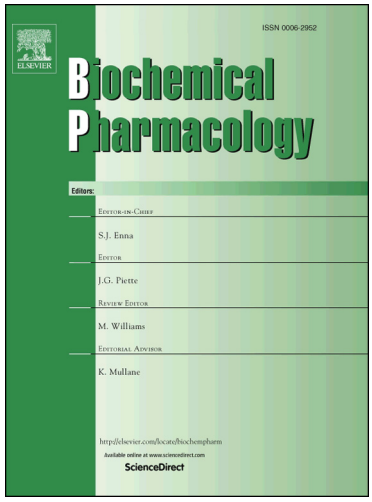
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Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1

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Abstract

Background: Ageing is associated with progressive endothelial senescence and dysfunction, and cardiovascular risk. Circulating endothelial microvesicles (MVs) are pro-senescent and pro-inflammatory endothelial effectors in acute coronary syndrome. Omega 3 PUFAs intake was claimed beneficial in cardiovascular prevention.

Purpose: To investigate whether the intake of the omega-3 formulation EPA:DHA 6:1 by middle-aged and old rats reduces the shedding of pro-senescent microvesicles from cultured spleen leukocytes (SMVs) and clarify the underlying mechanisms in target coronary primary endothelial cells (ECs).

Methods: Middle-aged male Wistar rats (M, 48-week old) received 500 mg/kg/d of either EPA:DHA 6:1, EPA:DHA 1:1, or vehicle (CTL) for 7 days, old rats (72-week old) for 14 days. Spleen-derived leukocytes were prepared and cultured for 24 h and MVs collected from supernatants (SMVs). Cultured ECs were prepared from freshly isolated porcine coronary arteries. Senescence-associated β-galactosidase activity (SA-β-gal) was assessed by C12FDG, protein expression by Western blot analysis, oxidative stress by dihydroethidium using confocal microscopy, and procoagulant MVs by prothrombinase assay. The pro-senescent potential of SMVs from middle-aged rats (M-SMVs) was analyzed by comparison with young (Y, 12-week) and old (O) rats.

Results: The shedding of SMVs significantly increased with age and was inhibited by EPA:DHA 6:1 intake that also prevented ROS accumulation in spleen. Incubation of ECs with 10 nM SMVs from middle-aged and old but not those from young rats induced premature senescence after 48 h. The pro-senescent effect of M-SMVs was

prevented by Losartan and associated with endothelial oxidative stress. M-SMVs induced an up-regulation of senescence markers (p16, p21, p53), pro-atherothrombotic (VCAM-1, ICAM-1, tissue factor) and pro-inflammatory markers (pNF-κB, COX-2) and proteins of the angiotensin system (ACE, AT1-R). Conversely, endothelial NO synthase was down-regulated. Intake of EPA:DHA 1:1 and 6:1 by middle-aged rats decreased SMV shedding by 14% and 24%, respectively. Only EPA:DHA 6:1 intake abolished the M-SMVs-induced endothelial senescence and reduced the prosenescent action of O-SMVs by 45%. Protection of ECs was not observed in response to SMVs from EPA:DHA 1:1 treated rats.

Conclusion: Ingestion of EPA:DHA 6:1 by middle-aged or old rats, respectively abolished or limited both the shedding of SMVs and their pro-senescent, pro-thrombotic and pro-inflammatory effects in ECs, most likely by triggering the local angiotensin system. EPA:DHA 6:1 may help to delay ageing-related endothelial dysfunction.

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1. Introduction

Cardiovascular diseases (CVDs) account for 39.6% of age-related chronic diseases [1]. The age-driven production of reactive oxygen species (ROS) within the vessel wall is a well-known contributor to endothelial dysfunction and vascular tone alteration [2]. Endothelial senescence is an emerging cause of the age-related progressive vascular dysfunction. It is however also early detected in middle-aged individuals at thrombogenic vascular sites, such as in coronary arteries, which are prone to flow disturbance. Senescence is characterized by an irreversible cell cycle arrest, morphological changes and a pro-atherogenic endothelial phenotype, favoring a loss of endothelial-dependent vascular function and progressive cardiovascular damages accompanying hypertension or atherosclerosis [3, 4]. In senescent endothelial cells (ECs), Reactive oxygen species (ROS) from mitochondrial sources and generated by up-regulated cytoplasmic NADPH oxidase, cyclooxygenases (COXs) initiate redox-sensitive processes that prompt endothelial dysfunction thereby shifting the hemostatic features of the healthy endothelium towards a pro-inflammatory, procoagulant and vaso-constricting phenotype. In addition, P53 and down-stream cyclin-dependent kinase inhibitors, P21 and P16, are up-regulated while the cytoprotective endothelial nitric oxide synthase (eNOS) is down-regulated. Ultimately, senescent ECs promote the release of endothelial microvesicles able to disseminate a pro-senescent signal [5, 6].

Microvesicles (MVs), are sub-micron plasma membrane vesicles shed by cells in response to stress. MVs convey active cytoplasmic proteins, lipids as well as miRNA that characterize the cell lineage and stress at the origin of their release [7]. In blood, they circulate as surrogate and pathogenic markers of primary and secondary cardiovascular risk [8]. Indeed, circulating MVs constitute a storage pool of effectors

of multiple cell origin orchestrating vascular cell cross-talk. MVs are pro-coagulant because they expose phosphatidylserine (Phtdser), an anionic phospholipid that catalyzes the assembly of blood coagulation complexes, and when stemmed from endothelial cells and monocyte, tissue factor (TF), the cellular initiator of blood coagulation [9]. MVs of endothelial origin from patients with acute coronary syndrome (ACS) have been shown to induce coronary endothelial cell dysfunction, premature senescence and thrombogenicity through the activation of the local angiotensin system [6].

Interestingly, leukocyte-derived MVs (LMVs), were shown crucial to thrombus growth and vascular remodeling [10]. Their elevated plasma level was correlated with the Framingham score in patients with primary and secondary cardiovascular adverse outcomes [11]. LMVs are recruited at the surface of the inflamed endothelium, constitute an additional source of TF and mediate cell interactions within the thrombus [12, 13]. In human atherosclerotic plaques, 55% of MVs originate from leukocytes [14]. Animal models of atherothrombosis or ischemia reperfusion indicate that LMVs contribute to tissue remodeling by limiting the endothelial NO and promoting redox-sensitive pathways, among which the up-regulation of pro-inflammatory cytokines and of adhesion proteins favoring diapedesis. In mice, the injection of LMVs from diabetic patients alters the vascular tone by reducing the acetylcholine-evoked endothelial relaxation of the aorta [15, 16]. Altogether, TF^{+}-MVs prompt coagulation and exacerbate inflammation through multiple amplification loops involving TF-driven cell responses [17]. Interestingly, MV shedding seems linked to the lateral lipid organization of the plasma membrane, lipid rafts favoring their TF enrichment when released from activated macrophages [18].

The lipid composition of the plasma membrane of immune cells influences

membrane fluidity and down-stream production of lipid mediators. In humans consuming western diet, polyunsaturated fatty acids (PUFAs) in the plasma membrane of circulating neutrophils, lymphocytes, monocytes are mainly composed of 10%-20% of arachidonic acid, 0.5%-1% eicosapentaenoic acid (EPA) and 2%-4% docosahexaenoic acid (DHA). Intake of omega-3 PUFAs enhances the EPA /DHA membrane proportion at the expense of the n-6 PUFAs, mainly arachidonic acid and derived metabolites, alters the membrane order, and raft-dependent downstream pathways [19].

Clinical trials and animal models have shown that EPA and DHA reduce the risk of cardiovascular diseases [20, 21]. Daily consumption of omega-3 PUFAs is protective against primary or secondary cardiovascular events [22]. While EPA and DHA attenuate redox-driven DNA damages in aorta cells [23], vasoprotection by omega-3 PUFAs was shown dependent on the EPA/DHA ratio. We previously showed that maximal vaso-relaxation of healthy pig coronary artery is only obtained with omega-3 EPA:DHA 6:1 as compared to omega-3 EPA:DHA 1:1 [24]. Intake also improves the endothelial dysfunction and reduces systolic blood pressure by 53% in angiotensin-II-induced hypertensive rats [25].

