

Phenotyping of circulating extracellular vesicles (EVs) in obesity identifies large EVs as functional conveyors of Macrophage Migration Inhibitory Factor



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ABSTRACT

Objective: Obesity-associated metabolic dysfunctions are linked to dysregulated production of adipokines. Accumulating evidence suggests a role for fat-derived extracellular vesicles (EVs) in obesity-metabolic disturbances. Since EVs convey numerous proteins we aimed to evaluate their contribution in adipokine secretion.

Methods: Plasma collected from metabolic syndrome patients were used to isolate EV subtypes, namely microvesicles (MVs) and exosomes (EXOs). Numerous soluble factor concentrations were measured successively on total, MV- and EXO-depleted plasma by multiplexed immunoassays.

Results: Circulating MVs and EXOs were significantly increased with BMI, supporting a role of EVs as metabolic relays in obesity. Obesity was associated with dysregulated soluble factor production. Sequential depletion of plasma MVs and EXOs did not modify plasma levels of these molecules, with the exception of Macrophage Migration Inhibitory Factor (MIF). Half of plasma MIF circulated within MVs, and this MV secretory pathway was conserved over different MIF-producing cells. Although MV-associated MIF triggered rapid ERK1/2 activation in macrophages, these functional MV-MIF effects specifically relied on MIF tautomerase activity.

Conclusion: Our results emphasize the importance of reconsidering MIF-metabolic actions with regard to its MV-associated form and opening new EV-based strategies for therapeutic MIF approaches.

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Keywords Obesity; Extracellular vesicles; Microvesicles; Exosomes; Adipokines; MIF

1. INTRODUCTION

White adipose tissue (WAT) is recognized as an endocrine organ that secretes many factors, which orchestrate metabolic responses at the organismal level. Obesity is associated with a dysregulated production of these “adipokines”, known to actively participate to the development of insulin-resistance (IR) as well as chronic inflammation [1]. Recent evidence has highlighted the potential role of extracellular vesicles (EVs) in the development of metabolic diseases [2]. EVs are membrane-derived vesicles released in the extracellular environment and recovered in many body fluids. EV constitute conveyors of biological information and transfer their bioactive cargo in target cells and, thereby, contribute to numerous diseases [3].

Different EV subtypes exist based on their cellular compartment origin and size [4]. Multivesicular-body derived EVs (classically called exosomes, EXOs) are small EVs (30–100 nm of diameter) whereas plasma membrane-shed microvesicles (MVs) designate larger EVs between \approx 100 nm up to 500 nm. Distinguishing EV subpopulations is important since intracellular mechanisms leading to MV and EXO production are distinct, and each EV subtype presents a specific protein signature [5,6], suggesting different effects on target cells.

EXOs derived from obese WAT regulate inflammation pathways as well as insulin-sensitivity [7–9]. Others and we have demonstrated the ability of adipocytes to secrete MVs and EXOs, which convey adipokines that might contribute to EV metabolic responses [5,8].

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Abbreviation: EVs, extracellular vesicles; MVs, microvesicles; EXOs, exosomes; MIF, Macrophage Migration Inhibitory Factor

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The objective of this study was to evaluate EV contribution in the secretion of factors known to participate to the development of obesity-associated metabolic dysfunctions. Through the analysis of plasma samples from metabolic syndrome (MS) patients successively depleted from MVs and EXOs, we identified MVs as specific conveyors of functional Macrophage Migration Inhibitory Factor (MIF) cytokine. MV-MIF effects specifically rely on MIF tautomerase activity, underlying the need to reconsider MIF-activated pathways with regard to its MV-associated form.

2. MATERIALS AND METHODS

2.1. Human studies

Institutional ethics committee approved the study and consent was obtained from each patient. Patients were included from the NUMEVOX cohort, referenced at Clinicaltrials.gov (N°: NCT00997165). The investigation conforms to the principles outlined in the Declaration of Helsinki. Patients were eligible for inclusion if they met criteria for the National Cholesterol Education Program-Adult Treatment Panel III; they had at least three of the following five criteria: (i) waist circumference >102 or 88 cm for men and women, respectively; (ii) systolic and diastolic pressures $\geq 130/85$ mmHg; (iii) fasting glycemia ≥ 1.1 g/l; (iv) triglycerides ≥ 1.5 g/l; and (v) high-density cholesterol lipoprotein <0.4 g/l in men or <0.5 g/l in women (Table A.1). Patients with a history of cardiovascular disease (CVD), preexistent chronic inflammatory disease, and cancer were excluded.

Thirty four patients with MS (18 females/16 males) were included from the NUMEVOX Cohort at the Department of Endocrinology and Nutrition of Angers University Hospital. Normal controls consisted of 22 subjects (13 females/9 males) who met less than two of the MS criteria (70% without any component of MS). Three groups were defined, based on patient BMI (in $\text{kg}\cdot\text{m}^{-2}$): Control (BMI <27), overweight (27 < BMI <30), and obese (BMI >30).

2.2. Animals

Four-week-old lean (ob/+) or obese (ob/ob) mice (Jackson Laboratories; stock 000632) were used for plasma collection following intracardiac puncture. Animal care and study protocols were approved by the French Ministry of Education and Research and the ethics committee N°6 in animal experimentation and were in accordance with the EU Directive 2010/63/EU for animal experiments.

