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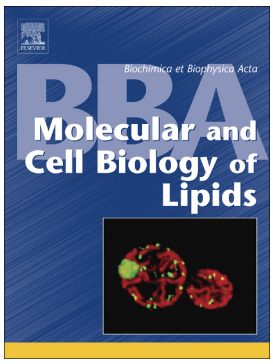

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PII: S1388-1981(19)30113-1
DOI: https://doi.org/10.1016/j.bbalip.2019.06.010
Reference: BBAMCB 58484

To appear in: *BBA - Molecular and Cell Biology of Lipids*

Received date: 6 February 2019
Revised date: 23 May 2019
Accepted date: 13 June 2019

Please cite this article as: M. Holopainen, R.A. Colas, S. Valkonen, et al., Polyunsaturated fatty acids modify the extracellular vesicle membranes and increase the production of proresolving lipid mediators of human mesenchymal stromal cells, BBA - Molecular and Cell Biology of Lipids, https://doi.org/10.1016/j.bbalip.2019.06.010

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Polyunsaturated fatty acids modify the extracellular vesicle membranes and increase the production of proresolving lipid mediators of human mesenchymal stromal cells

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Running Title: PUFA supplements affect MSC lipid signaling

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ABSTRACT

Human mesenchymal stromal/stem cells (hMSCs) are used in experimental cell therapy to treat various immunological disorders, and the extracellular vesicles (hMSC-EVs) they produce have emerged as an option for cell-free therapeutics. The immunomodulatory function of hMSCs resembles the resolution of inflammation, in which proresolving lipid mediators (LMs) play key roles. Multiple mechanisms underlying the hMSC immunosuppressive effect has been elucidated; however, the impact of LMs and EVs in the resolution is poorly understood. In this study, we supplemented hMSCs with polyunsaturated fatty acids (PUFAs); arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, which serve as precursors for multiple LMs. We then determined the consequent compositional modifications in the fatty acid, phospholipid, and LM profiles. Mass spectrometric analyses revealed that the supplemented PUFAs were incorporated into the main membrane phospholipid classes with different dynamics, with phosphatidylcholine serving as the first acceptor. Most importantly, the PUFA modifications were transferred into hMSC-EVs, which are known to mediate hMSC immunomodulation. Furthermore, the membrane-incorporated PUFAs influenced the LM profile by increasing the production of downstream prostaglandin E_2 and proresolving LMs, including Resolvin E2 and Resolvin D6. The production of LMs was further enhanced by a highly proinflammatory stimulus, which resulted in an increase in a number of mediators, most notably prostaglandins, while other stimulatory conditions had less a pronounced impact after a 48-hour incubation. The current findings suggest that PUFA manipulations of hMSCs exert significant immunomodulatory effects via EVs and proresolving LMs, the composition of which can be modified to potentiate the therapeutic impact of hMSCs.

Highlights

- Cell membrane phospholipids of hMSCs accept supplemented PUFAs with different dynamics
- Extracellular vesicle membranes of hMSCs can be modified with PUFA supplementation
- hBMSCs produce proresolving lipid mediators
- PUFA supplementation and inflammatory stimuli impact the lipid mediator profile

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Keywords

Specialized proresolving mediator, prostaglandin E_2, phospholipid, cell therapy

Nonstandard Abbreviations

COX, cyclooxygenase; EV, Extracellular vesicle; hBMSC, human bone marrow-derived mesenchymal stromal/stem cell; LM, lipid mediator; LOX, lipoxygenase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PLA, phospholipase; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SM, sphingomyelin; SPM, specialized proresolving mediator.

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1. INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are used for the experimental treatment of immunological disorders, such as graft-versus-host disease and Crohn's disease, with promising results [1–3]. However, the clinical use of these cells is hampered by an insufficient understanding of their mechanisms of function. The MSC mode of action resembles the resolution of inflammation, i.e., the active dampening of inflammation [4], and they modulate immune cells by expressing and secreting various factors, such as the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase [5], adenosine-producing CD73 [6–8], prostaglandin (PG)E_2 [9], and extracellular vesicles (EVs) [10,11]. According to a new intriguing mechanism MSCs are required to undergo apoptosis in the patient to exert their therapeutic response [12]. Apoptosis is essential for efferocytosis, the clearance of dead and dying cells, which is carried out by macrophages during the resolution of inflammation [13].

A failure in the resolution of inflammation has been associated with the pathogenesis of inflammatory disorders, such as inflammatory bowel disease [14] and asthma [15,16]. Different lipid mediators (LMs) play key roles in different phases of inflammation. PGs are traditionally considered to be proinflammatory LMs, but they also initiate LM class switching, which results in a decrease in 5-lipoxygenase (LOX)-derived proinflammatory LMs and an increase in 15-LOX-derived proresolving LMs [17]. The specialized proresolving mediators (SPMs), which include the resolvins, protectins, maresins, and lipoxins, regulate inflammation at pico- to nanomolar concentrations by counter-regulating the production of proinflammatory mediators, inducing efferocytosis, and polarizing macrophages towards a more anti-inflammatory phenotype [18,19]. A myriad of enzymes, including phospholipases (PLAs), cyclooxygenases (COXs), LOXs, and cytochrome P450s, are involved in LM biosynthesis from their precursor polyunsaturated fatty acids (PUFAs) [20]. These PUFAs, such as arachidonic acid (AA), eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) may be attached in membrane phospholipids (PLs), which can be deliberated from the membrane prior to the biosynthesis to LMs [20–25].

The plasma membrane is a dynamic interface between the cell and the environment and the site of intercellular communication. One of the represented mechanisms of cellular communication is to secrete EVs, which are surrounded by the PL bilayer and transport a variety of protein and lipid molecules, including PLA_2 enzymes, LMs and their monohydroxy pathway markers [26–31]. These bioactive components of EVs are thought to regulate immunological responses, and therefore the EVs have been regarded as an option for cell-free therapeutics [29]. MSC-derived EVs (MSC-EVs) have been shown to elicit similar immunosuppressive functions to the cells and, thus, have been suggested to mediate the therapeutic effect of MSCs [11,32,33].

Our research group has previously demonstrated that the membrane n-3/n-6 PUFA ratio correlates with the functionality of human bone marrow-derived MSCs (hBMSCs), as an increase in n-3 fatty acids was associated

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with an improved immunosuppressive capacity [34]. We have previously shown that the PL profile of hBMSCs can be modified by PUFA supplementation [35], and others have reported that proinflammatory stimuli may alter the PL profile [36]. The importance of specific PUFA manipulations on MSC functions has been highlighted by the findings of Tsoyi and colleagues, who observed that MSCs preconditioned with carbon monoxide and DHA improved the survival of mice in a sepsis model when compared to cells preconditioned with carbon monoxide and AA [37]. Moreover, mammalian MSCs have been found to produce SPMs [37–39], although the data on human MSCs is sparse, reporting only the production of lipoxin A_4 (LXA_4) [38]. The hBMSCs have a limited ability to convert C18 PUFAs to the highly unsaturated (4-6 double bonds) C20-22 fatty acids due to their inadequate desaturase activities, and therefore, the cells must acquire the C20-22 PUFA precursors required for SPM biosynthesis from the environment [35].

In this study, we investigated the changes in lipid metabolism of hBMSCs and their EVs in response to different supplemented PUFAs, which serve as precursors for multiple LMs and SPMs. In more detail, we investigated the dynamics of PUFA incorporation into membrane PLs by monitoring the total fatty acid profile and the appearance of specific polyunsaturated species into the main PL classes of the cells, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Our aim was to elucidate whether these PL membrane modifications of hBMSCs would be reflected in the compositions of the EVs they secrete and, ultimately, the impact of this phenomenon on the LM profiles. Our findings demonstrate that hBMSCs produce LMs and suggest that their SPM profiles may contribute to hBMSC protective actions.

2. MATERIALS AND METHODS

2.1 Ethics and bone marrow donors

The Ethical Committee of Northern Ostrobothnia Hospital District or the Ethical Committee of the Hospital District of Helsinki and Uusimaa approved all the patient protocols. The use of human material conformed to the principles outlined in the Declaration of Helsinki. After acquiring written consent, aspirates were collected from the iliac crest or upper femur methaphysis of adult patients. hBMSCs were isolated from the obtained bone marrow, and primary cell lines were established as previously described [40,41].

2.2 Cell culture of hBMSCs

Primary hBMSC lines established from four different donors were used, and passage four cells were thawed and plated on 10 cm or 15 cm plates (Nunclon^{TM} Delta Surface, Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1,000 cells/cm^2. The cells were cultured at 37 °C in a 5% CO_2 humidified incubator in proliferation

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medium: minimum essential α-medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 mM HEPES (all from Thermo Fisher Scientific). The medium was replaced once during cultivation. The cells were washed with 5 mL Cell Therapy Systems Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific), detached with 1.5 mL TrypLE™ Express (Thermo Fisher Scientific) when the confluence reached 80%, and passaged once for the following experiments. The cell number and viability were calculated using a NucleoCounter® NC-100™ (ChemoMetec, Lillerod, Denmark).