Still, the beneficial effects of omega-3 PUFAs in cardiovascular health are debated. Clinical trials such as DART, GISSI-Prevenzione, GISSI-HF, JELIS have shown beneficial effects of omega-3 PUFAs in reducing the risk of cardiovascular diseases, whereas, others such as ALPHA OMEGA, SU.FU.OM3, ASCEND, VITAL did not observed promising proofs of reduction [26, 27]. Most recently, the REDUCE-IT trial which included hyperlipidemic patients with previously established cardiovascular diseases and receiving statin therapy, showed that 4 g/day ingestion of icosapent ethyl EPA led to a 19% reduction in plasma triglycerides and a 25%

drop in major cardiovascular events after 4.9 years follow-up [28, 29].

Strikingly, mechanisms of endothelial protection by omega-3 PUFAs against LMVs remain yet to be characterized. In post-myocardial 60 years-old patients, elevated levels of circulating MVs of leukocyte and platelet origin were slightly but significantly lowered by a 12-week supplementation with 5.2 g/day omega-3 PUFA (33% EPA 60%DHA w:w) given 6 month later [30], while MV procoagulant potential was reduced *in vitro* and involved both TF-dependent and -independent coagulation [30]. However, it still remains unclear whether omega 3 PUFA prevent the release of LMVs or rather shift noxious LMVs into cytoprotective actors and if so, which leukocyte population becomes beneficial to the ageing endothelium.

In vitro, omega-3 PUFAs directly modulate ion channels endocytosis, trigger PPAR-α or HNF-4α nuclear receptor dependent-responses, G-protein mediated-activation and ultimately ERK ½ kinases and NF- ƙB signaling. They also may alter cell responses by incorporating into the plasma membrane, where they are converted by COXs and LOXs into key anti-inflammatory specialized pro-resolving lipid mediators, including D- and E-series resolvins and protectins, maresin, [31-33].

This study was aimed at investigating whether the intake of the omega-3 EPA:DHA 6:1 new formulation modifies the ability of ageing immune cells to release MV, and whether it prevents their pro-senescent action towards the endothelium. MVs were isolated from the spleen of middle-aged (48 weeks), young (12 weeks) and old (72 weeks) rats. The ability of the spleen-derived leukocytes MVs (SMVs) to promote premature endothelial senescence was examined in young primary coronary endothelial cells (ECs).

2. Materials and Methods

2.1 Chemicals

All the chemicals and solvants, unless indicated, were from Sigma-Aldrich (Sigma-Aldrich, Saint Quentin Fallavier, France). Losartan was obtained from Merck Research Laboratories (Boston, MA, USA).

2.2 Ethics statement

Wistar rats (Janvier-labs, Le Genest-St-Isle, France) were kept in animal facility with controlled temperature (22 °C), 12-h light/dark cycle and were given free access to standard food and water. Experiments were consistent with the guidelines on animal care and use in laboratory, published by US institute of health (NIH, publication no. 85-23, revised 1996) and were authorized by French Ministry of Higher Education and Research and by the local ethic committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg). All experiments were performed in a registered animal yard at the Faculty of Pharmacy (Authorization number E-67-218-26).

2.3 Preparation of Omega-3 PUFAs

Purified formulations of omega-3 EPA and DHA were obtained from Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). EPA:DHA ratios of 6:1 and 1:1 (w/w) were prepared by mixing each EPA:DHA according to their relative purity under nitrogen flux to avoid oxidation, and stored in amber colored glass vials at 4°C. In several experiments (impact of Losartan on SMV-mediated endothelial senescence, determination of SMV phenotypes), the EPA:DHA 6:1 solution was replaced by the

content of Vascazen capsules (Pivotal Therapeutics Inc) and compared to the content of Omacor capsules (EPA:DHA 1.2:1).

2.4 Rat treatment

Middle-age male wistar rats (M, 48-week old) were weighed and assigned to four groups (9 rats/group). Rats were given 500 mg/kg/day oral dose of either EPA:DHA 6:1, EPA:DHA 1:1, corn oil as an isocaloric control without omega-3 or tap water for a week. Young (Y, 12-weeks old) and old (O, 72-weeks-old) male wistar rats, respectively treated for 7 and 14 days, were used for comparative purpose. After treatment, rats were weighed and euthanized by I.P injection of pentobarbital (150 mg/kg).

2.5 Spleen-derived leukocytes culture (splenocytes)

Freshly isolated rat spleens were washed with sterile PBS (phosphate buffered saline, Lonza, USA), cleaned of any fat debris and weighed. Under sterile conditions, spleens were cut using razor blades into five or six large pieces further homogenized in PBS using the plunger of 2 mL syringes and filtered through a strainer (100-µm Nylon, Falcon, USA) over a 50-mL tube. Filtrate was then centrifuged at 450g for 5-min. at room temperature (RT). The pellet was gently re-suspended in 3 mL ammonium chloride potassium (ACK) erythrocyte lysis buffer (0.15M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4), mixed under gentle shaking for 3 min, re-centrifuged (450g, 5min, RT) and re-suspended with RPMI medium-1640 (Gibco, life technologies limited, UK) supplemented with L-glutamate (2 mM), penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 mg/mL) and 15% fetal bovine serum (FBS) (Gibco, Saint Aubin, France). Cells were counted, seeded at 3 x 10^6

cells/mL in T75 flasks and incubated in humidified incubator at 37 °C, with 5% CO2 for 24 hours.

2.6 Isolation, quantification and characterization of splenocyte microvesicles

After 24 hours culture, supernatants were collected under sterile conditions and splenocytes were counted using Trypan blue to determine the number of surviving cells. Splenocytes and cell debris were discarded by centrifugation at 450g,15min, RT. Supernatants were centrifuged twice at 14,000g, 60min, 4 °C, and washed SMVs pelleted and concentrated in Hanks Balanced Salt Solution (HBSS, without phenol red, without Ca^{+2} and Mg^{+2}, Lonza, Belgium) and stored at 4°C for a maximum of 1 month. Prothrombinase assay was performed to quantify SMVs after their capture on Annexin-A5 coated micro-wells using a microplate spectrophotomer in kinetic mode. Annexin-5 has high affinity for phosphatidylserine (PhtdSer) exposed at the surface of MVs. In this assay, PhtdSer is the rate-limiting factor of the generation of thrombin from prothrombin detected at 405 nm using a chromogenic substrate (PNAPEP-0216, cryopep, Montpellier, France). SMV concentration was referred to as nanomolar PhtdSer equivalent (nM phtdser), by reference to a standard curve constructed with synthetic vesicles with known concentration of PhtdSer [34]. The cell origin of SMVs was characterized by capturing SMVs onto biotinylated antibodies against leukocytes CDs before quantification by pro-thrombinase assay as described [35] using the following IgGs : anti-CD45 for leukocyte common antigen, anti-CD3 for T lymphocyte population, anti-CD4 for T helper cells, anti-CD8b for T cytotoxic cells, anti-CD161a for natural killer cells, anti-CD25 for IL-2 receptor T cells and splenic dendritic cells, anti-CD31, mainly for

endothelial cells, anti-CD11b/c for monocyte/macrophage and granulocyte, anti-CD11b for neutrophil. Biotinylated monoclonal antibodies were insolubilized onto the streptavidin-coated microtitration plates and incubated with SMVs. All antibodies were purchased from BD Pharmingen, San Jose, USA. The MV concentration was obtained by subtracting the OD values measured using a control istotype biotinylated IgG.

2.7 Primary coronary artery endothelial cell culture

Primary coronary artery endothelial cells (ECs) were prepared from left circumflex coronary arteries of pig hearts, collected from local slaughterhouse (COPVIAL, Holtzheim, France) as described by [6]. Briefly, left circumflex coronary arteries were dissected out of freshly slaughtered pig hearts, cleaned of any adhesive conjunctive tissues and flushed with PBS without calcium to remove all the remaining blood. Cleaned coronary arteries were then treated with Collagenase type I (Gibco, life technologies corporation, USA) solution at 1 mg/ml prepared in MCDB-131 medium (Gibco, life technologies limited, UK), supplemented with streptomycin (100 U/ml), penicillin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM, all from Lonza, St Quentin en Yvelines, France) for 15 min at 37°C. ECs were then extracted into 50-mL falcon tubes by circular massage of arteries with frequent flushing with medium. The collected medium containing ECs was then centrifuged (450g, 5min, RT), supernatant was discarded and cells were re-suspended with complete MCDB-131 medium supplemented with 15% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM). ECs of three different coronary arteries were cultured in adherent T25 flask in humidified incubator at 37 °C, with 5% CO2. After 6 hours, cells were washed with PBS to

remove any non-adherent cells and fresh complete medium was added (P0 ECs). Thereafter, first passage ECs (P1ECs) were grown for 72 hours with medium changed every 48 hours.

