transported by the extracellular vesicles.

The free fatty acid concentration reported in the literature ranges from 450 μ M to 100 μ M (eg. for humans [41]; for mice [42]). The hBM-MSC cells containing between 5 and 10% of the total fatty acids [42, 43], under normal BM-MSC circulating PUFA concentration is estimated to be in the 10-40 μ M. Therefore, we decided to feed the cells with a PUFA concentration of 10 μ M. As the so called free fatty acids are circulating as complexes with lipid binding proteins, we combined the PUFA with bovine albumin (BSA).

Independently from the cell type, the supplementation of the culture media with 10 μ M BSA associated-PUFAs, increased the oxilipin production by a factor 10 to 100.

According to the lipid mediator biosynthetic cascade, EPA was mostly converted, in cell, into 18-HEPE. More surprisingly, it activated also the COX pathway as PGE2 and PGD2 levels increased significantly. DHA was converted as attempted in 17- and 14-HDoHE and, more surprisingly, also activated the production of 18-HEPE, but not the COX-pathway. Neither EPA nor DHA was further converted into E resolvins and D resolvins respectively. As expected, the supplementation with AA, an ω -6 fatty acid, supplied the production of prostaglandins PGE2 and PGD2 but also the formation of 15-HETE, an AA metabolite produced by the 15-LOX and /or the CYP450. Again, even if it was in a less measure compared to DHA, AA seems also to activate the production of 18-HEPE.

We never were able to observe robust amounts of Resolvins in all the cells types.

Even after AA supplementation, and a huge cellular increase, the extracellular vesicle cargo was poorly loaded with PGs. After AA or EPA addition, the extracellular vesicle contained an important load of 18-HEPE and the amount of LXB4 was maintained at the normal level (around 0.1 ng/mg cell proteins). Extracellular vesicles -as observed under standard nutrition- are efficient hydroxylated-lipid (HDoHE and HEPE) transporter, as the HEPE content in extracellular vesicles is higher compared to the one of the parent cells.

Figure 8

3.7. The PUFA response is dose dependent

The cells were grown in culture media supplemented with different concentrations of AA or DHA. The oxilipins profiles were measured in the extracellular vesicles fraction produced over 24h. For the DHA, the profiles are constant in composition, but differed in abundancy. At 1 μ M, the PUFAs demonstrated no activity and increase linearly between 10 and 50 μ M. The situation differs for AA, as the prostaglandins are efficiently produced at 1 μ M. In contrast, the HETE evolve in the same manner than the ones produced after DHA supplementation. The lipid mediators observed at 1 μ g of PUFAs were similar in composition and concentration to the ones observed without PUFA supplementation.

Table 2

4. DISCUSSION

Omega-3 fatty acids are associated to the prevention of cardiovascular risks. Our knowledge of pathways and cells targeted by these molecules are limited. In this study, we provide an assessment of cellular and vesicular contents of oxilipins in basal conditions and after n-3 PUFA supplementation. We observed that each cells models analyzed exhibited efficient transport of lipid mediators in their extracellular vesicles that may constitute the principal vehicles for inflammatory signalization mediated by oxylipin. BM-MSCs and cardiomyoblasts, two cell types associated with beneficial effect

during myocardial injuries, showed in standard conditions, similar vesicular oxilipin profiles despite different cellular contents. Human and rat BM-MSCs had abundant cellular level of the prostaglandin PGE₂, PGF_{2a} and PGD₂ derived from the arachidonic cascade. However, neither thromboxane nor lipoxins appear in quantity at beside prostaglandins while TXB is expressed in these cells. Prostaglandins exert various effects on cardiovascular system through the activation of specific G protein coupled receptors. The PGE₂-EP₃ interaction triggers acute inflammation (vascular permeability, oedema, leukocyte infiltration) [44, 45], whereas the PGE₂-EP₄ mediated cardioprotection during myocardial ischemia. Moreover, although all 3 prostaglandins were found in rat mesenchymal cells, their extracellular vesicles presented only PGE₂ indicating a selective secretion. Similarly, the extracellular vesicles produced by different cell-types share important amounts of LXB₄ (as compare to the cells). Unfortunately, only few is published concerning the biological effect of LXB₄. H9C2 and MSCs contained also large amount of 9- and 13- HODE, these oxygenated polyunsaturated fatty acids are metabolite of linoleic acid formed by enzymatic or free radical-mediated oxygenation and mediate acute nociception and/or inflammatory hyperalgesia[46]. It is noteworthy that 18-HEPE faintly observable in extracellular vesicles was tremendously increased after EPA and DHA supplementation. 18-HEPE has been shown to inhibit macrophage-mediated pro-inflammatory activation of cardiac fibroblasts and prevent cardiac pathology [47].