2.2.1 PUFA incorporation experiments

For the PUFA incorporation experiments, hBMSCs were passaged onto 10 cm plates at a density of 1,000 cells/cm^2, and the medium was replaced once during cultivation. The hBMSCs were supplemented with different PUFAs as previously described [35] when the cultures reached 80–90% confluence. In brief, the initial medium with 10% FBS was replaced with proliferation medium containing only 5% FBS to limit the fatty acid content available for the hBMSCs. After the medium change, the cells were supplemented with ethanol (purity ≥ 99.5%, Altia Industrial, Rajamäki, Finland) as a control, or with the PUFAs AA (20:4n-6), EPA (20:5n-3) or DHA (22:6n-3) (all from Cayman Chemical, Ann Arbor, MI, USA) bound to fatty acid-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at 50 µM final concentration in the cell culture medium. The PUFA stock solutions were made in ethanol. The PUFA-supplemented cell cultures were incubated at 37 °C in a 5% CO_2 humidified incubator. Following incubations of 2, 6, and 24 h, the cells were washed two times with 5 mL cold PBS (Sigma-Aldrich), harvested, snap frozen, and stored at -70 °C.

2.2.2 hBMSC-EV experiments

For the collection of EVs, hBMSCs were passaged into the two-chamber type of Corning® CellSTACK® cell culture chambers (Sigma-Aldrich) at a density of 1,000 cells/cm^2 in 250 mL proliferation medium. Half of the medium was replaced once. When they had reached 80–90% confluence, the hBMSCs were first supplemented with PUFAs for 24 h as described in section 2.2.1, and then they were washed three times with 100 mL DPBS and one time with 75 mL a-MEM. The cells were then incubated for 48 h in 200 mL serum-free α-MEM, detached with 33.7 mL TrypLE™ Express and collected as described above. The conditioned cell culture medium was centrifuged at 2,000 g for 10 min to remove cell debris. The supernatant was ultracentrifuged with an Optima™ MAX-XP Ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA) at 100,000 g for 2 h +4 °C using a MLA-50 rotor (k-factor = 92, Beckman Coulter). The pelleted EVs were suspended to PBS and combined. The samples were further ultracentrifuged at 100,000 g for 2 h +4 °C using a MLS-50 rotor (k-factor

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= 71, Beckman Coulter) and suspended in 100 µL PBS or Millipore water for mass spectrometric analysis and immunoblotting, respectively. A 10 µL aliquot was transferred to Protein LoBind tubes (Eppendorf, Hamburg, Germany) for Nanoparticle Tracking Analysis. The samples were immediately snap frozen and stored at -70 °C.

2.2.3 hBMSC incubations for lipid mediator analysis

For the LM analysis, hBMSCs were cultured as for the EV collection described above but passaged onto 10 cm plates with 10 mL proliferation medium. The cells were supplemented with the different PUFAs for 24 h, washed three times with 7 mL DPBS, and then incubated for 48 h in 9 mL of serum-free α-MEM. Cells supplemented with AA and DHA were also incubated under the 4 stimulatory conditions for 48 h in serum-free medium after PUFA supplementation. Condition 1, transforming growth factor (TGF)-β1 (Thermo Fisher Scientific) 5 ng/mL and interleukin (IL)-10 (Thermo Fisher Scientific) 10 ng/mL, an anti-inflammatory stimulus; Condition 2, interferon (IFN)-γ (Sigma-Aldrich) 25 ng/mL and lipopolysaccharide (LPS, Sigma-Aldrich) 10 ng/mL, induces regulatory macrophage polarization [42]; Condition 3, IFN-γ 10 ng/mL and tumor necrosis factor (TNF)-α (STEMCELL Technologies, Vancouver, BC, Canada) 15 ng/mL, a classical licensing stimulus, which primes MSCs to become effective immunomodulatory cells [43]; Condition 4, TNF-α 10 ng/mL, IL-1β 10 ng/mL (Sigma-Aldrich), and LPS 100 ng/mL, a very powerful inflammatory stimulus with a high amount of LPS, which has been shown to affect SPM production in a co-culture of neutrophils and choroid-retinal endothelial cells [44]. The cell incubations (including cells and conditioned media) were collected, snap frozen, and stored at -70 °C.

2.3 EV quantification and size determination

The particle concentration and size distribution of EV samples was determined using Nanoparticle Tracking Analysis. Data were recorded using camera level 14, and 3 videos of 90 seconds were recorded, manually mixing the sample with a syringe between measurements. If necessary, the samples were diluted with 0.2 µm filtrated PBS. Data analysis was performed with a threshold of 5 and gain of 10. The used LM14C model was equipped with a 70 mW violet (405 nm) laser (Malvern Instruments Ltd., Malvern, UK) and sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan), and the data were recorded and analyzed with NanoSight software version 3.0 (Malvern Instruments Ltd.).

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2.4 Immunoblotting

hBMSC-EV pellets from control treatment were prepared for Western blot analysis by drying the EV suspensions with Savant™ SPD111V SpeedVac™ Concentrator (Thermo Fischer Scientific) and suspending the pellets in 15 µL DPBS containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland), prepared by dissolving 1 tablet of the inhibitor in 10 mL of DPBS. Due to a small amount of sample material, EV samples were loaded with an equal volume. Platelet-derived EVs were used as controls, and 30 µg of the controls were loaded onto the gels. The samples were prepared with 4x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 10% 2-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. Then, the samples were loaded onto Mini-PROTEAN TGX Stain-Free protein gels with a 4-20% gradient (Bio-Rad) together with Precision Protein Plus WesternC Blotting Standard (Bio-Rad). The gels were run for 50 min at 170 V in 1× Tris/Glycine/SDS Buffer (Bio-Rad), and the proteins were blotted for 20 min with 1.3 A up to 25 V using a semi-dry blotting machine Trans-Blot Turbo (Bio-Rad), 1× Transfer Buffer (Bio-Rad) including 20% methanol (Merck, Darmstadt, Germany), and Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-Rad), where the original 0.2 µm nitrocellulose membrane was replaced with a 0.45 µm nitrocellulose membrane (Bio-Rad). Transfer of the proteins was confirmed by imaging the gels and membranes using the ChemiDoc Touch Imaging System (Bio-Rad), followed by 1 h of membrane blocking at room temperature with 6% milk solution (Valio, Helsinki, Finland) prepared in 1 × Tris-buffered saline (Sigma-Aldrich) containing 0.05% Tween20 (Sigma-Aldrich).

Antibodies against CD9 (Becton Dickinson, Franklin Lakes, NJ, USA, clone M-L13), CD41 (Beckman Coulter, clone sz22), CD63 (Becton Dickinson, clone H5C6), CD73 (Abcam, Cambridge, UK, ab124725), cytosolic PLA_2 ($cPLA_2$, Abcam, ab58375), and secretory PLA_2 ($sPLA_2$, Abcam, ab23705) were diluted 1:250 (CD9 and CD63), 1: 1:500 ($cPLA_2$), 1:1000 (CD73, $sPLA_2$), or 1:10000 (CD41) in 1 × Tris-buffered saline containing 2% milk and 0.05% Tween20 and incubated overnight. The membranes were first rinsed and then washed with Tris-buffered saline containing 0.05% Tween20 3 × 10 min followed by incubation with goat anti-mouse or anti-rabbit IgG (H + L)-HRP conjugated secondary antibodies (Bio-Rad) containing Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) diluted 1:3000 and 1:10000, respectively, to Tris-buffered saline containing 2% milk and 0.05% Tween20. After incubation, the membranes were first rinsed and then washed 2 × 10 min in Tris-buffered saline containing 0.05% Tween20 and 10 min in Tris-buffered saline, followed by the addition of 1 mL of Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA) per membrane, mixed 1:1 as instructed. After a 1-min incubation at room temperature, the chemiluminescence of the membranes was captured using the ChemiDoc Touch Imaging System.

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2.5 Fatty acid analysis

Fatty acids of hBMSCs were identified and quantified from transmethylated lipid extracts as described previously [35]. Cell samples supplemented with PUFAs for 2, 6, and 24 h were extracted according to Folch et al. [45], evaporated into dryness under a nitrogen gas stream, and transmethylated as recommended by Christie[46]. In brief, the samples were heated in 1% H_2SO_4 (Sigma-Aldrich) in methanol (LiChrosolv®, Merck) at a temperature of 96 °C under a nitrogen atmosphere for 120 min. The fatty acid methyl esters (FAMEs) formed were recovered with hexane (LiChrosolv®, Merck) in two steps, dried overnight in anhydrous Na_2SO_4 (EMSURE®, Merck**)**, and analyzed using a gas chromatograph (Shimadzu GC-2010 Plus, Kyoto, Japan) equipped with an auto injector (AOC-20i), flame ionization detector (FID), and ZB-wax capillary column (30 m, 0.25 mm ID, 0.25 μm film, Phenomenex, Torrance, CA, USA). The FAME identification was based on the retention time, use of authentic standard mixtures of known composition and confirmatory recordings of mass spectra (GC-2010 Plus with GCMS-QP2010 Ultra, Shimadzu, equipped with a similar column as in the GC-FID system). Quantifications were based on FID responses, which were corrected according to the theoretical response factors [47] and calibrations with quantitative FAME standards (Supelco, Bellefonte, PA, USA). The fatty acid proportions were calculated as the mol%, and the fatty acids were marked using the following abbreviations: [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g., 22:6n-3 for DHA).