2.8 Treatment of ECs by splenocyte MVs

Following trypsinization (Trypsin, Gibco, Life Technologies SAS, St Aubin, France) P1ECs were seeded in 6-well plate at 65-75% confluency and incubated with either SMVs (10 or 30nM PhtdSer eq.) for 6 or 48 hours, H_2O_2 (100 µM for 1h and after washing, 23h in standard culture medium for senescence, or 300 µM for apoptosis for 1 h). In some experiments, pharmacological modulators were added prior to SMVs e.g. NADPH oxidase inhibitor (VAS-2870, 5 µM), a cyclooxygenase inhibitor (indomethacin, 30 µM), mitochondrial combined inhibitors (myxothiazol, potassium cyanide and rotenone, 1 µM each) for 30 min or AT1R inhibitor (Losartan, 1 µM) for 1 h before application of SMVs.

2.9 Measurement of Senescence-Associated β-galactosidase activity

The fluorogenic cell permeable substrate C12FDG (5-dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, ThermoFisher, Illkirch, France) was used to determine the senescence-associated β-galactosidase activity (SA-β-gal) in treated ECs by flow cytometry, as described previously [6]. Briefly, ECs were alkalinized (pH raised to 6) with chloroquine (300 µM) for 1 h, followed by 1 h incubation with C12FDG (33 µM), a fluorogenic substrate of SA-β-gal [36]. Cells were thereafter washed with ice-cold PBS, harvested with trypsin and freshly analyzed using the CellQuest software (FACScan, Becton Dickinson, San Jose, CA, USA). Light scattering parameters were set to eliminate dead cells and subcellular debris. The

green C12-fluorescein signal was measured and SA-β-gal activity estimated using the mean fluorescence intensity (MFI) of the cell population. Auto-fluorescence gains were determined in unlabeled cells and set at the first logarithmic decade.

2.10 Western blot analysis

After SMV treatment, ECs were washed with cold PBS, and proteins were extracted by RIPA lysis buffer (20mMTris/HCl, 150mM NaCl, 1mM Na3VO4, 10mM sodium pyrophosphate, 0.01mM okadaic acid, 20mM, a tablet of protease inhibitor (Roche, Basel, Switzerland), and 1% Triton X-100 (Euromedex, Souffelweyershem, France). Proteins (15 µg or 20 µg) were separated by 10 % or 12 % SDS-PAGE at 100 V for 2 hours and further electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, VWR, Fontenay-sous-Bois, France) at 100 V for 2 hours. Membrane non- specific binding sites were blocked in Tris-buffered saline (TBS) solution containing 5% BSA (Bovine Serum Albumin) and 0.1% Tween-20 (Euromedex) for 1 h at room temperature. Proteins of interest were probed with specific primary antibodies in blocking solution, rabbit monoclonal anti-VCAM-1 (ab215380, 1:1000, Abcam, UK), mouse monoclonal anti-ICAM-1 (ab171123, 1:1000, Abcam), rabbit polyclonal anti-phosphorylated NF-kB (ab86299, 1:1000, Abcam), rabbit monoclonal anti-COX-1 (ab109023, 1:1000, Abcam), rabbit polyclonal anti-COX-2 (ab15191, 1:1000, Abcam), mouse monoclonal anti-tissue factor (4509, 1:1000, Sekisui Diagnostics, Germany), mouse monoclonal anti-eNOS (610297, 1:1000, BD Biosciences, France), mouse monoclonal anti-p21 (sc-817, 1:1000; Santa Cruz Biotechnology, USA) and rabbit polyclonal anti-p53 (sc-6243, 1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-p16 (250804, 1:500, Abbiotec, USA) and rabbit polyclonal anti-ACE (250450, 1:500, Abbiotec), rabbit

polyclonal anti-AT1R (sc-1173, 1:500, Santa Cruz Biotechnology), at 4°C overnight. Membranes were washed three times with TBS-T (TBS-Tween, Euromedex) and incubated with peroxidase-labelled secondary antibodies; anti-rabbit (7074s) and anti-mouse (7076s), (3:10000, Cell signaling technology, USA) for 60 min, at room temperature. Pre-stained markers (protein ladder, Euromedex) were used for molecular mass determination. Immunostaining was revealed by chemiluminescence solution (ECL, Bio-Rad laboratories, USA). The chemiluminescence signal was recorded with ImageQuant LAS4000 system (GE Healthcare Europe GmbH, Velizy-Villacoublay, France) and analyzed using ImageQuant TL software (version 8.1, GE Healthcare). Quantitative normalization with respect to housekeeping protein (mouse monoclonal anti-GAPDH, ab125247, 1:1000, Abcam) was done for each protein of interest.

2.11 Characterization of oxidative stress in spleen tissue and ECs

Oxidative stress in spleen tissue or ECs was determined using DiHyroEthidium (DHE), a redox-sensitive fluorescent probe. Spleen tissues, embedded in histo-molds containing Tissue-Tek optimum cutting temperature (OCT) compound (Sakura 4583, Leiden, Netherlands) and snap-frozen in liquid nitrogen, were cryosectionned (25 µm) and mounted on slides. For ECs, P1ECs were seeded in Labtek chambers (Millicell EZ slide, Ireland) and incubated with 10nM PhtdSer eq. SMVs in humidified incubator at 37 °C, with 5% CO2 for 6 hours. Slides (spleen tissue or ECs) were then incubated with DHE (2.5 µM) for 30min, at 37ºC in a light protected humidified chamber. Slides were washed three time with PBS and mounted under coverslip using fluorescence mounting medium (DAKO, USA), dried in dark and analyzed by confocal microscopy (Leica SP2 UV DM IRBE; Leica, Heidelberg, Germany) with a

20X magnification lens. Level of oxidative stress was quantified using the Image J software.

To identify the sources of oxidative stress, ECs were pre-treated for 30 min, with an NADPH oxidase inhibitor (5 μM VAS-2870), a cyclooxygenase inhibitor (30 μM indomethacin), antioxidant (1mM N-acetylcysteine), or a mixture of mitochondrial inhibitors of the respiratory chain (myxothiazol, potassium cyanide and rotenone, 1 μM each) prior addition of DHE alone (tissue) or combined with SMVs (ECs).

2.12 Measurement of endothelial apoptosis

Apoptosis was measured by flow cytometry using Annexin-5 and propidium iodide double labeling. ECs treated by 10 nM or 30 nM SMVs for 48 h were incubated with fluorescent Annexin-5 (5 μg/mL ImmunoTools, Friesoythe, Germany) and 2.5 μg/ml propidium iodide (Milteny Biotec SAS, Paris, France) in dark for 15 min, at room temperature. Early apoptotic cells were defined as Annexin-5 stained and PI unlabeled. Fluorescence acquisition was performed by Guava EasyCyte Plus FlowCytometry System (Millipore). Analysis was constructed from a minimum of 2000 events.

2.13 Statistical Analysis

Data are expressed as mean ± standard error mean (S.E.M) for n different experiments and analyzed by Graphpad Prism 5. Statistical variance between two groups was determined by applying unpaired-T test. Differences were considered statistically significant at $P < 0.05$.

3. Results

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3.1 Microvesicle shedding from primary splenocytes increases with age and is associated with enhanced pro-senescent potential

Using freshly harvested rat spleens from young (Y), middle-aged (M) and old (O) rats as a convenient source of primary spleen-derived leukocytes, thereafter termed splenocytes, we measured the microvesicle (SMV) shedding in cell supernatants after 24h culture. The proportion of surviving splenocytes was reduced by half in old compared to young splenocyte cultures (figure 1c), while SMV shedding significantly increased with age (3.3 folds in O, 1.7 folds in M *vs*. Y, $p < 0.01$). After 48h incubation with 10 nM SMVs, premature endothelial senescence of young primary coronary endothelial cells at passage 1 (P1ECs) was indicated by significantly enhanced SA-β-gal activity only in response to SMVs from old and middle-aged rats (O: 2 folds, M: 1.5 folds *vs.* untreated P1 ECs, $p < 0.01$). Conversely, SMVs from young rats had no significant effect (figure 1b). On line with other reports, [37] we also identified an age-related ROS formation in the corresponding spleen tissues with a significant amount of ROS in middle-aged rats by DHE staining (M: 1959.91 A.U. *vs.* Y: 1492.75 A.U., figure 2). Altogether, data suggest a progressive alteration of splenocytes with ageing that is associated with redox-sensitive mechanisms and leads to an increased shedding of pro-senescent SMVs.