2.3. Circulating EV isolation from blood samples

Peripheral blood (≈ 20 ml) was collected from non-MS or MS subjects on EDTA-coated tubes, following an overnight fasting (Vacutainers; Becton Dickinson) using a 21-gauge needle to minimize platelet activation and was processed within 2 h. After a 15-min centrifugation at $260 \times g$, platelet-rich plasma (PRP) was separated from whole blood, and recentrifuged for 15 min at $1500 \times g$ to obtain platelet-free plasma (PFP). Two hundred microliters of PFP were frozen and stored at -80 °C until subsequent use for MV characterization by flow cytometry. Remaining PFP was subjected to two series of centrifugations, each at $21,000 \times g$ for 45 min to pellet MVs. The PFP depleted of MVs was further ultracentrifuged (rotor MLA-50, Beckman Coulter Optima MAX-XP Ultracentrifuge) two times at $100,000 \times g$ for 1 h to isolate EXOs. At each step, a fraction of PFP, PFP without MVs and PFP depleted of total EVs was kept and stored at -80 °C for multiplexed analysis of soluble factors. Efficient MV removal from PFP samples was checked by flow cytometry. Both MV and EXO pellets were resuspended in NaCl and stored at 4 °C (for MVs) and -20 °C (for EXOs)

before further analysis. Similar protocol was used to isolate EVs from ob/+ or ob/ob mouse blood samples.

2.4. Flow cytometry assays

MV phenotyping was based on the detection of membrane-specific antigens representative of their cellular origins on EVs. Irrelevant human immunoglobulin G (IgG) was always used as an isotype-matched negative control for each sample, and subtracted from the value obtained. For numeration studies, 8 μl of PFP were incubated with 10 μl of FITC-conjugated specific antibody or with 5 μl for others fluorophore-conjugated antibodies (Beckman Coulter). Annexin V-binding was used to numerate phosphatidylserine (PS)-expressing circulating MVs (3 μl of Annexin V/5 μl PFP) using Annexin V-FITC kit according to manufacturer's instructions (Miltenyi Biotec). After 30 min at RT, samples were diluted in 300 μl of sterile 0.9% NaCl or Annexin-V labeling buffer, respectively. Then, an equal volume of sample and Flow-Count fluorospheres (Beckman Coulter) was added and samples were analyzed in a flow cytometer 500 MPL system (Beckman Coulter). Data obtained are expressed either as percent of total MV analyzed, or as the number of MVs detected per μl of plasma.

2.5. Nanoparticle tracking analysis (NTA)

EXO concentrations and size were determined by NTA analysis, as previously described [5]. EV preparations were diluted in sterile NaCl 0.9% before NTA analysis (NanoSight NS300, Malvern Instruments).

2.6. Protein arrays

Human adipokine array kit (#ARY024, R&D systems) were incubated with 150 μg of each EV subtype (MV or EXOs), and processed according to manufacturer's protocol (R&D systems). Protein signals were revealed using ChemiSmart 500 imager (Vilber Lourmat). Signal intensity of spots (measured as pixels number) was quantified using Image J Software and expressed as pixels/ μg of protein loaded on membrane. Only detectable protein signals are presented.

2.7. Multiplex immunoassays

Plasma concentrations for multiple secreted proteins were performed on plasma samples (PFP, PFP w/o MVs and PFP w/o EVs) through the combination of multiplex-assays according to manufacturer's protocol (R&D systems and BioRad), and analyzed on the Bioplex system. Percents of total MIF protein secreted either associated to EVs or under soluble forms were calculated based on MIF concentrations measured in the different plasma samples fractions, as follow:

$$\% \text{ of MV-associated MIF: } \frac{([\text{MIF}]_{\text{in PFP}}) - ([\text{MIF}]_{\text{in PFP w/o MV}})}{([\text{MIF}]_{\text{conc in PFP}})} * 100$$

% of EXO-

$$\text{associated MIF: } \frac{([\text{MIF}]_{\text{in PFP w/o MV}}) - ([\text{MIF}]_{\text{in PFP w/o EV}})}{([\text{MIF}]_{\text{in PFP}})} * 100$$

$$\% \text{ of soluble MIF: } 100 - (\% \text{ of MV-associated MIF} \\ + \% \text{ of EXO-associated MIF})$$

2.8. Protein lysates, SDS-PAGE and immunoblotting

Cell lysates or EV lysates were prepared and migrated on SDS-PAGE for western blot as previously described [5]. Ten μg of protein

lysates were diluted in Laemmli Buffer 4X in reducing conditions, and heated at 95 °C for 10 min before SDS-PAGE. Appropriate HRP-conjugated or IRDye® (LI-COR Biosciences) secondary antibodies were used respectively for protein detection following enhanced chemiluminescence (Immunocruz, Santa Cruz) using a Chemi-Smart imager (Vilber Lourmat) or digital fluorescence visualized by Odyssey CLX system (LICOR).