The incubation of MSCs with arachidonic acid enhanced the cell production in prostaglandins but no minor thromboxanes or leukotrienes amounts could be observed. Surprisingly, although these compounds were over-produced by the cell and efficiently transported by the extracellular vesicles, the eicosanoid profile was not deeply modified by AA supplementation. Indeed, PGD₂ and PGE₂ were increased by 1.5 fold and 3.2 fold respectively when arachidonic acid was introduced at 50 μ M as compared to 1 μ M suggesting a saturation in transport and/or packaging processes.

In MSCs incubated with media enriched in EPA or DHA, the profile of the cells and the extracellular vesicles are in good accordance. Interestingly, resolvins could not be detected in significant amounts in cells, but few were found in extracellular vesicles following DHA addition. It is also note full that the EPA cascade mainly ended with the 18-HEPE and the DHA cascade with 17- or 14-HDoHE. This point out, that the 5-LOX (encoded by alox5) is not active and was confirmed by absence of alox 5 expression measured RT-PCR in both H9C2 and BM-MSCs. The resolvins obtained with high concentration of DHA may result from residual non enzymatic oxygenation. In the cardiovascular system, leukocytes represent the main source of 5-LOX.

The low abundance of all lipid mediators in the ultra-centrifugated culture media demonstrated their selective enrichment in extracellular vesicles. Indeed, the oxilipins characterized in the extracellular vesicle-fraction are two magnitude more concentrated than the ones analysed in the medium. Moreover, it cannot be excluded that even in the medium, the few amounts of mediators reflect

residual unprecipitated or collapsed extracellular vesicles. This points out that the lipid mediators are specifically included in the extracellular nanovesicles, making extracellular vesicles specific shuttles of cell communications.

Excepted from the very recent publication [33], no publication had reported on the composition of free fatty acids transported by extracellular vesicles (EV). Actually, several works have focused on one or the other of the mediators and in particular on prostaglandins (and prostacyclins) [48] or leukotriene [49]. Only, rare publications consider a large panel of these compounds whom balance is at the origin of the activity.

Hoplainen et al. determined that after supplementation of hMSCs with polyunsaturated fatty acids these were incorporated into the main membrane phospholipid classes with different dynamics, with phosphatidylcholine serving as the first acceptor. They also demonstrated that the PUFA modifications were transferred into hMSC-EVs, which are known to mediate hMSC immunomodulation [eg. 50]. These membrane-incorporated PUFAs increased the production of downstream prostaglandin E₂ and Resolvins (E2 and D6). The authors demonstrated that the production of LMs was enhanced by a highly pro-inflammatory stimulus, which an increase number of mediators, especially prostaglandins, while other stimulatory conditions had less a pronounced impact. In this work, we analyzed and compared the whole profile of lipid mediators after PUFA supplementation and compared the cells with their derived extracellular vesicles. Our results are globally in accordance with Hoplainen et al., but revealed that EPA and DHA are even more efficient to increase the production of lipid mediators as huge amounts of the intermediates 18-HEPE and 14- or 17-HDoHE are obtained. We demonstrated that the limited impact of these two PUFA on the resolving profiles was mostly linked to the low expression of the 5-LOX. We also demonstrated that the PUFA modifications were transferred into the EVs, but we demonstrated that this occurs in a specific manner, as the vesicular lipid mediator profile differed from the cell ones. To our knowledge, it is the first report on lipoxins transport by extracellular vesicles. Moreover, we could measure that even in this class of compounds, a selection occurs as more LXB4 is found in the extracellular vesicles compared to the cells.

Finally, the focus of the literature is almost on BM-MSC, but we demonstrated here that the inflammatory signalization is also handled by other stromal cells. Therefore, it appears as very interesting to analyze the cooperative effect of the different members of the pathological micro-environment to increase our understanding on the benefic effect of the cell therapies.