Since omega-3 PUFA were reported to incorporate in rafts and modulate the plasma membrane lateral organization of lymphocytes [38], we investigated the impact of an EPA:DHA short-term intake on SMVs and first focused on splenocytes freshly isolated from middle-aged rats.

3.2 EPA:DHA 6:1 intake reduces oxidative stress in middle-aged rats with no incidence on spleen or total bodyweight

No significant changes were observed in spleen or total body weight of middle-aged rats after 7 days of treatment with either corn oil or omega-3 (figure 3a-b). Treatment with EPA:DHA 6:1, but not corn oil or EPA:DHA 1:1, significantly lowered the age-related amount of ROS by 19.5% in spleen tissues (EPA:DHA 6:1 : 1575.9 *vs*. control : 1959.91 A.U., $p<0.05$, figure 3c). Pharmacological inhibition by Indomethacin (IND), VAS-2870, and mitochondrial respiration complex inhibitors (MIT) reduced the amount of ROS in spleen tissue, indicating multiple sources of mitochondrial and cytoplasmic origins (figure 3d).

3.3 EPA:DHA 6:1 intake by middle-aged rats prevents the splenocyte shedding

Intake of omega-3 EPA:DHA 6:1 and EPA:DHA 1:1, but not of corn oil, significantly reduced the shedding of procoagulant SMVs measured by prothrombinase assay in the splenocyte supernatants, by 24% and 14%, respectively, suggesting that omega-3 PUFA significantly limit plasma membrane remodeling *in situ* (figure 4a). The shedding of neutrophils, T lymphocytes, natural killer cells, monocytes/macrophages and granulocytes, was approximately reduced by half, reaching a ~ 60% drastic decrease for natural killers and T lymphocytes (figure 4b and table 1), thereby suggesting that EPA:DHA 6:1 strongly targets cells of the innate immune system. By comparison, the low $CD31^{+}$-SMVs shedding remained statistically unchanged, confirming that, in this model, immune cells are the main contributors to MV release

and prime targets of omega-3 PUFA. No statistical difference was observed with respect to splenocyte survival (figure 4c).

3.4 EPA:DHA 6:1 intake blunts the endothelial pro-senescent impact of splenocyte-derived MVs

Young primary coronary endothelial cells (P1ECs) were incubated for 48 h with 10 nM PhtSer eq. of washed SMVs isolated from either one of the 4 different M-rat subsets (SMV_{CTL}, SMV_{CO}, $SMV_{1:1}$, $SMV_{6:1}$). SMV_{CTL} significantly increased the SA-β-gal activity by 55% in P1ECs ($p<0.05$, SMV_{CTL} vs. P1ECs), reaching values similar to those induced by H_2O_2, a known inducer of premature endothelial senescence (figure 5a). SMV_{CTL} also induced significant up-regulation of senescence markers p53 (1.6 fold) and down-stream p21 and p16 (1.7 and 2 folds, respectively), thereby confirming the pro-senescent potential of SMVs detected by SA-β-gal activity (figures 5b, and above). Intake of EPA:DHA 6:1, but not of corn oil or EPA:DHA 1:1, abolished the pro-senescent effect of SMVs by SA-β-gal activity (17.7 A.U. *vs* 10.8 A.U., $p<0.05$, $SMV_{6:1}$ *vs.* SMV_{CTL}), an observation that was also confirmed by assessment of the expression of p53, p21 and p16 senescence protein markers.

In addition, the fact that splenocyte supernatants depleted of SMV_{CTL} by high-speed centrifugation, did not induce significant SA-β-gal activity, confirmed that SMV_{CTL} were the true inducers of premature senescence in the cell medium. Of note, none of the SMV subsets affected the degree of apoptosis in P1ECs, confirming the specific pro-senescent nature of the signal delivered by SMVs (figures 5a, 5c).

3.5 EPA:DHA 6:1 intake by middle-aged rats prevents the endothelial pro-inflammatory and pro-thrombotic responses induced by SMVs

We previously had reported that endothelial senescence is characterized by a pro-inflammatory and procoagulant phenotype [6]. We therefore examined the eventual protection by omega-3 PUFA intake using the above SMV-mediated endothelial senescence model. Endothelial pro-inflammatory and pro-coagulant protein markers were assessed by western blot after treatment for 6h or 48h with 10 nM washed SMV_{CTL}, SMV_{CO}, $SMV_{1:1}$, or $SMV_{6:1}$. After 48 h incubation, SMV_{CTL} induced the up-regulation of COX-2, but not of COX-1, by 3 folds, ICAM-1 and TF by 1.5 fold, VCAM-1 by 1.6 fold (figure 6). Interestingly, a significant 1.9 fold rise in phosphorylated NF-kB was detectable as early as 6 h after the addition of SMVs, pointing at a pivotal role of the inflammatory signaling in the progression of senescence. Conversely, the expression of pro-inflammatory and procoagulant makers was abolished in the presence of $SMV_{6:1}$ but not corn oil or $SMV_{1:1}$, indicating a specific effect of EPA:DHA 6:1 ($p<0.05$ $SMV_{6:1}$ *vs.* SMV_{CTL}, figure 6).

3.6 EPA:DHA 6:1 intake by middle-aged rats limits oxidative stress in SMVs-treated ECs

Since the presence of ROS was early detected in middle-aged spleen and because oxidative stress contributes to endothelial senescence via reduced NO formation [4], we further examined whether 10 nM SMVs could act as early contributors to oxidative stress in ECs and whether omega 3 intake would be beneficial. After 6h incubation, SMV_{CTL} significantly increased the amount of ROS by 2 folds in young P1ECs (SMV_{CTL}: 2673.65 A.U. *vs.* untreated P1ECs: 1315.37 A.U, $p< 0.01$). Consistent with the whole spleen tissue measurements (figure 3), P1ECs showed significantly lowered amounts of ROS in response to $SMV_{6:1}$, but not to SMV_{CO} or $SMV_{1:1}$, suggesting that omega-3 EPA:DHA 6:1 intake prevents the SMVs-induced

oxidative stress (figure 7). Pharmacological inhibition by Indomethacin, VAS-2870 and the mitochondrial respiration complex inhibitors significantly blunted the SMVs-induced formation of ROS, indicating that COXs, NADPH oxidase and mitochondria contribute to the SMVs-induced oxidative stress. Consistent with the observation, western blot analysis indicated that SMV_{CTL} down–regulated eNOS by 30% in P1ECs after 48 h (SMV_{CTL} *vs*. untreated ECs, $p<0.05$, figure 7). Altogether, intake omega-3 EPA:DHA 6:1, but not of corn oil or omega-3 EPA:DHA 1:1, was a cytoprotective treatment against SMVs-induced endothelial dysfunction.

3.7 EPA:DHA 6:1 intake by middle-aged rats reduces the SMVs-mediated activation of local angiotensin system in ECs

Because the local angiotensin system contributes to the induction of endothelial senescence and is associated with increased endothelial expression of the angiotensin converting enzyme (ACE) and its angiotensin type-1 receptor (AT1R) [4], we investigated the impact of omega-3 intake. The expression of ACE and AT1-R was significantly up-regulated in P1ECs treated with SMV_{CTL} for 48h (SMV_{CTL} *vs.* untreated P1EC, $p<0.05$, figure 8a). Only $SMV_{6:1}$ showed reduced ACE and AT1-R expression by 44% and 45%, respectively, suggesting that EPA:DHA 6:1, but not corn oil or EPA:DHA 1:1, prevent the SMV-mediated activation of the local angiotensin system. Furthermore, the SMV_{CTL}-induced SA-β gal activity was reduced to baseline in the presence of Losartan, an AT1R antagonist, thereby indicating the pivotal role of the angiotensin system in the SMV-induced premature senescence (figure 8b).

Altogether, our data indicate that SMVs from middle-aged rats are pro-senescent endothelial effectors acting via redox-sensitive pathways, and that their noxious effects can be abolished by a 7-day omega-3 EPA:DHA 6:1 intake.