2.9. Cell culture

Wild-type (WT) and MIF knockout (MIF^{-/-}) Mouse embryonic Fibroblasts (MEFs) (kindly provided by R. Bucala, Yale University, USA), RAW 264.7 macrophages and 3T3-L1 adipocytes (kind gift from I Dugail, INSERM UMRS 1166, Paris, France) were cultured in DMEM 4.5 g/l glucose supplemented with 1% P/S and 10% FBS. MEFs were moreover supplemented with 25 µg/ml Geneticin. 3T3-L1 were differentiated as previously described [5]. T-CEM lymphocytes were cultured in X-VIVO-15 medium. 48 h-serum-free cell culture supernatants, or following 24 h stimulation with Actinomycin-D (1 µg/ml) for T-CEM lymphocytes were collected in order to concentrate EV subtypes according to our previous validated procedure [5].

2.10. PKH26-EV internalization

MVs isolated from WT and MIF^{-/-} MEF supernatants were labeled with PKH26 dye using the red fluorescent cell linker kit (Sigma Aldrich) according to manufacturer's protocol. Briefly, 100 µg MVs diluted in PBS were added to 0.5 ml Diluent C. In parallel, 4 µl PKH26 dye was added to 0.5 ml Diluent C and incubated with the MV solution for 4 min at RT. To bind excess dye, 2 ml 0.5% bovine serum albumin/PBS was added. The labeled MVs were washed at 21,000×g for 30 min, and the MV pellet was suspended with PBS and used for uptake experiments.

Twenty µg of PKH26-labeled MVs (20 µg/ml) were incubated for 0, 0.5, 1, 2, and 4 h on MIF^{-/-} MEFs cultured on glass coverslips. MV internalization was imaged using a LSM 710 confocal (Zeiss). Internalization kinetics graph represents the mean fluorescent intensity (MFI) measured by Image J software on 30 different cells per MV condition (10 cells taken for each condition from three different images).

2.11. Proteinase K protection assay

One hundred µg of plasma MVs were either incubated with 20 µg/ml Proteinase K (Sigma Aldrich) in the presence or not of 1% Triton X-100 or left untreated, in a final volume of 1 ml for 1 h at 37 °C. PMSF (5 mM) was added 5 min at 37 °C and proteinase K was inactivated by placing the mix assay at 90 °C for 10 min. MVs were re-pelleted at 13000×g for 30 min and resuspended in 100 µl of Laemmli Buffer. Twenty µl of MVs were loaded on SDS-PAGE gel for western blot.

2.12. Activation of ERK1/2 in RAW cells

RAW cells were plated in 12-well plate at 30,000 cells per well. After 24 h, medium was changed and cells were incubated with 20 µg/ml of MEF MVs or 200 ng/ml recombinant MIF protein (R&D systems) in the presence or not of 50 µM MIF antagonist Iso-1 for 2 h. MEF-MV effects (20 µg/ml) on ERK1/2 phosphorylation was also investigated in the presence of 80 µg/ml neutralizing mouse anti-MIF IgG1 mAb (mAB III.D.9) and their IgG control isotypes (kindly provided by R. Bucala, Yale University, USA).

2.13. Quantification and statistical analysis

Significant differences were assessed using GraphPad Prism software following statistical analysis by an ANOVA test corrected for multiple

comparisons by Tukey's test are indicated by *p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001. For linear correlations, Pearson coefficient (R) is indicated, and association is considered significant when p < 0.05.

3. RESULTS

3.1. Plasma EV subtypes are significantly increased in obesity

Plasma samples were collected from MS patients, who were stratified according to their BMI in two groups, overweight (27 < BMI < 30) and obese (BMI > 30), and compared to healthy patients (BMI < 27) (control). Clinical parameters revealed metabolic alterations in both overweight and obese groups (Table A.1). EVs were isolated from platelet-free plasma (PFP) samples by differential centrifugations allowing the separation of MVs (21 000×g) and subsequently EXOs (100 000×g) for each patient (Figure 1A). Plasma MV levels were significantly increased in obese patients compared to control patients, as well as in overweight group despite high variability (Figure 1B). MV distribution in healthy patients, according to their cellular origin, revealed the well-known predominance of platelet-derived MVs (CD41⁺) by comparison to MVs derived from endothelial cells (CD146⁺), erythrocytes (CD235⁺), leukocytes (CD45⁺) and monocytes (CD11b⁺) (Figure 1D). Nearly one-fourth of circulating MVs presented a pro-coagulant potential, as illustrated by their annexin-V positivity labeling (Figure 1D). Platelet- and endothelium-derived MVs were significantly increased in obese patients with a higher trend for MV pro-coagulant activity (annexin-V⁺) (Figure 1E–J). Plasma EXO concentrations, analyzed by NTA, were significantly increased both in overweight and obese patients compared to those of healthy patients, again with high dispersion of EXO concentrations in overweight patients (Figure 1C). No changes in EXO size were observed (Figure 1K). Positive associations were found between EV concentrations with patient BMI, HOMA-IR and other metabolic parameters suggesting that EVs might participate to obesity-associated metabolic complications (Table A.2). Similarly to humans, circulating EV subtypes analysis in obese rodents also demonstrated a significant increase of both MVs and EXOs in comparison to lean animals (Figure 1L) whereas respective MV and EXO size were not impacted by obesity (Figure 1M).