5. CONCLUSION:

In this study, we analysed the lipid profile of Rat BM-MSC and cardiomyoblasts (H9c2) secretome. We demonstrated that these cells used as models for cell-therapy, have a specific lipid secretome included

in their extracellular vesicles. At basal conditions, the EVs of both cell types were enriched in LXB4 and PGE2. After incubation of cells with the ω -3 and-6 PUFAs, EPA, DHA and AA respectively, the PUFAs derived metabolites were transferred into the EVs, in a specific manner, as the nanovesicles lipid mediator profile differed from the cell ones. Put together, these results indicate that extracellular vesicles are vehicles for lipid mediators and may open novel therapeutic opportunity to improve the benefit of cell therapy on post-infract.

ACKNOWLEDGMENTS:

We thank the lipidomic platform of the Metatoul network for the mass spectrometric analyses of lipid mediators, especially P. LeFaouder and J. Bertran-Michel.

FUNDINGS:

The INSERM, CNRS and University of Toulouse 3 financial supports allowed this work to be performed

AUTHOR CONTRIBUTIONS:

Pizzinat N.: collection of data, analysis of data, writing of manuscript and work conception

Ong-Meang V.: collection of data

Bourgailh-Tortosa F.: collection of data

Blanzat M.: collection of data, analysis of data, writing of manuscript

Perquis L.: collection of data

Cussac D.: writing of manuscript

Parini A.: work conception

Poinsot V.: work conception, collection of data, analysis of data and writing of manuscript

REFERENCES:

- [1] F. van den Akker, J.C. Deddens, P.A. Doevendans, J.P. Sluijter, Cardiac stem cell therapy to modulate inflammation upon myocardial infarction, *Biochimica et Biophysica Acta*, 1830 (2013) 2449-2458.
- [2] D. D'Amario, C. Fiorini, PM. Campbell, P. Goichberg, F.Sanada, H. Zheng, T. Hosoda, M. Rota, JM. Connell, RP. Gallegos, FG. Welt, MM. Givertz, RN. Mitchell, A. Leri, J. Kajstura, MA. Pfeffer, P. Anversa, Functionally competent cardiac stem cells can be isolated from endomyocardial biopsies of patients with advanced cardiomyopathies, *Circ. Res.* 108 (2011) 857-61
- [3] H. Martini, JS. Iacovoni, D. Maggiorani, M. Dutaur, DJ. Marsal, J. Roncalli, R. Itier, C. Dambrin, N. Pizzinat, J. Mialet-Perez, D. Cussac, A. Parini, L. Lefevre, V. Douin-Echinard, Aging induces cardiac mesenchymal stromal cell senescence and promotes endothelial cell fate of the CD90 + subset, *Aging Cell.* 18(2019), e13015.

- [4] G. van Niel, G. d'Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, *Nat. Rev. Mol. Cell Biol.* 19 (2018) 213–228
- [5] L. Blanc, M. Vidal, New insights into the function of RabGTPases in the context of vesicular secretion, *Small GTPases* 9 (2018) 95–106.
- [6] X. Yu, R Prekeris, GW Gould, Role of endosomal RabGTPases in cytokinesis, *Eur. J. Cell Biol.*, 86(2017), 25-35.
- [7] A. Waldenström, G Ronquist, Role of Extracellular vesicles in Myocardial Remodeling, *Circ Res.* 114 (2014) 315-324
- [8] J. Burrello, J. Burrello, S. Monticone, C. Gai, Y. Gomez, S. Kholia and G. Camussi. Stem cell-derived extracellular vesicles and immune modulation. *Front. Cell. Dev. Biol.* 4 (2016) 83.
- [9] M. Romano, Lipoxin and aspirin-triggered lipoxins, *Scient. World J.* 10 (2010) 1048-64.
- [10] A. Rinaldo-Matthis, JZ. Haeggström, Structures and mechanisms of enzymes in the leukotriene cascade, *Biochimie.* 92 (2010) 676-681
- [11] C. Lehmann, J. Homann, AK. Ball, R. Blöcher, TK. Kleinschmidt, D. Basavarajappa, C. Angioni, N. Ferreirós, AK. Häfner, O. Rådmark, E. Proschak, JZ. Haeggström, G. Geisslinger, MJ. Parnham, D. Steinhilber, AS. Kahnt, Lipoxin and resolvin biosynthesis is dependent on 5-lipoxygenase activating protein, *FASEB J.* 29 (2015) 5029-43.
- [12] R. Rajakariar, MM. Yaqoob, DW. Gilroy, COX-2 in inflammation and resolution, *Mol. Interv.* 6 (2006) 199-207.
- [13] CN. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, *Nat. Immunol.* 6 (2005) 1191-7
- [14] S. Uderhardt, G. Krönke, 12/15-Lipoxygenase during the regulation of inflammation, immunity, and self-tolerance, *J. Mol. Med.* 90 (2012) 1247–1256.
- [15] CN. Serhan, Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not?, *Am J. Pathol.* 177 (2010) 1576-91.
- [16] ME. Ertunc, GS. Hotamisligil, Lipid signaling and lipotoxicity in metaflammation: indications for metabolic disease pathogenesis and treatment, *J. Lipid Res.*, 57 (2016) 2099-2114.
- [17] J. Dalli, M. Zhu, NA. Vlasenko, B. Deng, JZ. Haeggström, NA. Petasis, CN. Serhan, The novel 13S,14S-epoxy-maresin is converted by human macrophages to maresin 1(MaR1), inhibits leukotriene A4 hydrolase (LTA4H), and shifts macrophage phenotype, *FASEB J.*, 27 (2013) 2573-83.
- [18] SK. Biswas, A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm, *Nat. Immunol.* 11 (2010) 889-96.
- [19] A. Keating, Mesenchymal stromal cells: new directions, *Cell Stem Cell.* 10 (2012) 709-716, Erratum in: *Cell Stem Cell.* 11 (2012) 136.
- [20] K. Le Blanc, D. Mougiakakos, Multipotent mesenchymal stromal cells and the innate immune system, *Nat. Rev. Immunol.* 12 (2012) 383.
- [21] DJ. Prockop, Concise review: two negative feedback loops place mesenchymal stem/stromal cells at the centre of early regulators of inflammation, *Stem Cell.* 31 (2013) 2042.