3.8 EPA:DHA 6:1 intake is beneficial to old rats

Because the shedding of SMVs was significantly higher in old rats, we also verified that a 14 days intake of only EPA:DHA 6:1, but not of corn oil, significantly reduced the ability of old splenocytes to shed SMVs. EPA:DHA 6:1 significantly reduced the shedding of SMVs by 39% while a symmetrical 22% splenocyte survival was observed as compared to treated rats ($p<0.05$ $O_{6:1}$ vs. O_{CTL}, figure 9a-b). Conversely, a 14-day corn oil ingestion had an inverse effect with a 25% fold increase in SMV release (figure 9a) and a reduced splenocyte survival (figure 9b). Using the SMV-induced senescence model, we observed a significant 35% reduction of the P1CEs SA-β-gal activity in the presence of $SMV_{6:1}$ (figure 9c).

4. Discussion

The present findings indicate that age is a strong and progressive trigger for the shedding of pro-senescent spleen-derived leukocytes microvesicles (SMVs) that prompt premature senescence in primary coronary endothelial cells, on line with our previous observation [39]. Senescence was characterized by SA-β-gal activity, pro-oxidative, pro-inflammatory and procoagulant and pro-atherogenic responses. Interestingly, the release of pros-senescent MVs was a characteristic feature of both old and middle-aged rat splenocytes, thereby suggesting the benefit of an early pharmacological control. We therefore investigated the possibility that a short-term omega-3 intake prevents the age-related SMV effects in middle-aged rats. The

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intake dose was equivalent to 5.67 g/day/70 kg body weight in humans [40], on line with the 0.18 to 10 g/day ranges given in clinical studies [41-44];

4.1 The ratio of EPA:DHA Omega 3 is critical in the prevention of the SMV-induced premature senescence.

Using an original SMV-endothelial cross-talk model, we compared two different formulations of omega 3 PUFA and clearly demonstrate that only the EPA:DHA 6:1 formulation has strong anti-senescent effects. Omega-3 EPA:DHA 6:1 limited the release of splenocyte SMVs and significantly blunted their pro-senescent properties whereas EPA:DHA 1:1 had no effect. This observation is consistent with a previous ex-vivo study in healthy pig coronary arteries showing that the EPA:DHA 6:1 is an optimal ratio to induce endothelium-dependent vaso-relaxation, predominantly involving eNOS and hence restoring NO availability [24]. Interestingly, in old (see companion article by Farooq A.M. et al.) or hypertensive rats EPA:DHA 6:1. Chronic intake was also associated to an improved endothelial-dependent vaso-relaxation measured in mesenteric arteries while limiting hypertension in the latter model [25]. Similarly, EPA:DHA 6:1 appeared also beneficial in old SMV-induced premature endothelial senescence. Our data also indicate that the oxidative stress was specifically reduced by EPA:DHA 6:1 in spleen tissues from which SMVs were harvested, thereby confirming organ targeting.

4.2 EPA:DHA 6:1 protection against age-related SMV-induced endothelial senescence and inflammaging.

As previously reported, inflammaging, characterized by the impairment of the immune system and chronic low-grade inflammation, contributes to persistent tissue

damages altering cells of both naïve and adaptive systems. Accumulation of senescent cells also impacts tissue repair and amplifies the senescence associated secretory syndrome (SASP) favoring pro-inflammatory and pro-oxidative loops and the secretion of cytokines and mediators acting as paracrine inflammatory effectors [45].

Endothelial-derived and splenocyte-derived MVs also act as cellular mediators of autocrine [5, 6] and paracrine induction of premature endothelial senescence [39]. It is therefore likely that pro-senescent MVs contribute to age-related senescence in clinical settings. While the proportion of pro-senescent MVs remains to be characterized, endothelial and leukocyte-derived MVs circulate at high levels in patients with cardiovascular diseases and chronic inflammatory disorders like type 2 diabetes [18, 46].

In the present study, EPA:DHA 6:1 omega 3 intake modifies the pattern of the cellular origin of spleen leukocyte-derived MVs and their spontaneous release from the innate and adaptive immune cells. Furthermore, in middle-aged rats, the pro-senescent features of SMVs leading to coronary endothelial senescence are abolished by a short-term EPA:DHA 6:1 intake, that completely blunts the endothelial dysfunction and SMV-borne pro-oxidative, pro-coagulant and pro-inflammatory potentials, most likely by reducing oxidative stress, enhancing NO bioavailability and preventing the activation of the local angiotensin system. In addition, the rapid blunting of the inflammatory stress via an early targeting of the NF-$_K$B activation would also contribute to prevent the up-regulation of TF, an early responsive gene with high thrombogenic potential [13].

Since endothelial senescence is promoted by flow disturbance favoring MV-endothelial interactions and early detected at coronary branches, it is tempting to

speculate that EPA:DHA 6:1 also blunts the pro-senescent signaling of LMVs at such endothelial sites prone to athero-thrombosis, thereby limiting the low grade but sustained endothelial inflammation in middle-aged and old rats and deferring the progression of cardiovascular events with age.

4.3 Ageing-related endothelial dysfunction and targets of EPA:DHA 6:1

We could identify NADPH oxidase cyclooxygenases (COXs) and the mitochondrial respiratory chain as the endothelial main sources of ROS accumulation during age-related SMV induced endothelial senescence. In a previous report from the team, EPA:DHA 6:1 was shown a potent inducer of NO formation targeting endothelial Src/PI3-kinase/Akt and MAPkinase pathways and eNOS activation [24]. Nevertheless, the EPA:DHA 6:1 direct targets remain poorly characterized, despite clear anti-inflammatory effects [19]. In the splenocyte membrane, non-exclusive mechanisms would be (i) the modification of the cavaeole lipid microenvironment [47] and/or membrane composition possibly leading to the accelerated endocytosis of TF, ACE or AT1, (ii) the modification of the lateral membrane proteo-lipidic domains (rafts) involved in pro-inflammatory signaling favoring cytokine release [48], thereby blunting the down-stream up-regulation of TF, ICAM, VCAM, or (iii) the sorting of noxious active proteins exported in the MV membrane. To the best of our knowledge, the impact of EPA:DHA on lipid transporters across the membrane bilayer remains unknown.

Of note, the EPA:DHA 6:1 intake was not associated with any sign of bleeding nor had any effect on SMV-induced endothelial senescence in healthy young rats, confirming the absence of safety issues after short-term intake and indicating that

ageing cells are specific targets of treatment. An eventual explanation is the reported enhanced degree of EPA incorporation in cells from old individuals [41].

4.4 Relevance to cardiovascular diseases

Angiotensin II is an inducer of premature endothelial senescence [49]. Although we demonstrated that EPA:DHA 6:1 protection against SMV-induced senescence was blunted after ECs pre-treatment with Losartan, an inhibitor of the AT1 receptor, the beneficial effect of EPA:DHA 6:1 and the ECs cytoprotection against SMV-induced premature senescence remains to be established in young and older hypertensive rats as already reported for endothelial-dependent vascular dysfunction. Indeed, a spleen-cardiac axis was recently proposed, pointing at the role of immune cells and chronic low-grade inflammatory state in cardiovascular diseases. In addition, in aged patients with chronic heart failure, splenomegaly and a higher rate of peripheral monocytes were indicative of non-responders to resynchronization therapy and higher prevalence of new hospitalization due to disease progression [50]. Furthermore, in a mouse model of prolonged cardiac ischemic insult, a cardio HMGB1-splenic RAGE signaling axis was evidenced during the infarct reperfusion, showing that cardiac tissues release mediators contributing to infarct exacerbation through the activation of splenic neutrophil in a FPR-1-dependent manner and their migration to the heart [51].

4.5 Conclusion

Ageing is associated with increased leukocyte ability to shed MVs that are strong promoters of age-associated endothelial dysfunction due to their pro-senescent, pro-inflammatory and pro-atherothrombotic properties. Short-term ingestion of the

omega-3 optimized formulation EPA:DHA 6:1 not only reduces such shedding but also protects the endothelium against their prosenescent potential, most probably by reducing oxidative stress, preserving eNOS and preventing the activation of the local angiotensin system. Moreover, spleen appears to be a convenient and rich source of leukocyte-MVs reflecting the immune competence of the individual and enabling the study of pharmacologigal monitoring. The data further suggest that omega-3 EPA:DHA 6:1 may help to prevent or delay the age-related endothelial dysfunction mediated by prosenecent leukocyte-derived MVs.