3.2. Plasma EV subtypes carry adipokines

In order to better define WAT-derived adipokines retrieved in circulating EVs, we used commercial protein arrays gathering soluble proteins known to participate to metabolic responses and also secreted by adipocytes. Among the 58 proteins tested, 29 were detected in MVs and EXOs isolated from plasma (Figure 2). It revealed the presence of a selective set of inflammatory proteins, proteases or protease inhibitors, matrix proteins, complement factors or others soluble factors in plasma EVs. We confirmed the presence of adiponectin both on circulating MVs and EXOs, as previously reported [5,8], whereas leptin or resistin were undetectable in analyzed EV preparations (Figure 2).

3.3. MVs constitute a major secretory pathway for MIF

In order to evaluate EV contribution in the secretion of adipokines, we proceeded to multiplex analysis of 22 secreted factors concentrations in platelet-free plasma (PFP) before and after sequential depletion of MVs and EXOs (see Figure 1A). Analytical measurement was validated on total plasma by confirming increased circulating levels of chemerin, FABP4/aP2, IL-6, leptin, MCP-1, MIF, PAI-1, TIMP-1, and visfatin in obese patients whereas adiponectin concentrations, as expected, negatively correlated with BMI (Table A.3). We also confirmed a positive correlation for some of these factors with the HOMA-IR index, in agreement with their role in the development of IR.