2.6 Phospholipid profiling

Total lipids of hBMSCs and hBMSC-EVs were extracted using the Folch method [45]. The lipid extracts were studied by direct infusion electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as previously described [35] using Agilent 6490 Triple Quad LC/MS with iFunnel technology (Agilent Technologies Inc., Santa Clara, CA, USA). In brief, the final lipid extracts in chloroform:methanol (1:2, v:v) (both LiChrosolv®, Merck) spiked with 7 internal standards [PC 14:1/14:1, PC 20:1/20:1, PC 22:1/22:1; PE 14:0/14:0 and 16:1/16:1, PS 14:0/14:0, and sphingomyelin (SM) 18:1/17:0 (all from Avanti Polar Lipids, Alabaster, AL, USA)] and 1% NH_4OH (Suprapur®, Merck) were infused into the MS at a flow rate of 10 μL/min. Specific precursor ion scans m/z 184 for PC and SM and neutral loss scans of 141 amu for PE, and 87 and 185 amu for PS, were employed to profile the membrane PL composition [48]. The spectra were processed using MassHunter Qualitative Analysis software (Agilent Technologies, Inc.), and the individual lipid species were quantified using the internal standards and free software called Lipid Mass Spectrum Analysis [49]. The acyl chain assemblies in each lipid species were identified in our previous analytical work on the hBMSCs (cultured for 9 days with different PUFA supplements) by detecting anionic fragments of the acyl chains [35]; however,

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the species in the present work are marked as follows: [sum of acyl chain carbons]:[sum of acyl chain double bonds] (e.g., 38:4 for species 18:0_20:4n-6). The results are described as mol% of each lipid species in its PL class, and the species exceeding 1.0 mol% are included in the figures.

2.7 Lipid mediator profiling

Incubations of hBMSCs were thawed on ice and 2 volumes of ice-cold methanol (Thermo Fisher Scientific) containing the internal standards d_8-5*S*-hydroxyeicosatetraenoic acid (HETE), d_5-resolvin D2 (RvD2), d_5-LXA_4, d_4-PGE_2, and d_4-leukotriene B_4 (all from Cayman Chemical), 500 pg each, was added to the sample. LMs were extracted and identified as described previously [50,51]. Briefly, the samples in methanol were incubated for 45 min at -20 °C for protein precipitation and centrifuged at 1900 g at 4 °C for 10 min. The methanol content of the supernatant was evaporated to less than 1 mL using a nitrogen gas stream, and the LMs were extracted with an automated Extra-Hera system (Biotage, Uppsala, Sweden) employing solid-phase extraction. The methyl formate eluates were concentrated and injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (LC-20AD HPLC (Shimadzu) and SIL-20AC autoinjector (Shimadzu) paired with QTrap 6500+ (ABSciex, Framingham, MA, USA) or QTrap 5500 (ABSciex). LMs were identified and quantified using multiple reaction monitoring of the precursor (Q1) and product (Q3) ions in negative ionization mode. Identification was conducted in accordance with published criteria, matching the retention time with authentic and synthetic standards (from Cayman Chemical, prepared in house or provided by Charles N. Serhan, Harvard Medical School, Boston, MA, USA) and identifying at least 6 diagnostic ions from the MS/MS spectra [50,51].

2.8 Statistical analysis

Nonparametric tests were applied due to the non-normal distribution of variables, and in cases with a low number of biological replicates, statistical tests were not conducted. When applicable, the results are expressed as medians with ranges or interquartile ranges depending on the number of biological replicates. The variation in fatty acid data at the 2, 6, and 24-h time points was analyzed using the Kruskal-Wallis test and ordered trends using the Jonckheere-Terpstra test. The analyses were conducted using IBM SPSS Statistics (Version 24), and *p*-values < 0.05 were considered statistically significant. The fold changes of the PL and LM data were determined by calculating the ratio of the values from the PUFA supplementation to those of the control treatment in biological replicates of each primary cell line. Principal component analysis (PCA) was conducted and visualized with centered and scaled PL mol% data (all lipid species per PL class were used as variables) and LM metabolome data as pg / incubation using R version 3.5.1 with the ggbiplot package [52,53].

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3 RESULTS

3.1 The incorporation dynamics of supplemented PUFAs differ between hBMSC membrane PLs

Supplementation with the PUFAs AA, EPA, and DHA showed their successful incorporation into the fatty acid profile (Fig. 1) and membrane PLs (Supplementary Figs. 1-3) after 2, 6, and 24 h of incubation. The specific PUFA supplementations resulted in an increase in the corresponding PUFAs, especially at the 24-h time point (Fig. 1 and Supplementary Table 1). Additionally, both AA (20:4n-6) and EPA (20:5n-3) supplementation caused an accumulation of the elongated forms of these PUFAs: adrenic acid (ADA, 22:4n-6) and n-3 DPA (22:5n-3), respectively. The PUFA supplementation caused a diminishing trend in the levels of both saturated and monounsaturated fatty acids (MUFAs) compared with the control. Moreover, AA supplementation decreased the levels of the n-3 PUFAs DPA ~0.55-fold and DHA ~0.45-fold when compared to the control. In contrast, both EPA and DHA supplementation decreased the levels of the n-6 PUFAs AA and ADA (EPA: ~0.50- and ~0.61-fold decrease, DHA: ~0.65- and ~0.54-fold decrease in AA and ADA, respectively). EPA supplementation also decreased the levels of DHA ~0.36-fold.

Incorporation of the exogenous PUFAs was also detected in the hBMSC PL species profiles, but the SMs species profiles of the cells were mainly unaffected by the supplementation (Fig. 2 and Supplementary Figs. 1-3). Extensive PUFA incorporation into the PC, PE, and PS classes was achieved after a 24-h incubation; however, different PL classes accepted the supplemented PUFAs with different dynamics (Fig. 2). The incorporation into PC was already visible after 2 h and increased steadily over time. In PE, the effect was observed to some extent at 6 h and clearly after 24 h. In the PS species profile, PUFA incorporation was only observed in the 24-h incubation and was limited to the polyunsaturated C40 species, which showed increased proportions.

3.2 PUFA modifications of hBMSC PL membranes are transferred to hBMSC-EVs

We investigated the hBMSC-EVs collected after cultivation of cells with PUFAs (24 h) and subsequently in serum-free medium (48 h). Neither the amount nor the size distribution of the particles in hBMSC-EV samples were altered by the PUFA supplementation (Fig. 3A and B). The hBMSC-EVs expressed the MSC surface marker CD73, while the expression of other markers tested (tetraspanins CD9 and CD63, platelet marker CD41, $cPLA_2$, and $sPLA_2$) was negligible (Fig. 3C and Supplementary Fig. 4). We profiled the membrane PL composition of the hBMSC-EVs (Fig. 4B), which corresponded to the PL profile of the donor cells (Fig. 4A) with certain changes. In PC, di-PUFA species comprising two long and polyunsaturated acyl chains were found

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enriched in EVs compared to the donor cells after PUFA supplementation (e.g., cells: ~2-fold and EVs: ~15-fold increase in the 42:10 species after EPA supplementation). Moreover, after AA and EPA supplementation, the levels of the 36:1 species, harboring saturated fatty acids and MUFAs, were decreased less in hBMSC-EVs than in the cells (EVs: ~0.73-fold and ~0.79-fold decrease following AA and EPA supplementation, and in the cells: ~0.45-fold and ~0.69-fold decreases, respectively). The changes in the PE species profile resembled those found in the PCs: the di-PUFA species were present with higher proportions in EVs than in the cells (e.g., an increase in the relative amount of 42:10 after AA supplementation in cells of ~2.4-fold and in EVs up to ~4.7-fold), and the levels of 36:1 (18:0_18:1 chains) did not decrease in the AA and EPA-supplemented hBMSC-EVs despite a clear decrease in the donor cells (AA supplemented cells: ~0.56-fold, EPA supplemented cells: ~0.76-fold decrease). In PS, the species containing C22 ADA, n-3 DPA, and DHA chains (40:4, 40:5, and 40:6, respectively) were present in cells at higher proportions than in their EVs after the corresponding PUFA supplementation (cells: ~5.5-, ~5.1-, and ~2.6-fold increase, EVs: ~2.1, ~1.8-, and ~1.6-fold increase in 40:4, 40:5, and 40:6, respectively).