- [22] Y. Deng, Z. Yang, T. Terry, S. Pan, DG. Woodside, J. Wang, K. Ruan, JT. Willerson, RA. Dixon, Q. Liu, Prostacyclin-producing human mesenchymal cells target H19 lncRNA to augment endogenous progenitor function in hindlimb ischaemia, *Nat. Commun.*, 7 (2016) 11276.
- [23] N. Muraoka, K. Nara, F. Tamura, H. Kojima, H. Yamakawa, T. Sadahiro, K. Miyamoto, M. Isomi, S. Haginiwa, H. Tani, S. Kurotsu, R. Osakabe, S. Torii, S. Shimizu, H. Okano, Y. Sugimoto, K. Fukuda, M. Ieda, Role of cyclooxygenase-2-mediated prostaglandin E2-prostaglandin E receptor 4 signaling in cardiac Reprogramming, *Nature Commun.* 10 (2019) 674. doi.org/10.1038/s41467-019-08626-y
- [24] G. Ozen, I. Gomez, A Daci, C. Deschildre, L. Boubaya, O. Teskin, BS. Uydeş-Doğan, PJ. Jakobsson, D. Longrois, G. Topal, X. Norel, Inhibition of microsomal PGE synthase-1 reduces human vascular tone by increasing PGI₂: a safer alternative to COX-2 inhibition, *Br. J. Pharmacol.* 174 (2017) 4087-4098. doi: 10.1111/bph.13939.
- [25] R. Bosviel, L. Joumard-Cubizolles, G. Chinetti-Gbaguidi, D. Bayle, C. Copin, N. Hennuyer, I. Duplan, B. Staels, G. Zanoni, A. Porta, L. Balas, JM. Galano, C. Oger, A. Mazur, T. Durand, C. Gladine, DHA-derived oxilipins, neuroprostanes and protectins, differentially and dose-dependently modulate the inflammatory response in human macrophages: Putative mechanisms through PPAR activation, *Free Radic. Biol. Med.* 103 (2017) 146-154.
- [26] J. Roy, C. Oger, J. Thireau, J. Roussel, O. Mercier-Touzet, D. Faure, E. Pinot, C. Farah, DF Taber, JP. Cristol, JC. Lee, A. Lacampagne, JM. Galano T. Durand, JY. Le Guennec, Nonenzymatic lipid mediators, neuroprostanes, exert the antiarrhythmic properties of docosahexaenoic acid, *Free Radic. Biol. Med.*, 86 (2015) 269-78.
- [27] E. Mourmoura, V. Chate, K. Couturier, B. Laillet, G. Vial, JP. Rigaudiere, B. Morio, C. Malpuech-Brugere, K. Azarnoush, L. Demaison, Body adiposity dictates different mechanisms of increased coronary reactivity related to improve in vivo cardiac function, *Cardiovasc. Diabetol.* 13 (2014) 54.
- [28] A. Habbout, N. Li, L. Rochette, C. Vergely, Postnatal overfeeding in rodents by litter size reduction induces major short- and long-term pathophysiological consequences, *J. Nutr.* 143 (2013) 553–62.
- [29] J -P Sergiel, L Martine, D Raederstorff, A Grynberg, and L Demaison, Individual effects of dietary EPA and DHA on the functioning of the isolated working rat heart, *Rev. Can. Physiol. Pharmacol.* 76 (1998) 728-736
- [30] KR. Zehr, MK. Walker, Omega-3 polyunsaturated fatty acids improve endothelial function in humans at risk for atherosclerosis: a review, *Prostaglandins & Other Lipid Mediators.* 134 (2018) 131–40.
- [31] L. Demaison, JP. Sergiel, D. Moreau, A. Grynberg, Influence of the phospholipid n-6/n-3 polyunsaturated fatty acid ratio on the mitochondrial oxidative metabolism before and after myocardial ischemia, *Biochim BiophysActa.* 1227 (1994) 53–9.
- [32] PL. McLennan, MY. Abeywardena, JA. Dallimore, D. Raederstorff, Dietary fish oil preserves cardiac function in the hypertrophied rat heart, *Br. J. Nutr.* 108 (2012) 645–54.
- [33] M. Holopainen, AR. Colas, S. Valkonen, F. Tigestu-Sahle, K. Hyvarinen, F. Mazzacuva, P. Lehenkari, R. Kakela, J. Dalli, E. Kerkela, S. Laitinen, Polyunsaturated fatty acids modify the extracellular vesicle membranes and increase the production of proresolving lipid mediators of human mesenchymal stromal cells., *BBA – Mol. Cell Biol. Lipids*, 1864 (2019) 1350–1362.