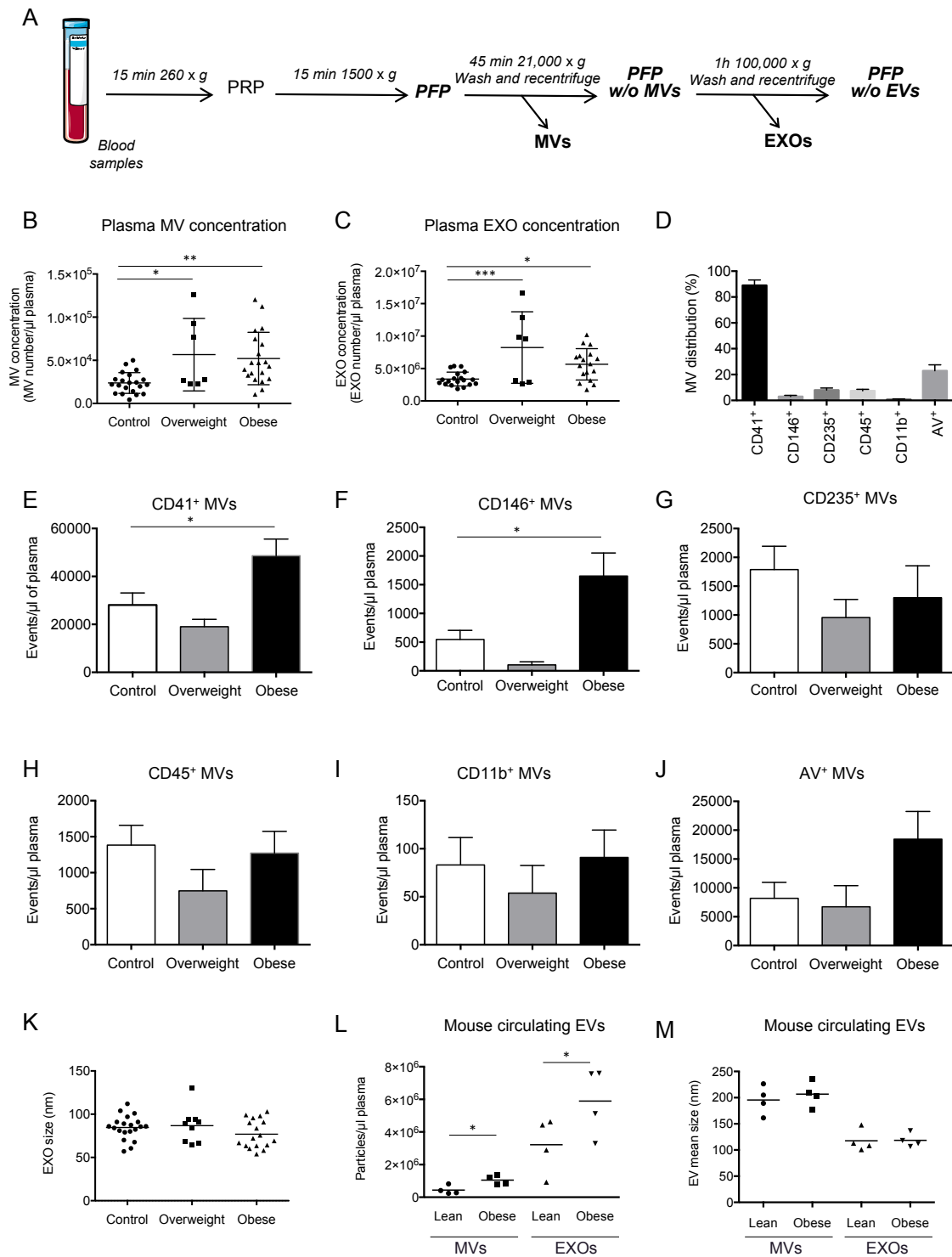


Figure 1: Plasma MV and EXO concentrations are significantly increased in obesity. **A.** Schematic protocol used to isolate EV subtypes, including MVs and EXOs, from plasma samples. Aliquots of platelet-free plasma (PFP), and successively depleted of MVs (PFP w/o MVs) then EXOs (PFP w/o EVs) were conserved for multiplex analysis. **B–C.** Plasma MV and EXO concentrations in control, overweight, and obese patients. Total circulating MVs (**B**) were quantified on PFP by flow cytometry and presented as MV number per μ l of plasma sample. EXO concentration (**C**) was estimated by NTA analysis on isolated EXO fraction. Control ($n = 21$), overweight ($n = 7$) and obese patients ($n = 20$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. **D.** Circulating MV distribution in plasma from control patients. Percent (%) of circulating MVs derived from platelets (CD41⁺), endothelial cells (CD146⁺), erythrocytes (CD235a⁺), leukocytes (CD45⁺), monocytes (CD11b⁺), or with procoagulant activity (annexin V⁺) estimated by flow cytometry. **E–J.** Circulating MV concentration levels derived from platelets (**B**), endothelial cells (**C**), erythrocytes (**D**), leukocytes (**E**), monocytes (**F**), or with procoagulant activity (**G**) were measured in control ($n = 20$), overweight ($n = 12$) and obese patients ($n = 21$). MV concentrations were expressed as events detected by flow cytometry per μ l of plasma. **K.** Plasma EXO mean size (nm) was measured by NTA on EXO isolated from control ($n = 20$), overweight ($n = 9$) and obese ($n = 17$) patients. **L–M.** Circulating plasma MV and EXO concentrations (**L**) and size (**M**) measured using NTA analysis in lean and obese mice ($n = 4$ per group). Significant differences ($p < 0.05$) between groups using a non-parametric ANOVA test corrected for multiple comparisons by Tukey's test are indicated by *.

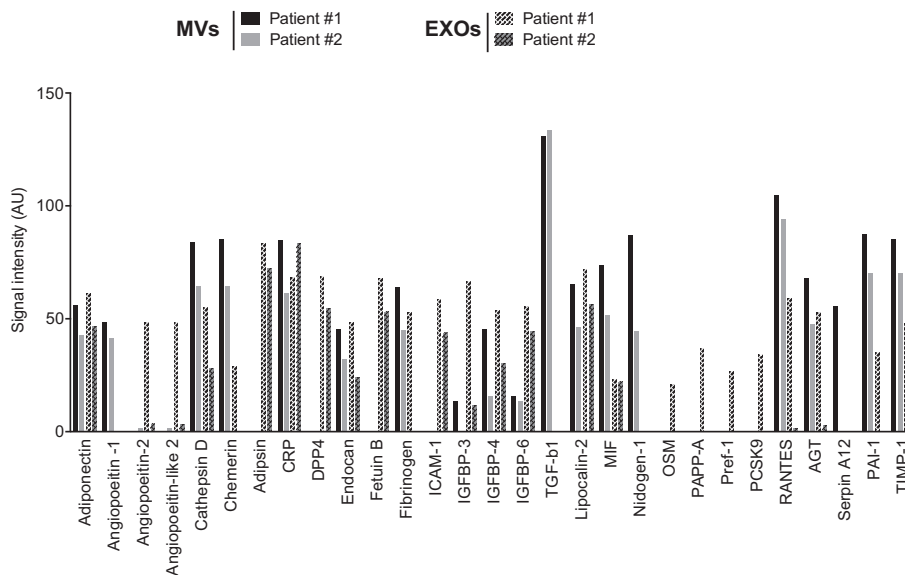


Figure 2: Proteome profiling of EV subtypes reveals the presence of numerous soluble factors. Protein arrays incubated with human plasma MVs or EXOs reveal that EVs convey many soluble factors. Results presented are mean of signal intensity (expressed as mean pixels per μg of protein) from two independent MV or EXO preparations prepared from two different obese patients, respectively.

The respective concentration of each soluble factor measured in PFP was compared with the one measured in PFP depleted from MVs (PFP w/o MVs) and in PFP depleted from all EVs (PFP w/o EVs) in the three patient groups (Table A.4). Depletion of MVs and/or EXOs from all plasma patients did not modify the overall circulating levels of these factors, at the exception of MIF (Table A.4 and Figure 3A). Of importance, more than half of plasma MIF circulated in association with EVs, with a preferential association to MVs (50.6% of total circulating MIF) (Figure 3B, control group). MIF secreted by adipocytes and by WAT-infiltrated immune cells may contribute to raising circulating MIF in obese patients (Table A.3). However, EV-associated proportions of MIF were unchanged in overweight or obese patients demonstrating that MIF constitutively used EV secretory pathway (Figure 3B). We confirmed specific MIF association with circulating human MVs isolated from patients by western blot (Figure 3C) whereas this cytokine was undetectable in human EXO fractions. β -actin and CD9 were used as MV and EXO protein markers, respectively, and MFG-E8 as a general EV marker, as previously described [5]. We also demonstrated specific MIF association with MVs derived from adipocytes and T lymphocytes, two cell types known to be major producers of this cytokine, underlying a conserved MV-oriented secretion mechanism (Figure 3D). Finally, MIF protein is located within MVs (and not at the MV surface) since MIF signal was still detected after proteinase K action but quenched following a combined treatment of proteinase K with Triton X100 (Figure 3E).