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Figures legends

Figure 1: Ageing enhances the release of pro-senescent MVs from splenocytes. *MVs were measured by prothrombinase assay in the supernatant of freshly isolated primary splenocytes after 24 h culture (n=5), simultaneously to splenocyte survival by trypan blue exclusion (n=4). MV pro-senescent ability was assessed by SA-β-galactosidase activity (SA-β-GAL) measured in young primary coronary endothelial cells at passage 1 (P1ECs) after incubation for 48 with 10 nM SMVs from young (SMVy), middle-aged (SMV_M) and old (SMV_O) rats h (n=4). SA-β-GAL was quantified by flow cytometry using the C12 FDG fluorogenic substrate of SA-β-galactosidase.*

** : p < 0.05, ** : p < 0.01*

Figure 2: ROS accumulation in the ageing spleen.

ROS were measured in snap-frozen samples by fluorescence microscopy using the DHE probe. Each analysis is the mean of measurements performed in 4 individuals (4 sections/individual). Upper panels: illustration of one separate fluorescence analysis, lower panels: cumulative data of n experiments performed likewise.

** : p < 0.05*

Figure 3: Omega-3 EPA:DHA 6:1 intake prevents the spleen ROS accumulation in middle-aged rats. *Rats were given either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control) for 7 days, (*500 mg/kg/day oral dose). ***3a-b:*** *Spleen (n=7) and body weight (n=9).* ***3c:*** *Spleen ROS were measured after DHE staining by fluorescence microscopy in snap-frozen tissue samples of 4 individuals (4*

sections/individual). ***3d:*** *assessment of ROS sources after incubation of each section with pharmacological inhibitors for 30 min before the addition of DHE probe. IND: indomethacin, NAC: N-acetyl cysteine, VAS: VAS2870, NADPH oxidase inhibitor, MIT: Mitochondrial respiratory chain inhibitors, Neg DHE: auto-fluorescence level, control: absence of pharmacological inhibitor. Upper panels: illustration of one separate fluorescence analysis, lower panels: cumulative data of n experiments performed likewise. * : p < 0.05*

Figure 4: Omega EPA:DHA 6:1 intake reduces SMVs shedding and modifies the pattern of their origin. 4a: *SMVs were measured by prothrombinase assay in the supernatant of freshly isolated splenocytes from middle-aged rats receiving a 7 day-treatment by either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) as an isocaloric control or tap water (control)(n=6).* ***4b:*** *the SMV cell origin was determined after capture onto specific antibodies and concentration further assessed by prothrombinase assay (n=3).* ***4c:*** *after 24 h culture, survival of splenocytes freshly isolated from treated and untreated rats was assessed by Trypan blue exclusion and expressed as a percentage (n=4). * : p < 0.05, ** : p < 0.01*

Figure 5: Omega EPA:DHA 6:1 14-day intake protects the primary coronary endothelial cells against pro-senescent SMVs from middle-aged rats. *Ten nM SMVs isolated from middle-aged rats treated for 7 days by either EPA:DHA 6:1 ($SMV_{6:1}$), EPA:DHA 1:1 ($SMV_{1:1}$), Corn oil (SMV_{CO}) or tap water (SMV_{CTL}) were incubated for 48 h with primary young endothelial cells (P1ECs).* ***5a:*** *SA-β-GAL activity was measured in ECs by flow cytometry using the C12FDG fluorogenic substrate and compared to that of untreated P1ECs or H_2O_2-treated ECs.*

Supernatants from splenocyte freshly isolated from untreated rat and depleted of SMVs (SN-SMVs) by high speed centrifugation, were also incubated with P1ECs (n=4). ***5b:*** *Western blot analysis of senescent markers p53 (n=5) and downstream p21 (n=3), p16 (n=7) in SMV-treated P1ECs after 48 h.* ***5c:*** *measurement of apoptosis in SMV-treated ECs by cytometry using annexin-5 and propidium iodide double staining, early apoptotic cells being defined as Annexin-5 stained and PI unlabeled. ECs were treated with either 10 nM or 30 nM SMVs for 48 h, or H_20_2 for 1 h as an apoptotic control inducer (n=4). Upper panels: illustration of one separate western blot, lower panels: cumulative data of n experiments performed likewise. * : p < 0.05, ** : p < 0.01*

Figure 6: Omega EPA:DHA 6:1 intake prevents pro-inflammatory, pro-thrombotic responses of young primary ECs in response to SMVs from middle-aged rats. *ECs were incubated with 10 nM SMVs from middle-aged rats submitted to 7-day intake of either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil or tap water ($SMV_{6:1}$,$SMV_{1:1}$,SMV_{CO}, SMV_{CTL}). Protein expression was assessed by western blot after 48 h incubation except for the early phosphorylated NF-κB detection that was performed after 6 h incubation (n=4). Upper panels: illustration of one separate western blot, lower panels: cumulative data of n experiments performed likewise.*

** : p < 0.05, ** : p < 0.01*

Figure 7: Omega EPA:DHA 6:1 intake by middle-aged rats prevents young ECs from SMV-induced oxidative stress and endothelial dysfunction. *ROS detection using the fluorescent DHE probe was performed in P1ECs after 6 h incubation with*

10 nM SMVs (n=4) and ROS sources were identified after incubation of each cell layer with pharmacological inhibitors for 30 min before the addition of the DHE probe.

*IND: indomethacin, VAS: VAS2870, NADPH oxidase inhibitor, MIT: Mitochondrial respiratory chain inhibitors. eNOS expression was assessed by western blot after 48 h incubation with SMVs (n=3). * : p < 0.05, ** : p < 0.01*

Figure 8: The AT1 receptor mediates SMV-induced endothelial senescence and omega EPA:DHA 6:1 intake by middle-aged rats prevents the SMV-induced up-regulation of ACE and AT1-R. *SMVs were isolated from middle-aged rats that were treated by either EPA:DHA 6:1 ($SMV_{6:1}$), EPA:DHA 1:1 ($SMV_{1:1}$), Corn oil (SMV_{CO}) or tap water (SMV_{CTL}) for 7 days.* ***8a:*** *P1ECs were incubated with 10 nM SMVs for 48 h and up-regulation of ACE (n=3) and AT1-R (n=5) was measured by western blot.* ***8b****: Prior to incubation of P1ECs with SMVs for 48 h, P1ECs were treated for 30 min with Losartan, a AT1-R inhibitor, and senescence was assessed by SA-β-GAL activity measured by flow cytometry using the C12FDG fluorogenic substrate (n=4). Upper panels: illustration of one separate western blot, lower panels: cumulative data of n experiments performed likewise. * : p < 0.05, ** : p < 0.01*

Figure 9: Omega EPA:DHA 6:1 intake by old rats for 14 days counteracts the shedding of pro-senescent SMVs. *Old rats were given either omega EPA:DHA 6:1 ($O_{6:1}$), Corn oil (O_{CO}) or tap water (O_{CTL}) for 2 weeks before assessment of the SMVs shedding (n=5)* ***(9a)****, splenocyte survival (n=5)* ***(9b)*** *and SMV-induced endothelial senescence assessed by SA-β-GAL activity using the CD12FDG fluorogenic substrate (n=3)* ***(9c)****. * : p < 0.05, ** : p < 0.01*

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Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1

CRediT author statement

Qureshi A. W. : Investigation, Data curation, writing-original draft

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El Habhab A. : Methodology, Data curation

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Farooq M.A. : Validation, Methodology