- [34] SJ. Watkins, GM. Borthwick, HM. Arthur, The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro, *In vitro Cell. Dev. Biol.* 47 (2011) 125-131.
- [35] W. Liang, M. Chen, D. Zheng, J. He, M. Song, L. Mo, J. Feng, J. Lan, A novel damage mechanism: Contribution of the interaction between necroptosis and ROS to high glucose-induced injury and inflammation in H9c2 cardiac cells, *Int. J. Mol. Med.* 40 (2017) 201-208.
- [36] F. Beg, R. Wang, Z. Saeed, S. Devaraj, K. Masoor, H. Nakshatri, Inflammation-associated microRNA changes in circulating extracellular vesicles of heart failure patients, *BMC Res. Notes.* 10 (2017) 751.
- [37] C Mias, O Lairez, E Trouche, J Roncalli, D Calise, MH Seguelas, C Ordener, MD Piercecchi-Marti, N Auge, AN Salvayre, P Bourin, A Parini, D Cussac. Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction, *Stem Cells.* 27 (2009) 2734-2743
- [38] Le Faouder, V. Baillif, I. Spreadbury, JP. Motta, P. Rousset, G. Chêne, C. Guigné, F. Tercé, S. Vanner, N. Vergnolle, J. Bertrand-Michel, M. Dubourdeau, N. Cenac, LC-MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 932 (2013) 123-33.
- [39] M Mathieu, L Martin-Jaular, G Lavieu, C Théry, Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication, *Nat. Cell Biol.* 21 (2019) 9-17.
- [40] DK Jeppesen, AM Fenix, JL Franklin, et al., Reassessment of Exosome Composition. *Cell.* 177 (2019) 428-445.
- [41] B. Burla, M. Arita, AK. Bendt, A. Cazenave-Gassiot, EA. Dennis, K. Ekroos, X. Han, K. Ikeda, G. Liebisch, MK. Lin, TP. Loh, PJ. Meikle, M. Oresic, O. Quehenberger, A. Shevchenko, F. Torta, MJO. Wakelam, CE. Wheelock, MR. Wenk, MS-based lipidomics of human blood plasma: a community-initiated position paper to develop accepted guidelines, *J. Lipid Res.* 59 (2018) 2001-2017.
- [42] M. Awada, A. Meynier, CO. Soulage, L. Hadji, A. Géoën, M. Viau, L. Ribourg, B. Benoit, C. Debard, M. Guichardant, M. Lagarde, G. Genot, MC. Michalski, n-3 PUFA added to high-fat diets affect differently adiposity and inflammation when carried by phospholipids or triacylglycerols, *In mice Nutrition & Metabolism*, 2013, 10-23
- [43] JK. Virtanen, JHY. Wu, S. Voutilainen, J. Mursu, TP. Tuomainen, Serum n-6 polyunsaturated fatty acids and risk of death: the Kuopio Ischaemic Heart Disease Risk Factor Study, *Am. J. Clin. Nutr.* 107 (2018) 427-435.
- [44] S. Donnini, L. Bazzani, M. Ziche, E. Terzuoli, Nitric Oxide and PGE-2 Cross-Talk in EGFR-Driven Epithelial Tumor Cells, *Crit. Rev. Oncog.* 21 (2016) 325-331.
- [45] K. Morimoto, N. Shirata, Y. Taketomi, S. Tsuchiya, E. Segi-Nishida, T. Inazumi, K. Kabashima, S. Tanaka, M. Murakami, S. Narumiya, Y. Sugimoto, Prostaglandin E2-EP3 signaling induces inflammatory swelling by mast cell activation, *J. Immunol.* 192 (2014) 1130-7.
- [46] H. Shapiro, P. Singer, A. Ariel, Beyond the classic eicosanoids: Peripherally-acting oxygenated metabolites of polyunsaturated fatty acids mediate pain associated with tissue injury and inflammation, *Prosta. Leukot Essent Fatty Acids.* 111 (2016) 45-61.
- [47] J. Endo, M. Sano, Y. Isobe, K. Fukuda, JX. Kang, H. Arai, M. Arita, 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload-induced maladaptive cardiac remodeling, *J. Exp. Med.* 211 (2014) 1673-87.