3.4. MV-MIF functional effects specifically rely on MIF tautomerase activity

MIF cytokine is an important mediator in both acute and chronic inflammation, and its secretion is significantly enhanced in inflammatory diseases. MIF signal transduction is initiated by binding to CD74/CD44, and leads to ERK1/2 activation, which mediates most of MIF proliferative and inflammatory effects [10]. In order to decipher the functionality of MV-associated MIF, we isolated EV subtypes from MEFs expressing or not MIF protein (Figure 4A,B). We again demonstrated that MIF specifically associated with MVs derived from

WT MEFs (Figure 4B). Similar internalization kinetics for MVs derived either from WT or MIF^{-/-} MEFs excluding a role of MIF in this process (Figure 4C). WT MVs induce rapid ERK 1/2 phosphorylation when incubated for 2 h on RAW macrophages, in a similar manner than recombinant MIF (Figure 4D). Conversely, no ERK activation was observed following MIF^{-/-} MV incubation (Figure 4D). Addition of neutralizing MIF antibodies within the culture medium during the WT MV incubation period did not prevent from ERK1/2 phosphorylation (Figure 4E). These results suggest that MIF within the MVs, inaccessible to MIF neutralizing antibody, is responsible for such an activation, which would likely rely on MV internalization rather than on a classical ligand/receptor interaction. MIF is also known to act as a D-dopachrome tautomerase, known to transduce several MIF biological activities. Addition of Iso-1, a cell-permeant MIF tautomerase activity inhibitor, during MV incubation period, abolished ERK activation induced by WT MVs therefore establishing the role of MV-associated MIF enzymatic activity for triggering ERK activation in recipient cells (Figure 4D).

4. DISCUSSION

EVs have recently gained interest as mediators of obesity-associated immuno-metabolic dysfunctions, suggesting that they could convey metabolic relays such as adipokines.

To our knowledge, our study is the first investigating such an advanced characterization of MVs and EXOs within the same obese patient. Both EV subtypes are significantly increased with obesity, in agreement with previous studies focused, separately, on circulating MVs [11] or EXOs [12]. Significant association of circulating MVs and EXOs with BMI suggest that WAT may constitute an important source for circulating EVs. However, estimation of adipose-derived EVs is rendered difficult by the lack of an adipose marker specifically secreted by EVs, which would reflect adipose-derived EV secretion. We confirmed adiponectin presence both on circulating MVs and EXOs. Nonetheless, decreased adiponectin expression in obese adipocytes leads to unreliable estimation of fat-derived EVs (data not shown).