Zobairi F. : Methodology, Data curation

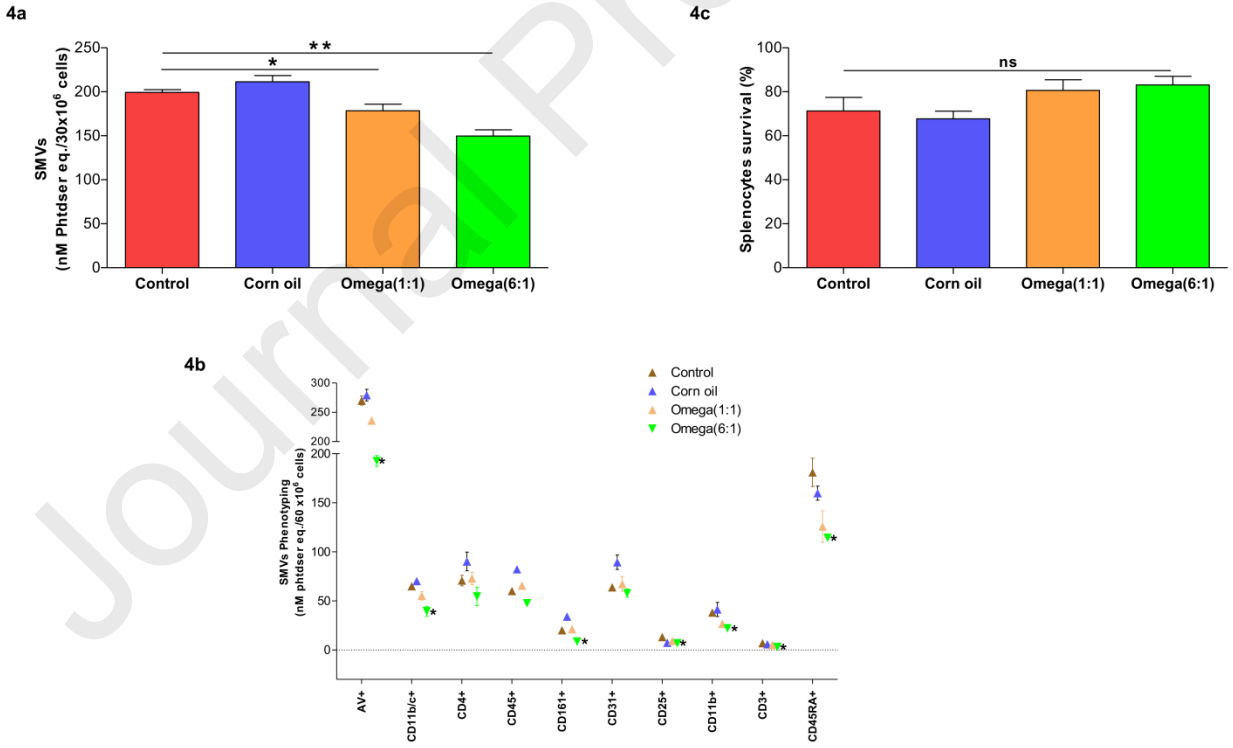
Hasan H : Validation

Amoura L. : Validation, Formal analysis

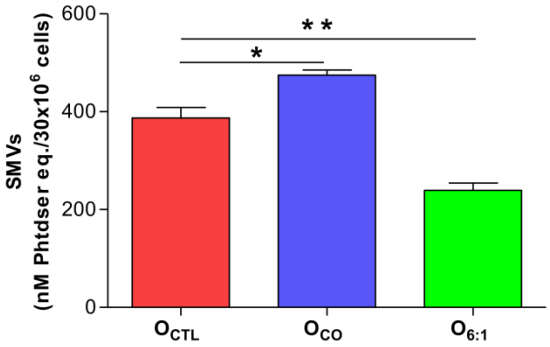
Auger C. : Project administration, Supervision, Review and Editing

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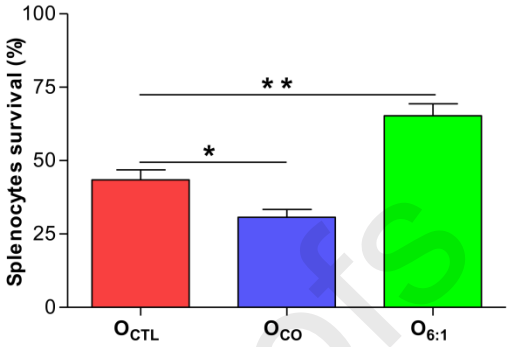
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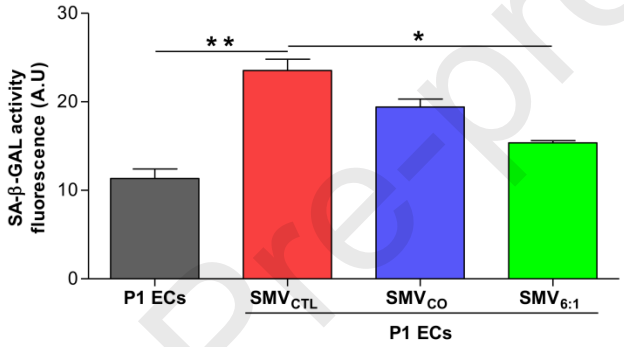
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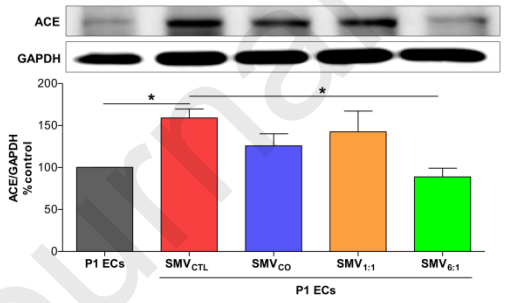
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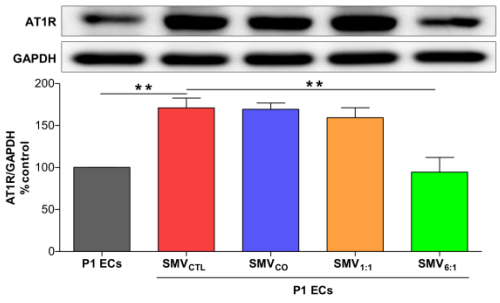


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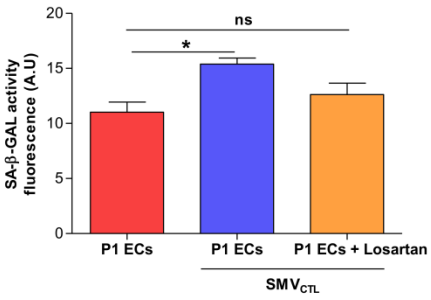


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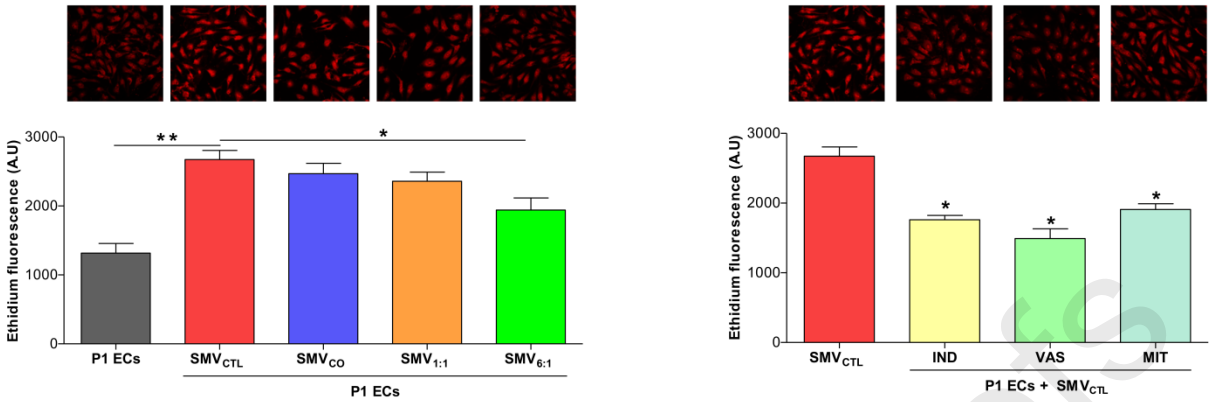