- [48] C. Subra , D. Grand, K. Laulagnier ,A. Stella, G. Lambeau, M. Paillasse, P. De Medina, B. Monsarrat, B. Perret, S. Silvente-Poirot, M. Poirot and M. Record, Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins, *J. Lipid Res.* 51 (2010) 2105-2120.
- [49] J Esser, U Gehrman, FL D'Alexendri, AM Hidalgo-Estévez, CE Wheelock, A. Scheynius, S. Gabrielsson, O. Radmark. Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration, *J. Allergy Clin. Immunol.* 126 (2010) 1032-1040.
- [50] D Ti, H Hao, C Tong, et al. LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b, *J. Transl. Med.* 13 (2015) 308-22.

FIGURE AND TABLE CAPTIONS

Figure 1: Top: Size distribution obtained from the intensity value with DLS analyzes of the extracellular vesicle fraction of H9c2 cells. Bottom: Approximated Size distribution in numbers, integrating that small particles are less capable to scatter the light.

Figure 2: Top: SEM images obtained after freeze fracture of the sample of extracellular vesicle fraction of H9c2 after 1:30h UC at 100 000g. Bottom: Immunodetection of Alix in purified extracellular vesicles fraction of human and rat BM-MSCs and H9c2 (left panel). Alix, one of the exosome specific marker, is present in important amounts in the exosomes. The right panel indicate the presence of a large protein distribution in the EV fraction.

Figure 3: Fatty acid profile measured in BM-BMSCs and H9c2 cells corresponding to rat BM-MSCs (dark) and to H9c2 (light grey). The results are expressed as ng/mg cell protein, n =7 and 12 experimental replicates per group respectively.

Figure 4: Comparison of the lipid mediator profiles obtained upon two different cultures of H9c2 cells After extraction of the isolated vesicles (pellets) and the 100 times freeze-dried concentrated medium (supernatant) obtained after ultracentrifugation.

Figure 5: Oxilipins profile obtained in pg/mg cellular proteins for rat BM-MSC (dark) and cardiomyoblaste (H9c2) (grey) extracellular vesicles. The extracellular vesicles collected after the two step-centrifugation were directly extracted by chloroform and analyzed using SRM LC-MS/MS. The relative quantification occurs through comparison with internal perdeuterated standards.

Figure 6: RT-PCR quantification of enzymes involved in lipoxin biosynthesis pathways without cell activation. mRNA were normalized to the housekeeping Rplp0 expression for each group. Data are presented in mean \pm SEM. ND: not detected.

Figure 7: Comparison of oxilipins profiles (in pg/mg cellular proteins) from a) rat BM-MSC cells (dark) and human BM-MSC cells (grey) b) in the EV fraction for each cell-type.