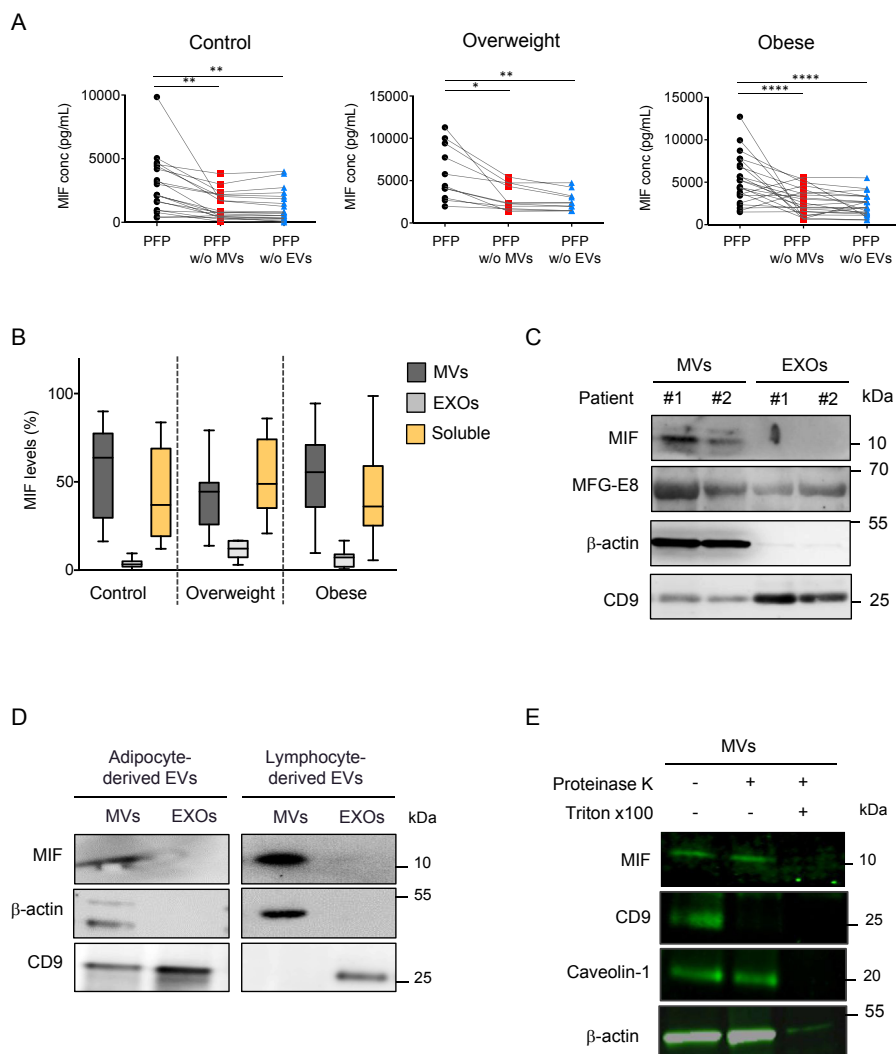


Figure 3: MIF specifically uses MVs as a secretory pathway. **A.** MIF concentration significantly decreased upon removal of plasma MVs. MIF concentration, successively measured in PFP, PFP w/o MVs, and PFP w/o EVs is presented in control patients (left, $n = 19$), overweight patients (middle, $n = 19$), and obese patients (right, $n = 19$). **B.** MV-associated MIF ratios are unchanged with obesity. Percent of EV-associated fractions and soluble MIF fraction was calculated based on MIF concentrations measured in PFP, PFP w/o MV, and PFP w/o EV. MVs carry around half of circulating MIF in control, overweight and obese patients. **C–D.** Western blots of EV preparations confirmed specific MIF association with MVs. MV and EXO preparations isolated from plasma patients (C) or from 3T3-L1 adipocytes and T-CEM lymphocytes supernatants (D) were respectively analyzed for MIF presence. MIF specifically associated with MVs, whatever EV cellular origin. β -actin and CD9 were respectively used as MV and EXO protein markers. **E.** MIF is located within MVs. Proteinase K treatment did not modify MV-associated MIF signal by comparison to untreated MVs excluding that MIF is exposed on MVs. Combined treatment of proteinase K and Triton X100 led to MIF signal disappearance, establishing that MIF is packed within MVs. The transmembrane protein CD9 and the cytoplasmic spindle-oriented caveolin are respectively used as positive controls of this protease K protection assay.

MV elevation in the obese state relies on a specific increase in platelet- and endothelium-derived MVs. These MV subtypes are known to actively participate to the development of CVD by modulating coagulation, inflammation, or vascular function [13]. We have previously demonstrated that intravenous injection of plasma MVs isolated from MS patients in mice induces endothelial dysfunction [14], thereby demonstrating active participation of these large EVs in the development of CVD. Besides, numerous clinical studies have established a relationship between elevated MV circulating levels and the development of type 2 diabetes mellitus (T2DM) [15]. A recent study also revealed higher EXO concentration in diabetic patients [16]. These authors demonstrated that IR state enhanced EV secretion identifying a vicious circle where obesity, a major factor risk for T2DM, could initiate EV

secretion that could be further entertained by IR and associated chronic inflammation.

Aiming at measuring EV contribution in the secretion of adipokines, we compared plasma levels of soluble factors before and after EV subtype depletion in a cohort of MS patients. Since most of concentrations of circulating factors were unchanged after EV retrieval, we concluded that their presence in EVs is likely related to default packing during EV secretion process. Conversely, half of plasma MIF cytokine specifically uses MVs as a conserved secretory process. MIF has been previously detected in EVs from adipocytes [8], in pancreatic ductal adenocarcinomas-derived EVs [17] or during sperm maturation [18], but no distinction was made between MV and EXO subpopulations. Specific MV association could depend on the ATP-