3.3 Incorporated PUFAs alter the downstream lipid mediator profile of hBMSCs

Incubations of hBMSCs, pre-cultured for 24 h with the PUFA supplements and then incubated for 48 h in serum-free medium, were studied to examine the LM profile. The hBMSCs produced multiple LMs that were identified employing previously published criteria by matching the retention times (Fig. 5A) and a minimum of 6 diagnostic ions from the MS/MS spectra of the analyzed mediators with corresponding authentic or synthetic standards (Fig. 5B) [50,51]. We observed trends that PUFA supplementation increased the production of downstream LMs and monohydroxy pathway markers even though there were variation in the profiles of primary hBMSCs from different donors (Fig. 5C and Supplementary Table 2). Assessment of the LM profiles using multivariate analysis demonstrated that the supplementation of hBMSCs with different PUFAs resulted in characteristic LM profiles, as depicted by the distinct clusters (Fig. 6). In more detail, AA supplementation increased the production of PGD_2, PGE_2, and $PGF_{2\alpha}$, and EPA supplementation increased the production of RvE2, while RvE3 provided variable results between individuals. DHA supplementation increased the levels of RvD4 and RvD6. All supplementations increased the levels of the corresponding monohydroxy pathway markers, and DHA supplementation also increased the production of hydroxyeicosapentaenoic acids (HEPEs).

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3.4 hBMSC lipid mediator profiles are regulated in a stimulus-dependent manner

We investigated the effects of stimulatory conditions on LM production by hBMSCs supplemented with AA and DHA for 24 h. hBMSCs were then cultured under one anti-inflammatory condition: TGF-β1 5 ng/mL and IL-10 10 ng/mL (Condition 1), and three proinflammatory conditions: IFN-γ 25 ng/mL and LPS 10 ng/mL (Condition 2); IFN-γ 10 ng/mL and TNF-α 15 ng/mL (Condition 3); or TNF-α 10 ng/mL, IL-1β 10 ng/mL, and LPS 100 ng/mL (Condition 4) for 48 h. The production of certain LMs and their monohydroxy pathway markers increased in the stimulatory conditions compared with the control treatment, however, there were variation in the LM profiles from different hBMSC donors (Fig. 7 and Supplementary Table 3). PGE_2 levels increased considerably under Conditions 1 (~8.7-fold), 3 (~8.6-fold), and 4 (~116-fold) in AA-supplemented cells, and under Condition 4 (~221-fold) in DHA-supplemented cells when compared to the control. With both supplements, Condition 4 increased the production of other prostaglandins, PGD_2 and $PGF_{2\alpha}$, and the monohydroxy pathway markers HETEs (~5-fold and 11.3-fold increase with AA and DHA, respectively) and hydroxydocosahexaenoic acids (HDHAs) (~2.1-fold and 2.3-fold increase with AA and DHA, respectively). Moreover, the levels of total SPMs increased with different supplementations, notably in Condition 4 (AA supplementation ~3.1-fold, and DHA supplementation ~2.2-fold increase) and Condition 3 (AA supplementation ~4.7-fold increase, while DHA supplementation resulted in ~0.8-fold decrease). Condition 3 had a marked impact on RvD4 production by increasing these levels in both AA and DHA-supplemented cells.

4. DISCUSSION

In this study, we established that the PL composition of hBMSC-EVs can be modified by supplementing their donor cells with PUFAs. The incorporation of exogenous PUFAs had different dynamics depending on the receiving PL class, as revealed in the 2, 6, and 24-h supplementations. The supplemented PUFAs first incorporated into PC, later into PE, and finally into PS. More importantly, we demonstrated that hBMSCs translate the changes in their membrane PL profile to their EVs and produce a variety of SPMs that are modified by the different PUFA supplements and inflammatory stimuli.

The overall PL profile of the control hBMSCs was consistent with our and other's previous reports [34–36]. Moreover, the fatty acid profile and the PL species composition following incubation with PUFAs were in accord with our previous work assessing later time points [35], and the most prominent incorporation of PUFAs was observed after 24 h of supplementation. In hBMSCs, AA was elongated into ADA, plausibly to limit the formation of highly bioactive downstream LMs [54,55]. ADA has been reported to elicit lower COX activity than AA, and thus the conversion to downstream LMs is less effective [54,55]. Moreover, EPA was elongated into n-3 DPA, which is also a precursor for multiple SPMs, and thereby the n-3 PUFA precursor pool was

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potentially altered towards an even more proresolving LM profile and cell signaling [22,25]. Additionally, due to the competition of n-3 and n-6 PUFAs for the same elongation and desaturation enzymes, AA supplementation reduced the levels of n-3 DPA and DHA, while both EPA and DHA reduced the levels of AA and ADA [56–58]. Mammalian cells use two main pathways for fatty acid incorporation into their PLs, the *de novo* Kennedy and remodeling Lands pathways. The latter process gives rise to the most preferred acyl combinations, often demonstrating saturated fatty acids or MUFAs in the sn-1 position and PUFAs in the sn-2 position of the molecule [59]. Here, we observed the formation of di-PUFA PL species presumably generated via the Kennedy pathway. This pathway has little positional preference for certain fatty acid structures and may also give rise to di-PUFA PL species, and it becomes important when the fatty acid concentration in the culture medium is in the μM range, and the Lands pathway is saturated [60].

Interestingly, we observed that the PUFA incorporation rates differed between the PL classes: the supplemented PUFAs incorporated first into PC and only later into PE and PS, while the SM class, which is in principle poor in PUFAs, remained largely unaffected. Efficient incorporation of the supplemented PUFAs into the PC species was already observed at 2 h, which is in agreement with findings from metabolic studies showing that in the low-capacity/high-affinity Lands cycle, lysoPC acyltransferases transfer the supplemented PUFAs to lysoPC, which serves as the first acceptor [60,61]. The PE species showed incipient remodeling at 6 h, which continued at the 24-h time point. Coenzyme A-independent remodeling enzymes, transacylases, transfer PUFAs from PC to PE, which may take several hours in primary cell lines but occurs in minutes in cancer cell lines [62–64]. A marked exception to the general observation of detecting elevated levels of the PE species with the supplemented PUFAs was the PE species 38:4 (mostly 18:0_20:4n-6, determined in our previous study [35]). The relative proportion of this biologically very active species, which provides AA to PLAs, is maintained at a constant 20 mol% irrespective of the incubation time with AA, perhaps to limit excess inflammatory signaling due to PGE_2 and other mediators produced from AA. Remodeling of the PS species was delayed the most, likely because they received their PUFAs mainly from PCs via transacylases between the 6 and 24-h time points, and direct biosynthesis via the Kennedy pathway was negligible since di-PUFA PS were undetectable. At 24 h, the chain elongation of PUFA precursors was highly progressed, and little AA or EPA was incorporated into PS, consisting mainly of only the elongated ADA and n-3 DPA. The incorporation of DHA was efficient among the PC, PE and PS species, but the relative amounts of di-PUFA species with DHA remained small. This finding suggests that DHA is not utilized to the same extent as AA or EPA in the Kennedy pathway. However, the reasons limiting the use of DHA for building di-PUFA species are unclear.

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Next, we investigated the effects of PUFA modifications on hBMSC-EV membranes at the 24-h time point, when the PUFAs were already incorporated into several PL classes. The PUFA supplementation had no effect on the amount of particles secreted or their size distribution. The hBMSC-EVs expressed the cell surface marker CD73 but the expression of tetraspanins CD9 and CD63, existing typically in the endosomal compartment, was negligible. These results are in agreement with our previous findings that constitutively produced umbilical cord blood-derived MSC-EVs lacked the expression of these tetraspanins and expressed specific Rab proteins, which indicated that these EVs would originate mainly from cell surface [33]. Taken together, these observations suggest that most EVs secreted by hBMSCs during starvation are derived from the cell surface.

The lipidome of hBMSC-EVs has previously been characterized in two reports. Vallabhaneni and colleagues demonstrated that hBMSC-EVs contain ceramides and diacylglycerols that were not investigated in this study [65]. Haraszti and colleagues were the first researchers to fully profile the PL species composition of hBMSC-EVs [66]; however, comparison of their results with ours is challenging due to differences in the reporting formats [66]. To our knowledge, we are the first group to demonstrate that modifications of the cell membranes can be transferred to the PL profile of hBMSC-EVs. The PL composition of the EVs resembled those of the cells, but with specific differences. The observed larger proportions of di-PUFA species of PC and PE in EVs compared with cells may arise from the high efflux propensity of these PLs, making them preferred substrates for PLA_2 type IVA cleaving PUFAs for the biosynthesis of LMs [67]. The higher levels of these di-PUFAs in hBMSC-EVs may enable more efficient biosynthesis of LMs due to the more abundant precursor availability. Additionally, previous studies have reported the accumulation of monounsaturated acyl chains, mainly due to an enrichment of 18:1, in the EV PL composition (reviewed recently by Skotland et al. [68]). Our study confirmed this observation since PE 36:1 and PS 36:1, both of which contain 18:1, were present in higher relative amounts in EVs than in the donor cells. Certain PL species plausibly move to EVs readily due to their high efflux propensities and their superior compatibility to the high curvature of the EV membrane, which may explain the differences in PL species composition between EVs and the corresponding cell membranes.