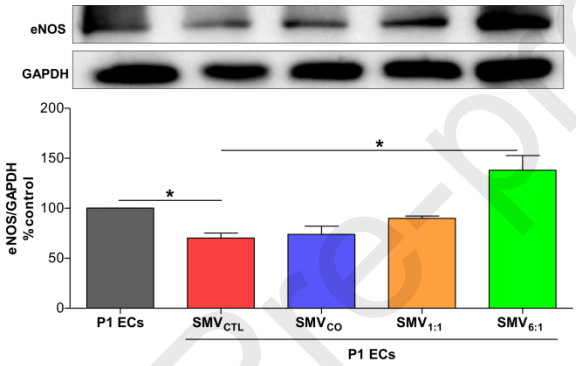


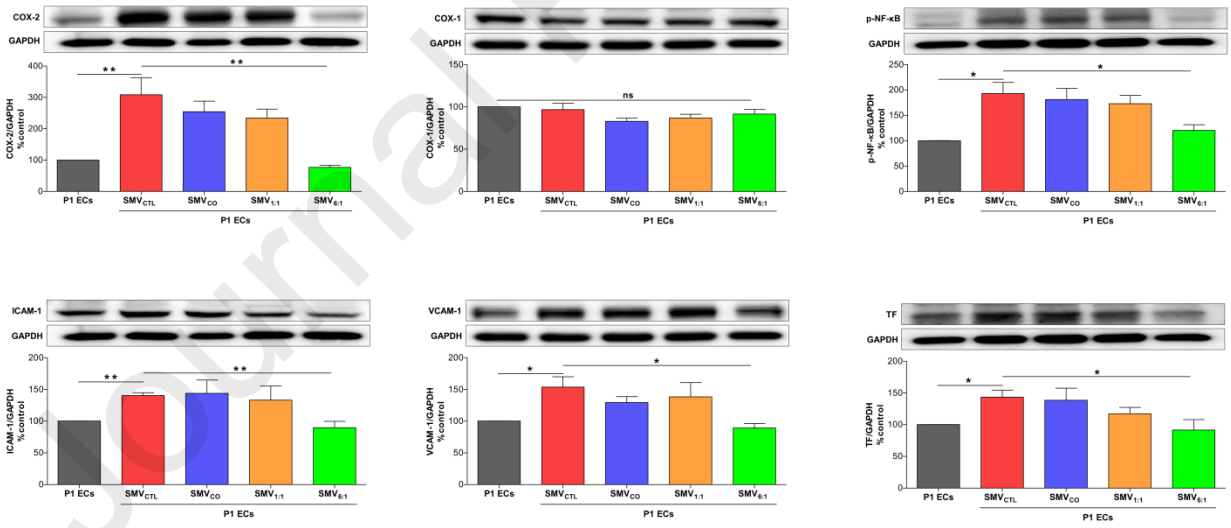
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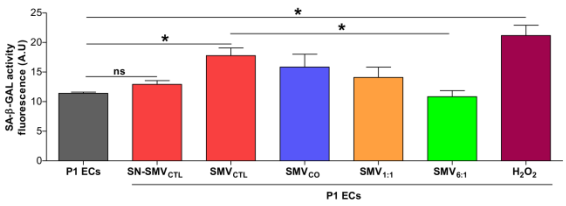




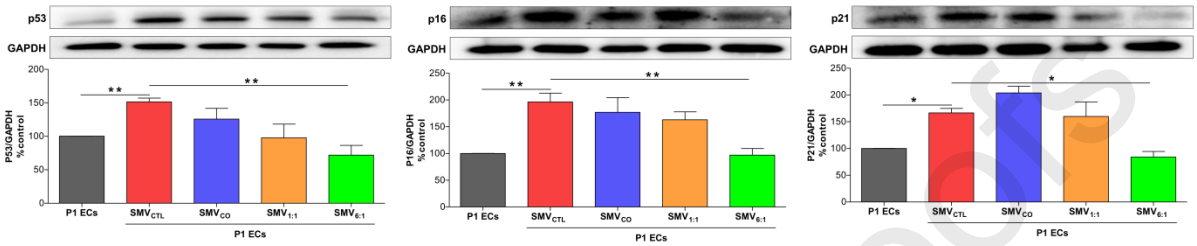


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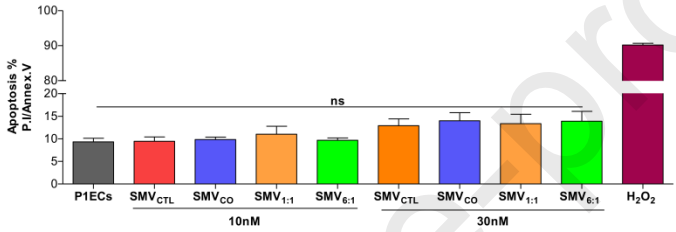
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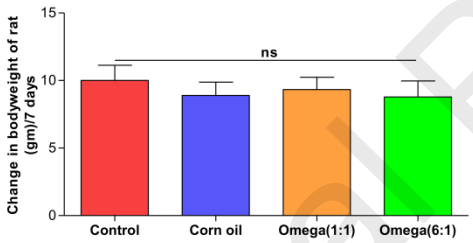
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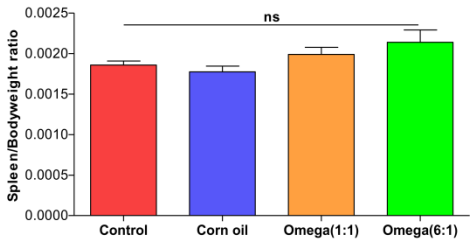
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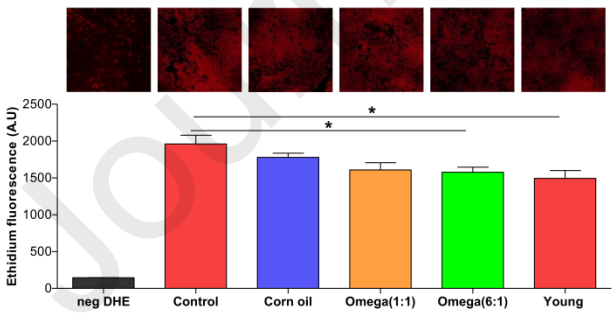
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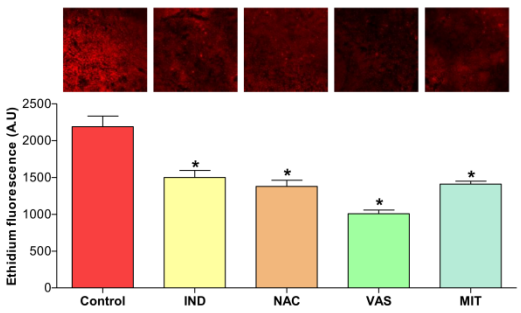
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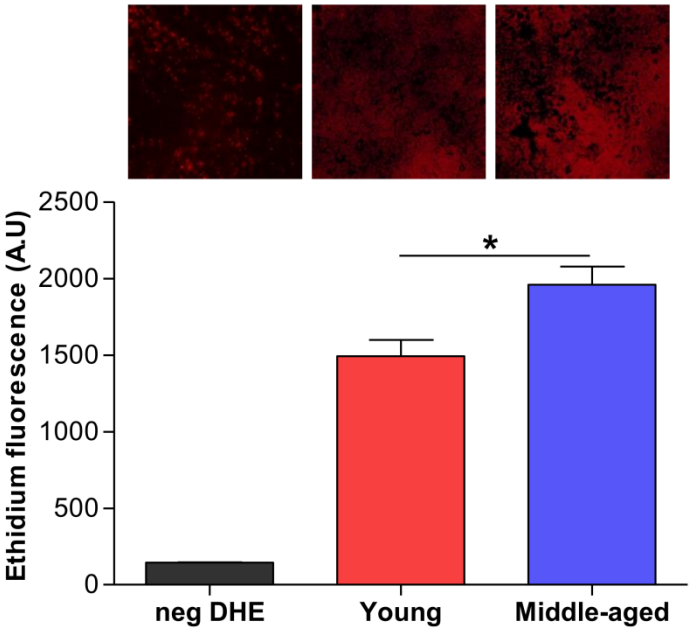
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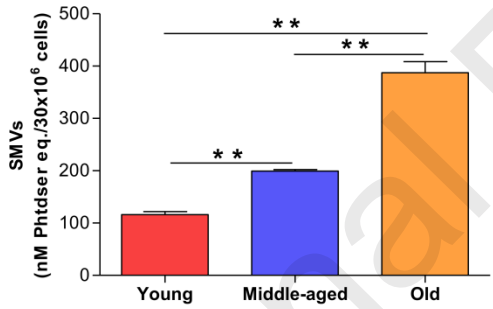
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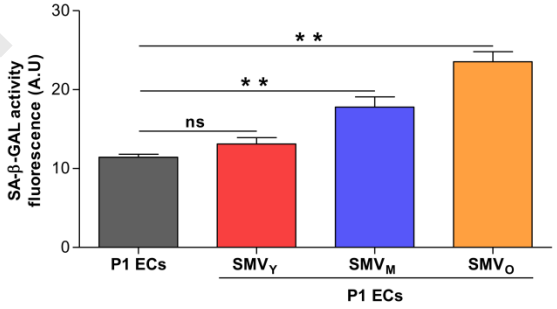
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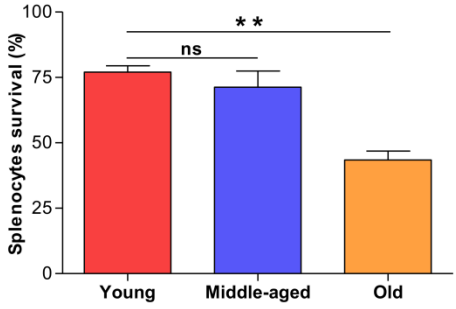
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