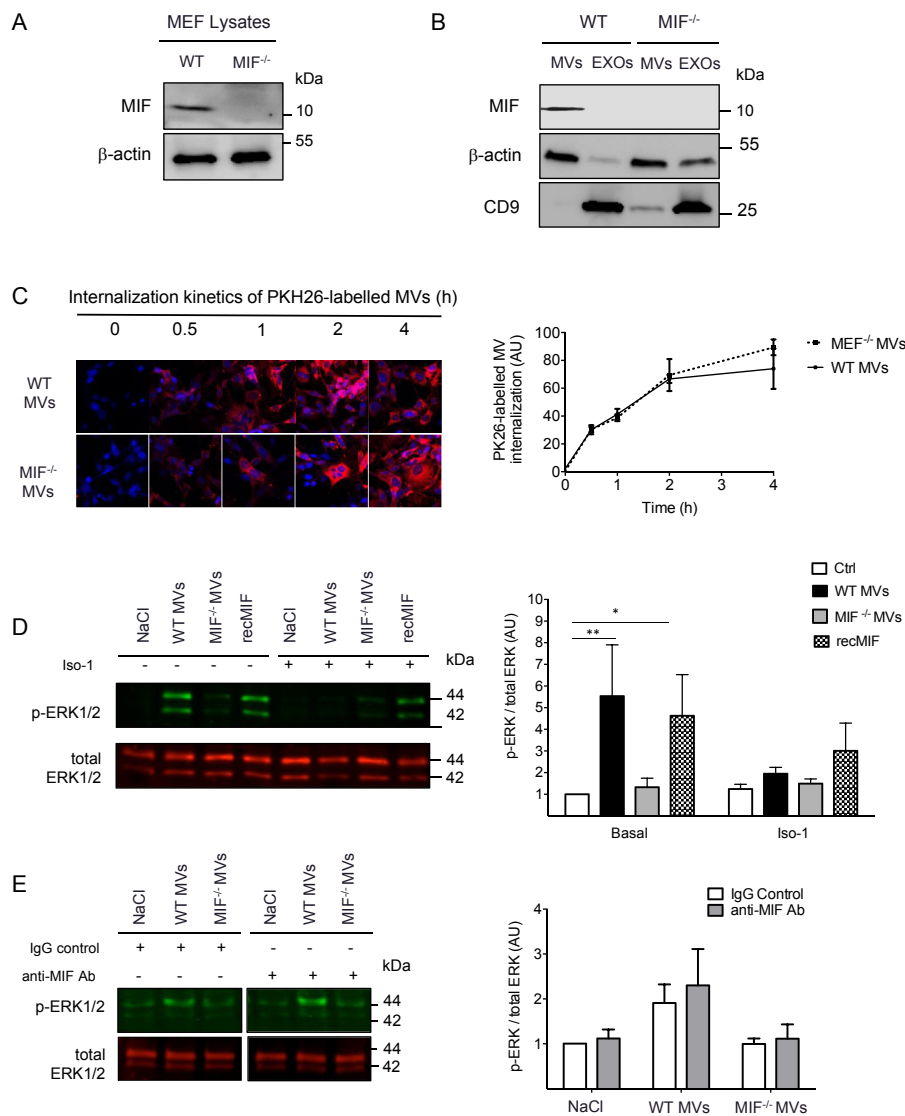


Figure 4: MV-associated MIF transduces ERK activation in macrophages. **A–B.** Production of MVs depleted or not of MIF from mouse embryonic fibroblasts (MEFs) derived from WT and knockout MIF embryos. MEF-derived EVs (B) were isolated from cell supernatants of MEFs expressing (WT) or not (MIF^{-/-}) MIF protein (A). β-actin and CD9 were respectively used as MV and EXO protein markers. **C.** MIF presence within MVs does not influence MV internalization. WT MVs and MIF^{-/-} MVs (20 μg/ml each) were both labeled with PKH26 membranous dye and MV internalization was imaged following different time of MV incubation (0, 0.5, 1, 2 and 4 h) on recipient cells. The absence of MIF does not influence the ability of recipient cells to internalize MVs. Mean Fluorescent Intensity (MFI) within 10 cells on three independent images was measured to quantify PKH26-labeled MV internalization. **D.** MV-associated MIF induces ERK phosphorylation via MIF tautomerase activity. MV-associated MIF (20 μg/ml) incubation induces a robust ERK1/2 phosphorylation in RAW cells, similar to the one observed following 200 ng/ml recombinant MIF (recMIF) treatment. Of note, MIF-depleted MVs (MIF^{-/-} MVs) are unable to mediate ERK1/2 activation. Addition of the inhibitor of MIF tautomerase activity, Iso-1 (50 μM), during MV incubation time, abolished the ability of WT MVs to induce ERK1/2 phosphorylation. Results presented were quantified from at least three independent experiments. **E.** Incubation of WT MVs with MIF-neutralizing antibodies does not modify their ability to induce ERK1/2 phosphorylation in macrophages. Treatment with neutralizing MIF antibodies or their control IgG isotypes (80 μg/ml each) was performed during MV incubation on RAW cells. Results presented were quantified from three independent experiments.

binding cassette transporter (ABCA1) since this transporter has been both involved in the export of MIF from monocytes [19] and in MV formation [20].

ERK signaling pathway has been implicated in the development of IR associated with obesity and T2DM. Indeed, ERK has been involved in adipose conversion [21] and ERK activity is abnormally increased in human and rodent adipose tissues in diabetic states [22,23]. Moreover, some diabetogenic factors have also been shown to influence insulin signaling through activation of ERK signaling cascade [24]. Of interest, targeting the ERK pathway with MEK (MAPK or ERK kinase)

inhibitor improved insulin sensitivity in a diabetic mouse model [25]. MIF actions mainly rely on ERK activation. MV-associated MIF presents the specificity to trigger ERK signaling in recipient cells through its tautomerase activity. Although MIF enzymatic activity is not well defined, its importance has been underlined in multiple disease states by mutational investigations or with irreversible inhibitors [26,27]. Therefore, new EV-based strategies based on MV-associated MIF might therefore appear promising in order to better understand MIF tautomerase activity as well as its involvement in modulating insulin signaling.

Altogether, our study emphasizes the importance of considering the role of EVs as metabolic relays in the development of obesity-associated metabolic complications. Particularly, MIF immune and metabolic responses would need to be reconsidered in the light of our results establishing specific cell responses when exposed to MV-associated form. Furthermore, this study opens new perspectives for MIF treatment using EV-based strategies, which will allow targeting metabolic responses linked specifically to MIF enzymatic activity.

AUTHOR CONTRIBUTIONS

SLL conceived, designed experiments, researched data, and wrote the manuscript. JA and MD conceived, designed experiments, researched data, and reviewed and edited the manuscript. MM, LV, and AF acquired data, analyzed data, and reviewed the manuscript. FG, SD, and JB recruited patients and reviewed the manuscript. GS analyzed data and reviewed the manuscript. OH, MCM, and RA reviewed and edited the manuscript.

SLL is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2018.10.001>.

APPENDIX

Metabol study group composition

CLINICS (Centre Hospitalo-Universitaire d'Angers, Angers, France)

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Brief Communication

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