After demonstrating that both hBMSCs and hBMSC-EV membranes incorporate vital precursors for SPMs, we further investigated the effects of these PUFA modifications on LM production. To allow relevant comparisons, we maintained the same experimental conditions as used for the EV membrane PL profiling. The main LM molecule produced by hBMSCs was PGE_2, which coincides with previous reports of PGE_2 as a central mediator of the therapeutic potential of MSCs [9,35,69,70]. Traditionally, PGE_2 has been classified as a proinflammatory mediator, but recent reports have linked it to multiple anti-inflammatory functions [9,58,69]. Interestingly, PGE_2 has also been found to induce LM class switching, a process that is vital for the induction of resolution

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[17], which merits investigation in light of the immunomodulatory response of hBMSCs [70]. Moreover, studies on SPM production by MSCs are lacking, the majority of which have focused on murine MSCs [37,39], and only LXA_4 has been reported to be produced by human MSCs [38]. A major finding of our study was that hBMSCs produced a variety of SPMs, including 15-epi-LXA_4, RvE2, RvD4, and RvD6, even in the absence of PUFA supplementation. Recently, we have reported that the level of secreted PGE_2 is enhanced in hBMSCs due to precursor AA supplementation [35]. Here we observed the same trend, in which precursor PUFA supplementation increased the production of downstream LMs in hBMSCs. Several studies have demonstrated a potentiated immunological phenotype following EPA or DHA supplementations [e.g., 37, 39, 71]. Interestingly, Abreu and colleagues recently demonstrated that EPA supplementation enhanced the therapeutic influence of BMSCs in asthmatic mice [39]. These observations highlight the importance of maintaining an optimal cell membrane fatty acid profile, which forms the precursor pool for LM biosynthesis. Since hBMSCs have an incomplete ability to metabolize C18 PUFA precursors to C20-22 PUFAs such as EPA and DHA, supplementing these precursors of SPMs during cell culture is essential to ensure the full therapeutic potential of the cells and their EVs [35].

The microenvironment and stimulatory conditions have profound effects on the immunomodulatory capacity of MSCs [4]. Here we exposed the AA and DHA-supplemented hBMSCs to anti- (Condition 1) and proinflammatory conditions (Conditions 2-4) to investigate the effects of these stimuli on SPM production. The effects of the given stimuli may have diminished by the end of our long (48 h) experimental window, which was initially chosen to correspond to the EV experiments, hence possibly hindering the detection of differences in LM production and also contributing to the variation observed in these results. Nonetheless, we observed moderate effects in LM production under Conditions 1-3 and a clear effect to PGs under Condition 4 regardless of the supplemented PUFA. Condition 4, with a high concentrations of LPS, is known to induce excessive Toll-like receptor 4 activation, and in this study resulted in a pronounced increase in PGs, potentially leading to a counter-regulatory action by also increasing the production of total SPMs and their precursors/pathway markers such as 15-HETE and 17-HDHA. As mentioned, the incubation time of the stimuli was long, and possibly, the initial response of the cells to the inflammatory stimuli may have already dampened after the 48-h incubation. Thus, the effects of the lower-grade stimuli (Conditions 1-3) to the LM production should be interpreted with caution due to the experimental setup. Even though the PL profiles of membranes in different primary hBMSCs remained stable, the observed variation in the LM results may arise due to differences in the activities of the biosynthetic enzymes of different hBMSC donors. Despite of these limitations, the key finding that hBMSCs produce SPMs stands firm. Taken together, we were able to elucidate that hBMSCs produce SPMs with and without an inflammatory stimulus, which has not been demonstrated prior to this study.

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MSCs are known to modulate the responses of various immune cells towards a more anti-inflammatory and immunosuppressive direction by, e.g., inhibiting the proliferation of T and B cells [72]. MSCs and MSC-EVs also modulate macrophages towards an anti-inflammatory and even proresolving phenotype [69,73]. It is important to note that proresolving processes are not immunosuppressive and, thus, do not inhibit the function of immune cells but rather promote the active return of homeostasis [74,75]. By elucidating the proresolving properties of MSCs, we may, therefore, uncover novel mechanisms underlying the MSC immunomodulatory properties, thus raising interest especially in trauma healing. We hypothesize that MSCs can act as promoters of resolution: by producing PGE_2, inducing LM class switching and secreting SPMs, MSCs may promote the onset of resolution. By increasing SPM production with PUFA supplementation, we are able to reinforce these MSC actions. The SPM content of hBMSC-EVs remains unknown, but it is tempting to speculate that hBMSC-EVs can act as inducers of SPM biosynthesis in other immune cells by carrying the raw material (PUFA-modified PLs) for their SPM biosynthesis. This idea is supported by a previous report, in which neutrophil-derived EVs were demonstrated to improve the biosynthesis of SPMs of acceptor macrophages [28].

To conclude, our results demonstrate that the PL composition of hBMSC membranes can be specifically modified and that these modifications are reflected by parallel modifications in the PL composition of hBMSC-EVs. When hBMSCs were supplemented with immunologically potent PUFAs, alterations in LM production occurred, the magnitude of which grew in response to highly proinflammatory stimuli. We also report the production of several SPMs by hBMSCs. Most importantly, with exogenous PUFA supplementation, we are able to induce profound changes in the downstream SPMs functioning in the resolution of inflammation. At a practical level, this study highlights the impact of the external fatty acid milieu during cell culture, which is currently underestimated in standard cell culture mediums lacking PUFAs such as EPA and DHA. The supplementation of PUFAs into the cell culture medium provides a natural, safe and convenient method to modify MSCs and MSC-EVs rather than undergoing genetic manipulations of the cells [76]. Thus, the PUFA supplementation approach during cell culture should be considered as an improvement of clinical MSC and MSC-EV therapy products with a more potent proresolving phenotype.

ACKNOWLEDGEMENTS

The authors thank Lotta Sankkila, Birgitta Rantala, and Sanna Sihvo for their excellent technical assistance, and Adj. Prof. M.D Matti Korhonen for providing hBMSC material for the study. The authors also acknowledge the EV CORE at University of Helsinki lead by Adj. Prof. Pia Siljander for the collaboration and expertise in the EV field.

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This work was supported by Clinical State Research Funding [EVO/VTR grant, Finland] (M.H.), Finnish Cultural Foundation (M.H.), and Business Finland [NANOSKIN grant no: 4708/31/2016] (S.V, S.L). This work is also funded by a Wellcome Trust Infrastructure Grant [Ref 101604/Z/13/Z], Academy of Finland [grant no: 287089], and Magnus Ehrnrooth Foundation (S.V.). J.D. received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme [grant no: 677542] and the Barts Charity [grant no: MGU0343]. J.D. is also supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society [grant 107613/Z/15/Z].

AUTHOR CONTRIBUTIONS

M.H., K.H., R.K., E.K., and S.L. designed the study. M.H. completed the cell culture and phospholipid analyses. F.T. conducted the fatty acid profile analysis and participated in the phospholipid data collection. S.V. conducted the NTA and Western blot analyses. P.L. contributed to the main hBMSC material for the study. M.H. and F.M. completed the LM data collection, and M.H., R.C., and J.D. conducted the LM data analysis. M.H., R.K., E.K., and S.L. interpreted the results and wrote the manuscript. S.V., F.T., K.H., R.C., F.M., P.L., and J.D. critically revised the manuscript and contributed to discussion. All authors approved the final manuscript.