Figure 8a: Oxilipins profile of PUFA-treated hBM-MSCs. Cellular content after a 24h treatment with 10µM AA or 10µM EPA or 10µM DHA on prostanoids (A), resolvins/ lipoxins (B) and derived oxilipins. ND: not detected in all conditions.

Figure 8b: Oxilipins profile of PUFA-treated hBM-MSCs. Extracellular vesicles content after a 24h treatment with 10µM AA or 10µM EPA or 10µM DHA on prostanoids (A), resolvins/ lipoxins (B) and derived oxilipins. ND: not detected in all conditions

Table1: Primer sequences used for PCR analysis.

Table 2: dose effect in the µM range of DHA and EPA over 24h on sub-confluent rat MSC cultures, on their metabolites. Values are given in pg/mg cell proteins. Values are the mean of two experience repeats producing similar results. In bold the specific metabolite for each polyunsaturated precursor.

Supplementary figure 1: Representative cytometric panels obtained with PI/annexin V staining of rat BM-MSCs grown in 10% SVF (left) and after 24h serum deprivation (right). Graph shows the results obtained from three different experiments.

Supplementary figure 2: Solvent testing for EV lipids extraction. Depending on the mediator families considered, the relative amounts may change (eg. hexane is most effective for extracting oxylipins but is effective for prostaglandins). However, the global profile remains similar.

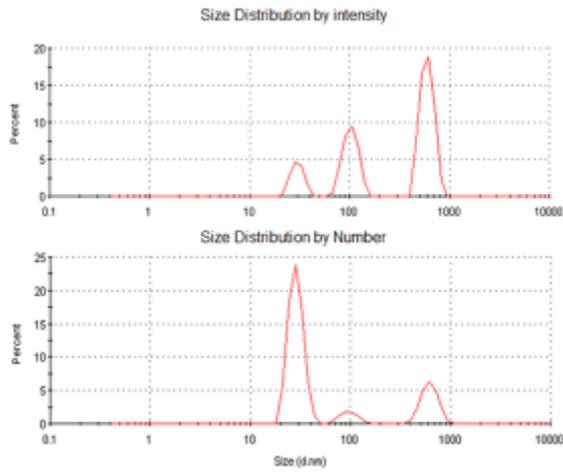


Figure 1

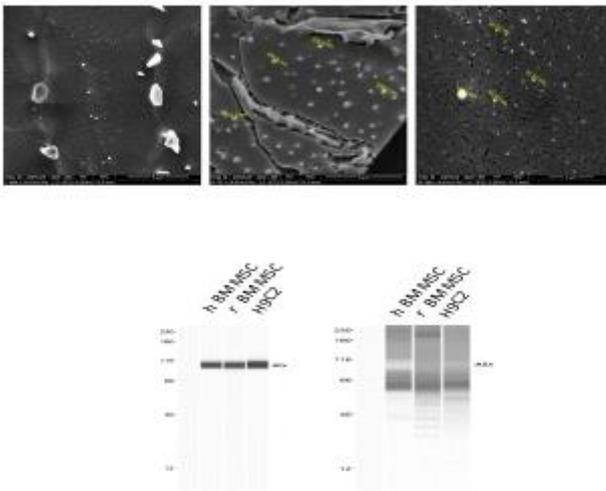


Figure 2:

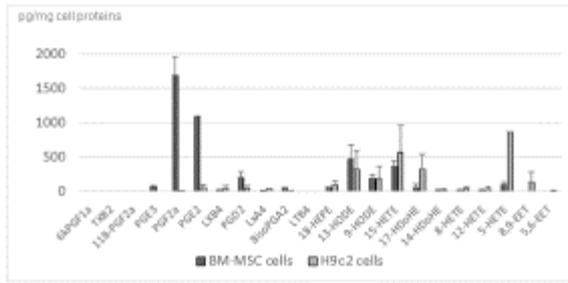


Figure 3

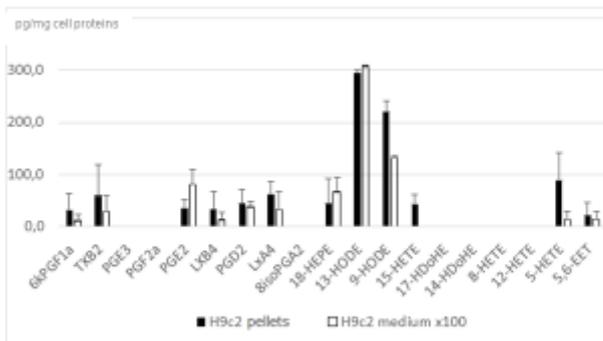


Figure 4

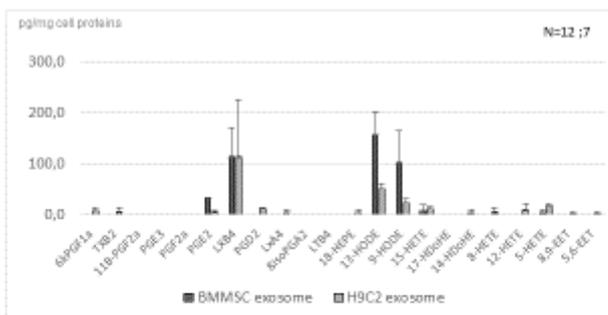


Figure 5

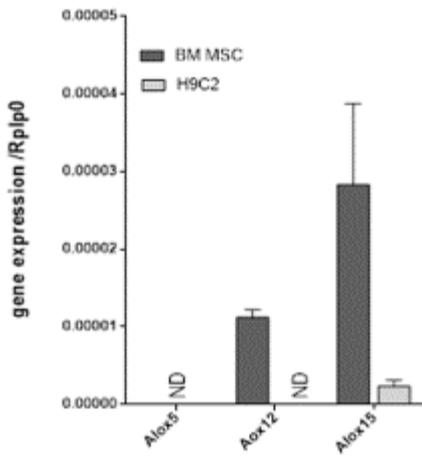


Figure 6

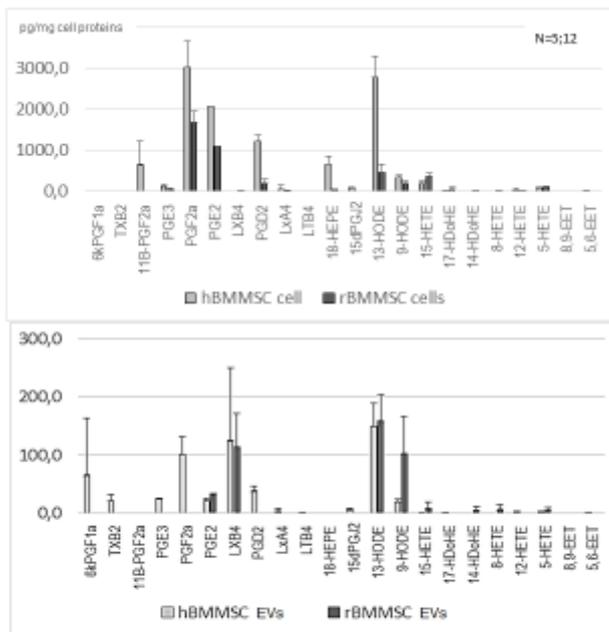


Figure 7: Comparison of oxilipins profiles (in pg/mg cellular proteins) from a) rat BM-MSc cells (dark) and human BM-MSc cells (grey) b) in the EV fraction for each cell-type.

gene	sequences	reference
Alox05	AGAGCGGCAGCTCAGTTTAG	NM_031010.2
	GGAACGGGTGTACAGGGG	
Alox12	AGA-GCG-GCA-GCT-CAG-TTT-AG	NM_012822.1
	GGA-ACT-GGT-GTG-TAC-AGG-GG	
Alox15	CTG-CAG-GAG-CTC-CAA-TTC-CA	NM_001105798.1
	AAC-GGA-TGT-GCG-GAA-CTA-GG	
Rplp0	GGAACGTGGGCTTTGTGTTC	NM_022402.2
	GTA CTGTGACCTCACACGGG	

Table1

	PGE2	PGD2	RvD3	RvD2	18- HEPE	17- HDoHE	14- HdoHE	15- HETE	12- HETE	9- HETE
AA										
1μM	7921	41923	0	0	1766	0	0	262	4835	143
AA										
10μM	26253	59892	0	0	1527	0	0	6436	55568	13371
AA										
50μM	25252	65141	0	0	1087	400	312	67040	80018	69773
DHA										
1μM	9818	25585	587	1324	2980	837	637	204	0	0
DHA										
10μM	344	424	1221	7380	41719	77494	71317	689	1905	326
DHA										
50μM	203	78	871	6563	24632	206757	184269	730	1503	319

Table 2