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FIGURES

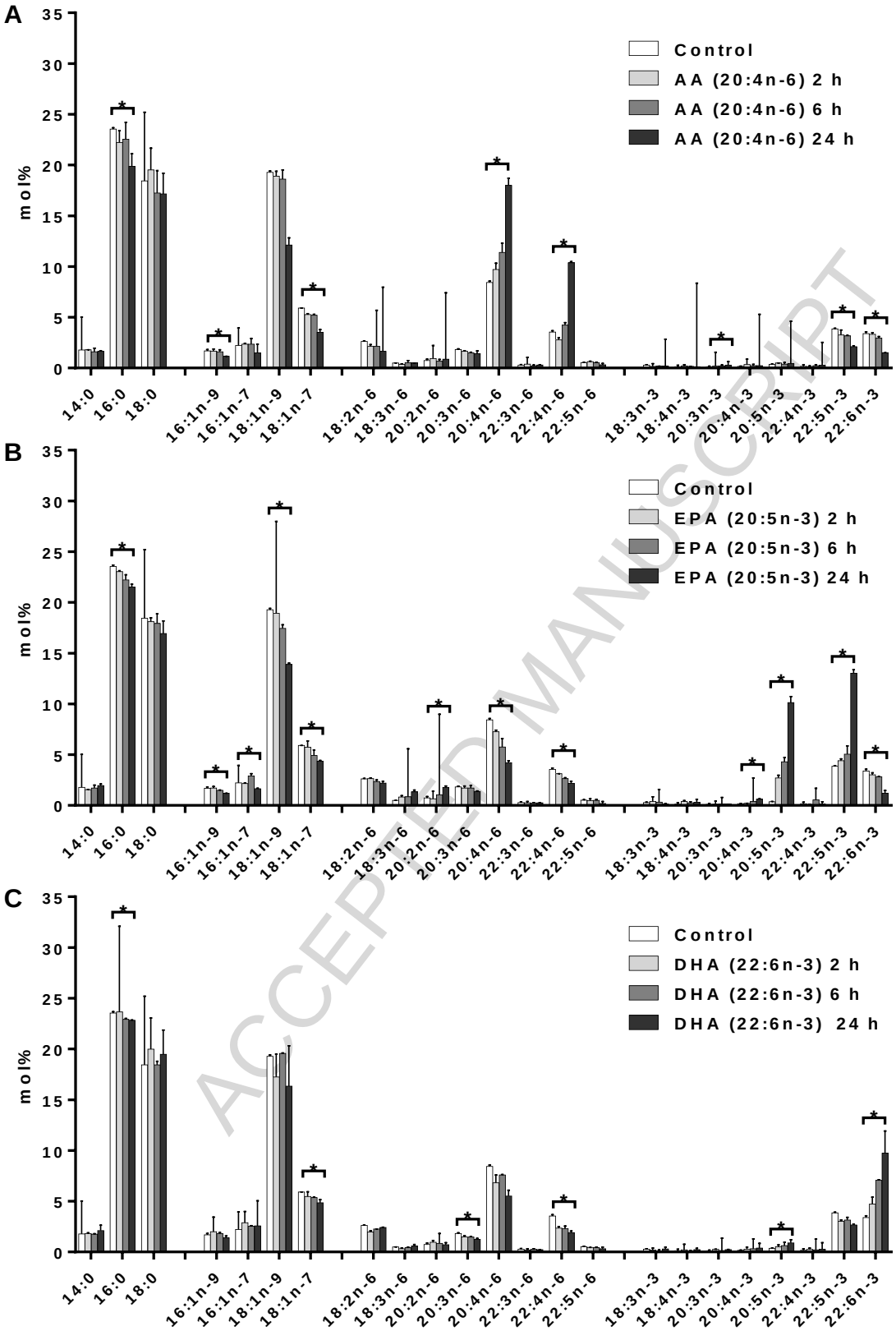

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Figure 1. Supplemented PUFAs alter the fatty acid profile of hBMSCs. hBMSCs were supplemented with AA (A), EPA (B), or DHA (C) for 2, 6, or 24 h and the fatty acid profile was analyzed by gas chromatography. The results are expressed as medians with ranges and as molar percentages (mol%); n = 3 experimental replicates per group; *, $p < 0.05$ using Jonckheere-Terpstra test for testing the trends.

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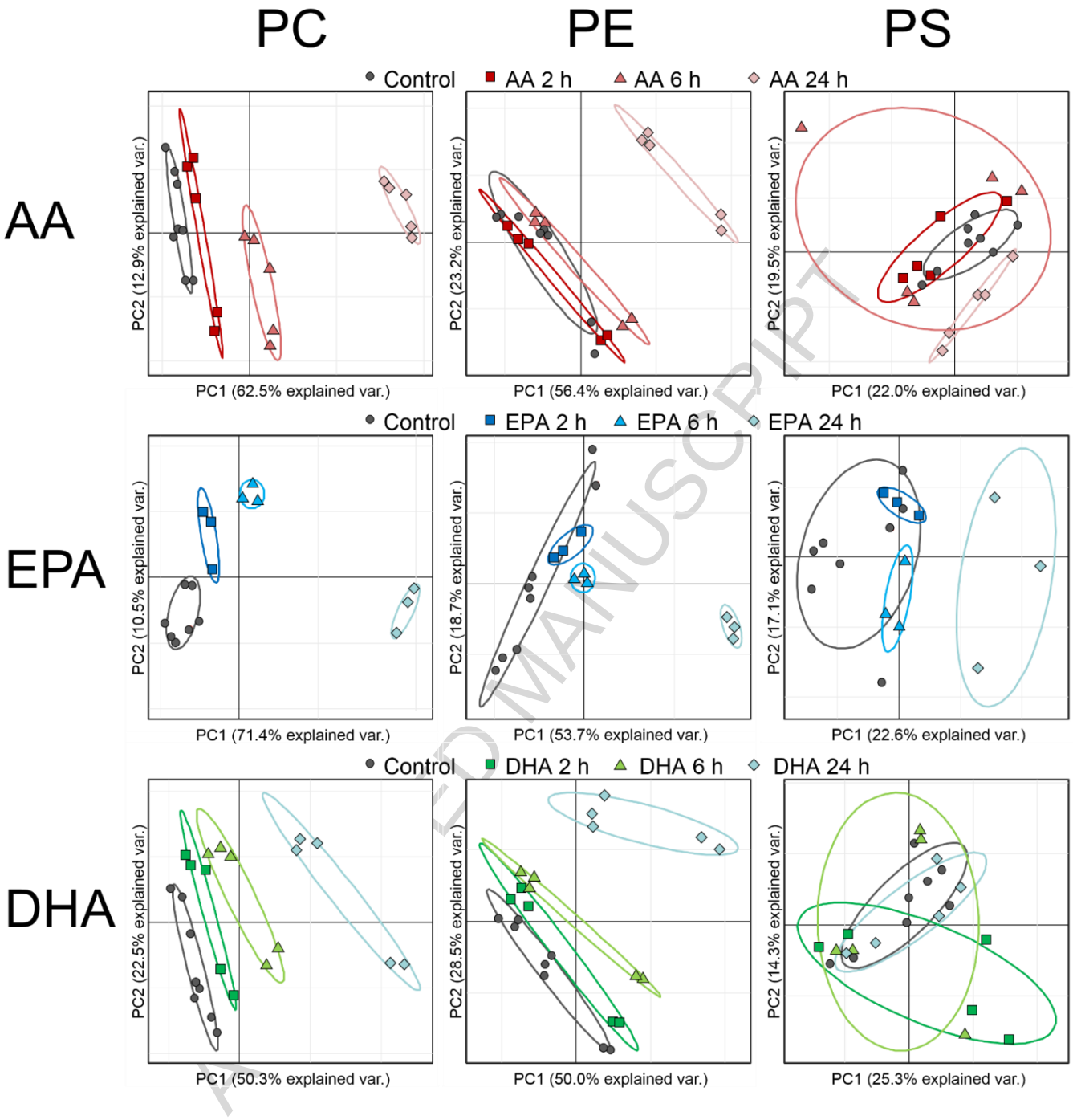

Figure 2. The incorporation dynamics of supplemented PUFAs differ between hBMSC membrane PLs. hBMSCs were supplemented with AA, EPA, or DHA for 2, 6, or 24 h and the consequent phospholipidome changes were analyzed by ESI-MS/MS. Principal component analysis (PCA) was conducted for the species profiles of each specified PL class, n = 8 (control), n = 5 (AA and DHA), or n = 3 (EPA) experimental replicates per group. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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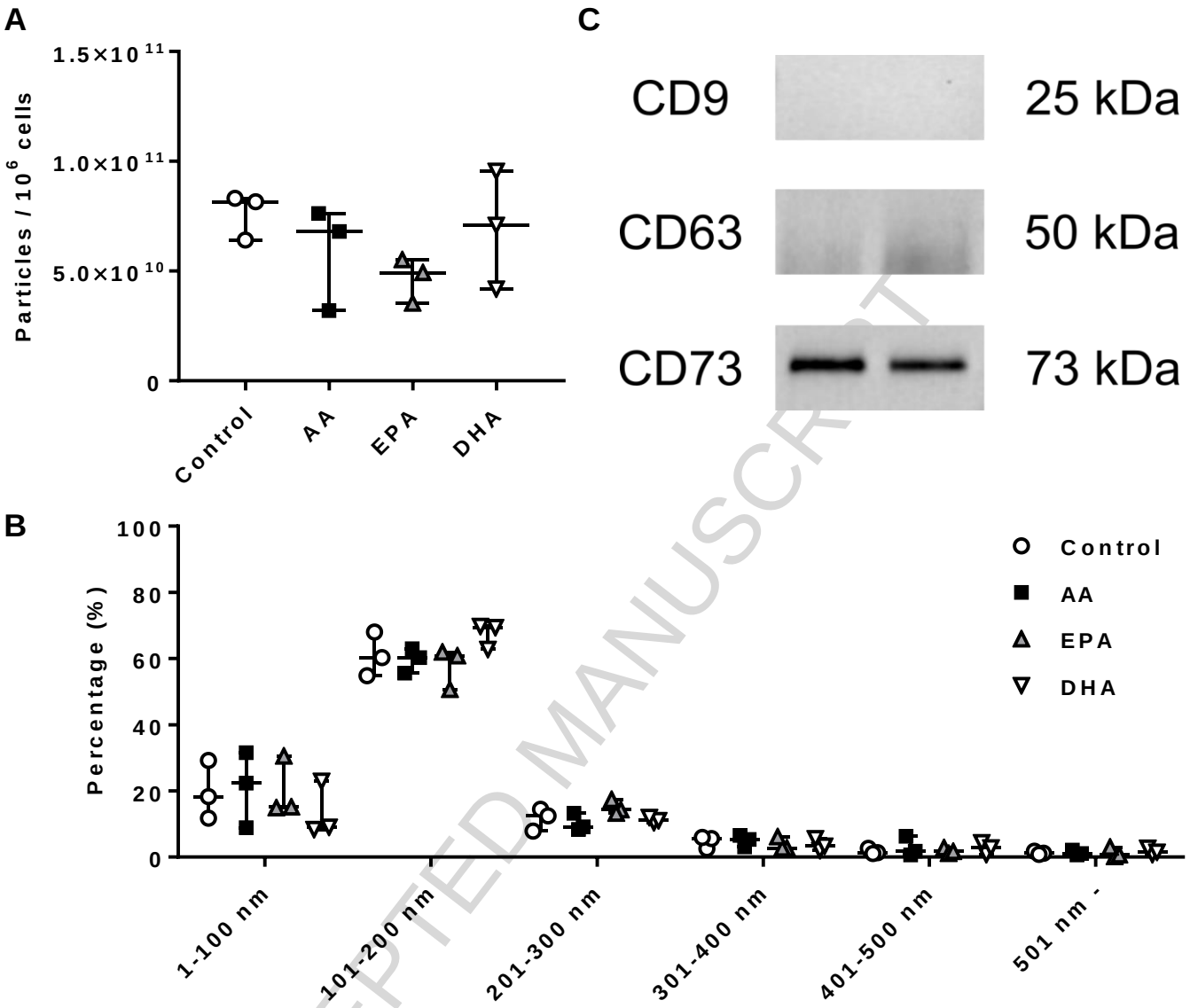

Figure 3. Characterization of hBMSC-EVs. hBMSCs were supplemented with AA, EPA, or DHA for 24 h, incubated in serum-free medium for 48 h, and EVs were collected via ultracentrifugation. The particle concentration per 10^6 hBMSCs (A) and the size distribution of the particles (B) in hBMSC-EV samples were analyzed by Nanoparticle Tracking Analysis; n = 3 biological replicates per group. The expression of CD9, CD63, and CD73 was analyzed from control EVs by Western blotting (C).

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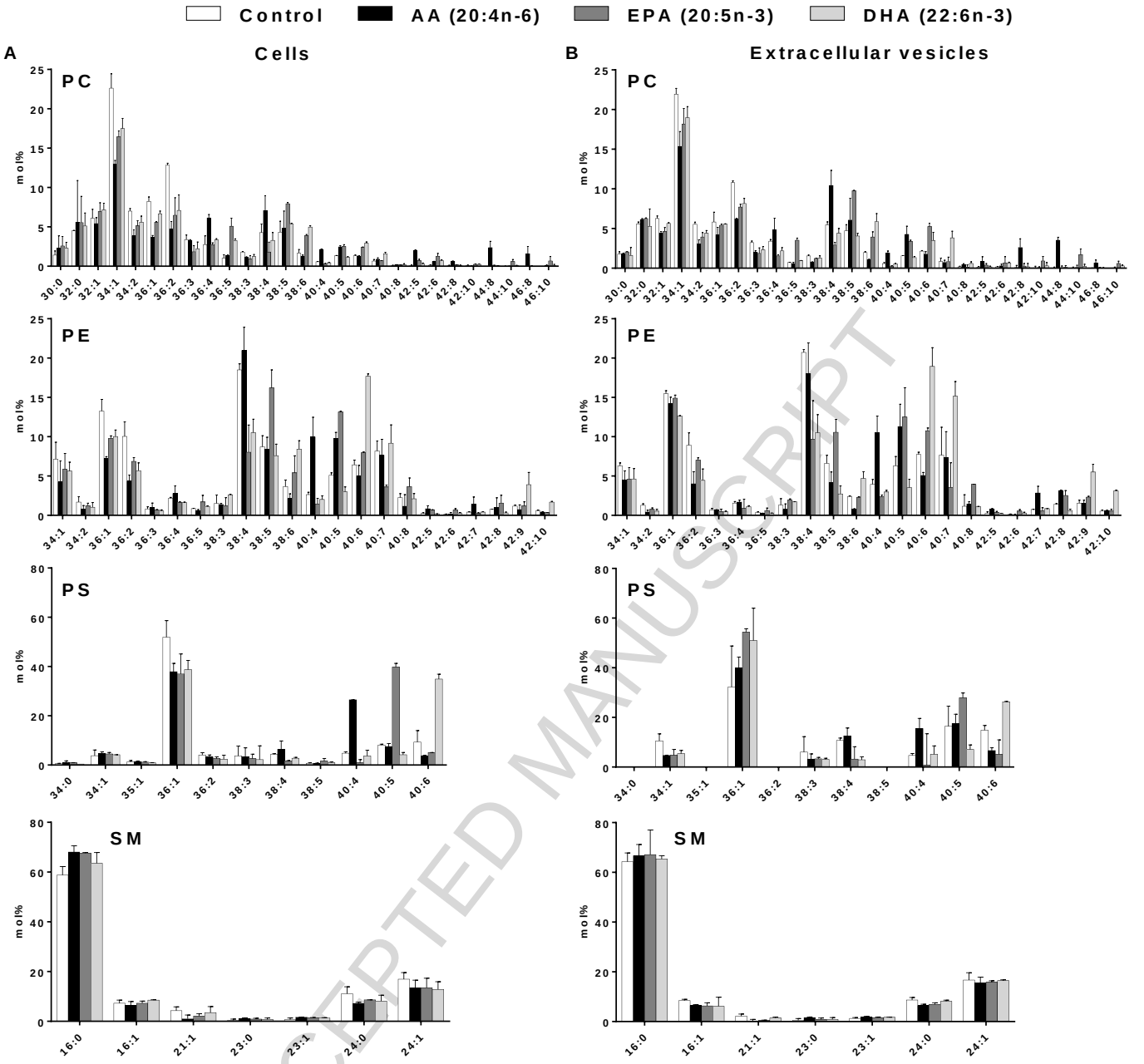

Figure 4. PUFA modifications of hBMSC PL membranes are transferred to hBMSC-EVs. The phospholipid profiles of hBMSCs (A) and hBMSC-EVs (B). Cells were supplemented with AA, EPA, or DHA for 24 h and then incubated for 48 h in serum-free medium. The hBMSC-EVs were collected from the cell culture medium via ultracentrifugation, and both EVs and cells were analyzed for the PL species profiles by ESI-MS/MS. The results are expressed as medians with ranges and as molar percentages (mol%), showing PL species with greater than 1 mol%; n = 3 biological replicates per group, except for PS AA n = 2. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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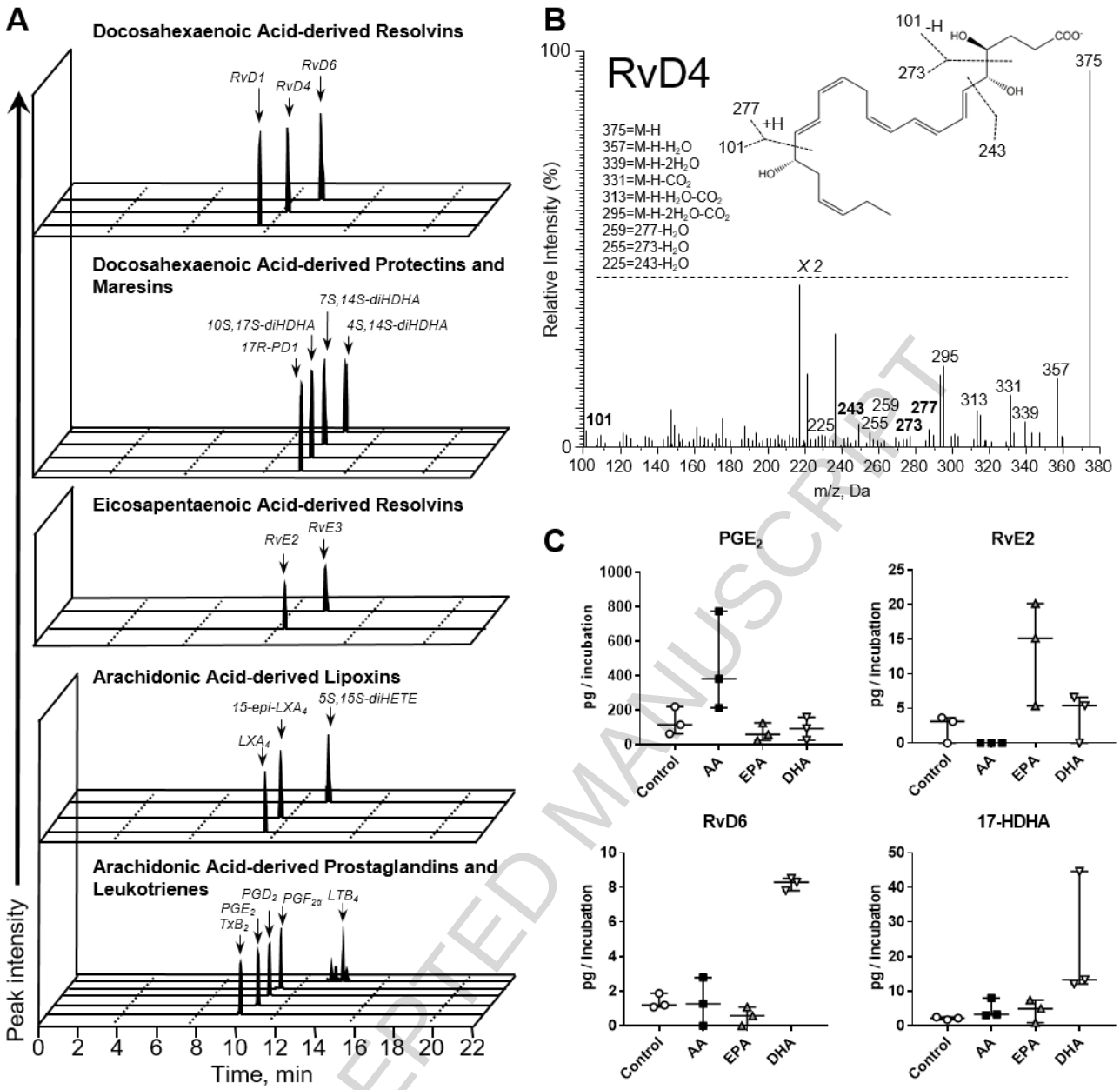

Figure 5. Representative multiple reaction monitoring chromatograms for the identified AA, EPA, and DHA metabolomes from the hBMSC incubations (A). Tandem mass spectrometry fragmentation spectra employed in the identification of resolvin (Rv)D4; peaks assigned to bolded m/z values indicate backbone breaks of the molecule (B). Specific examples of PUFA metabolomes following supplementation. hBMSCs were supplemented with AA, EPA, or DHA for 24 h, incubated for 48 h in serum-free medium, and then analyzed by LC-MS/MS. The results are expressed as pg per incubation (incubation volume = 9 mL); n = 3 biological replicates per group (C).

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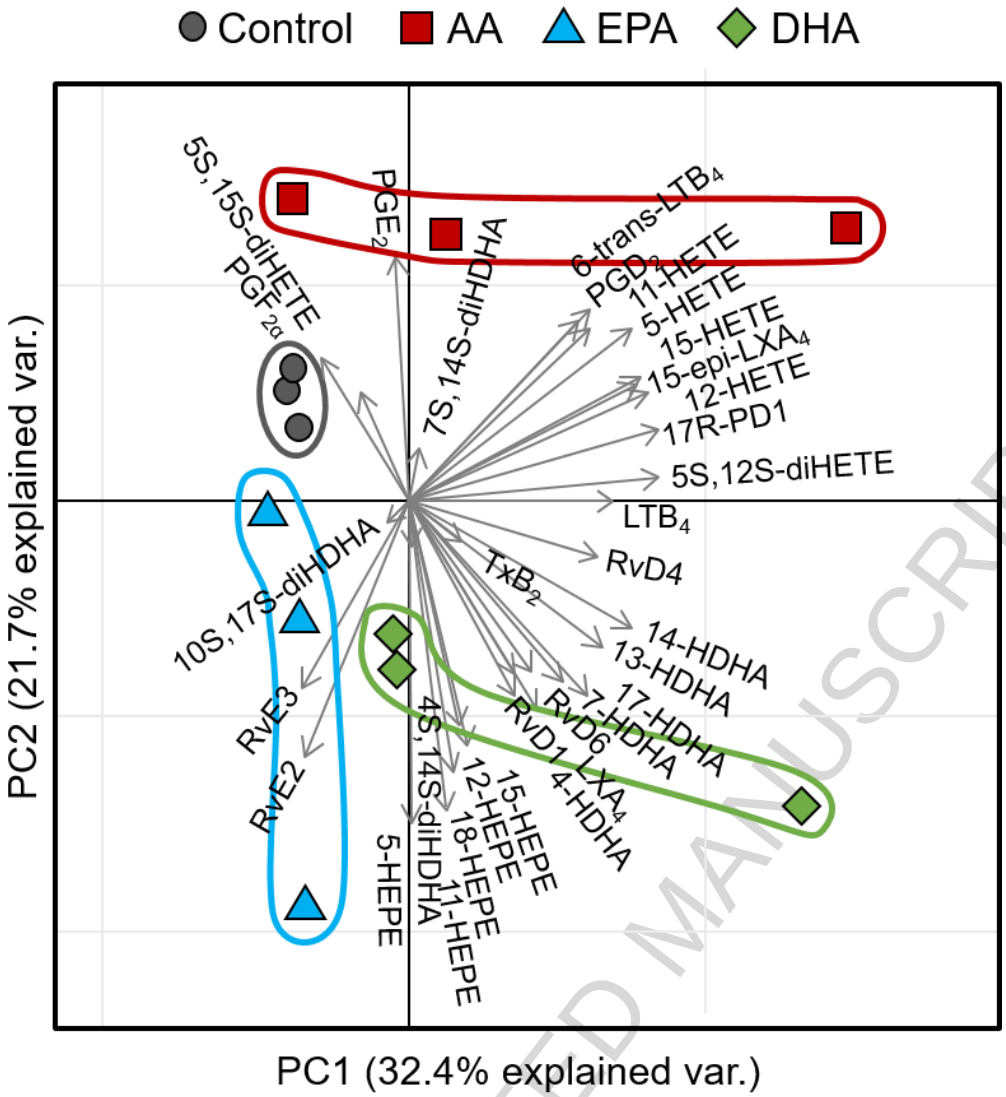

Figure 6. Incorporated PUFAs alter the downstream lipid mediator profile of hBMSCs. Principal component analysis of AA, EPA, and DHA metabolomes identified from the hBMSC incubations supplemented with AA, EPA, DHA, or control treatment, and then incubated for 48 h in serum-free medium, n = 3 biological replicates per group.

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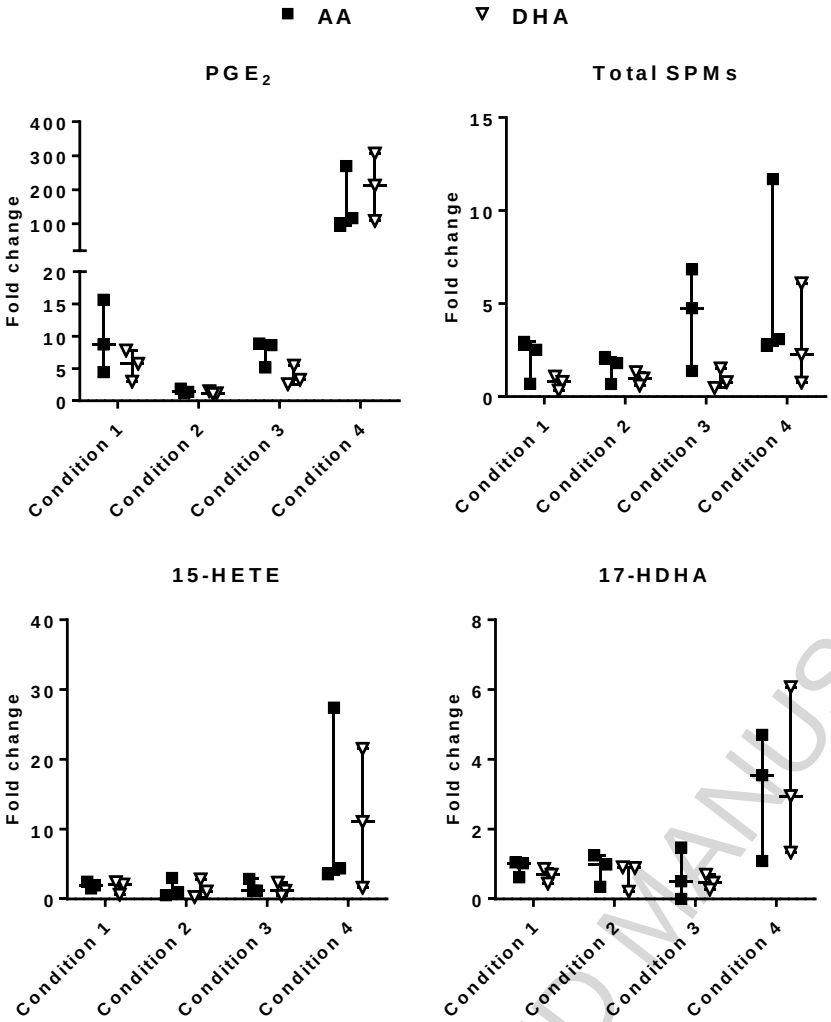

Figure 7. hBMSC lipid mediator profiles are regulated in a stimulus-dependent manner. hBMSCs were supplemented with AA or DHA for 24 h. The cells were then incubated with either TGF-β1 5 ng/mL and IL-10 10 ng/mL (Condition 1); IFN-γ 25 ng/mL and LPS 10 ng/mL (Condition 2); IFN-γ 10 ng/mL and TNF-α 15 ng/mL (Condition 3); or TNF-α 10 ng/mL, IL-1β 10 ng/mL, and LPS 100 ng/mL (Condition 4) for 48 h in serum-free medium, and analyzed by LC-MS/MS. The results are expressed as Fold change compared to the corresponding non-stimulated control; n = 3 biological replicates per group.

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