

Thèse de Doctorat

Marine MALLOCI

*Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université d'Angers
sous le sceau de l'Université Bretagne Loire*

École doctorale : Ecole Doctorale Biologie Santé

Discipline : Aspects moléculaires et cellulaires de la biologie

Spécialité : Biologie Cellulaire

*Unité de recherche : INSERM 1063 SOPAM : « Stress Oxydant et Pathologies
Métaboliques »*

Soutenue le 24 novembre 2017

Rôle des exosomes dans la dysfonction endothéliale associée au syndrome métabolique

JURY

Rapporteurs : **Fabienne FOUFELLE**, Directeur de recherche, Centre de recherche des Cordeliers Inserm UMRS 1138
Michel VIDAL, Directeur de recherche, UMR 5235-Université de Montpellier

Directeur de Thèse : **Carmen MARTINEZ**, Directeur de recherche, Université d'Angers

Co-directeur de Thèse : **Gilles SIMARD**, MCU-PH, Université d'Angers

Remerciements

Je tiens tout d'abord à remercier les Docteurs Fabienne Foufelle et Michel Vidal de m'avoir fait l'honneur d'accepter de juger mon travail de thèse en tant que rapporteurs.

A Carmen, je te remercie de m'avoir fait confiance pour ce projet de thèse et de m'avoir encadrée pendant ces trois années. Je te remercie également pour ta disponibilité et tes conseils tout au long de ce projet, qui m'ont énormément appris et permis de m'améliorer.

Je remercie également le professeur Gilles Simard pour son co-encadrement : merci pour votre disponibilité et votre expertise tout au long de ce projet.

Enfin, je tiens à remercier le Docteur Ramaroson Andriantsitohaina, de m'avoir accueillie au sein du laboratoire et d'avoir présenté mes résultats à l'international.

Un grand merci :

A ma famille qui m'a toujours soutenue durant ces trois années, et qui je l'espère sera fière du travail accompli.

Au Blond métal avec qui j'ai partagé les hauts et les bas de cette aventure.

Aux 3 super nanas du meilleur bureau (en toute objectivité) :

A Patricia, la dernière rescapée, pour ton aide durant ce projet, ton coaching et pour tous ces bons moments. J'espère que tu ne nous remplaceras pas trop vite ;)

A Émilie, merci d'avoir toujours été là, je te souhaite que des bonnes choses pour la suite, tu le mérites !

Et à Zainab, pour ta bonne humeur, tes encouragements et nos discussions scientifiques, je te souhaite le meilleur.

A Madlyne, d'avoir été une belle rencontre professionnelle et d'avoir participé à ce projet.

A Catherine et Maëva D, qui sont maintenant au soleil, pour ces moments d'amitié. A bientôt je l'espère, sur vos îles respectives !

Aux anciens doctorants et ceux de passage que j'ai eu le plaisir de rencontrer : en particulier Audrey, Ousama, Edward, Sylvain, Samuel, Mathieu, Camille, Xavi et Vanessa, et aux plus jeunes : Maëva V, Jérémy et Simon, merci pour votre soutien, votre disponibilité, vos conseils et d'avoir toujours été là pour décompresser autour d'un verre !

A tous les membres du laboratoire SOPAM, anciens et actuels, avec qui j'ai eu le plaisir de travailler.

En particulier, je tenais à remercier Luisa, Greg, Mireille, Soazig et Raffaella pour votre aide et vos conseils au cours de ce projet.

Enfin, à tous les doctorants que j'ai eu la chance de rencontrer grâce à DNA : félicitations à ceux qui ont déjà soutenu et bon courage pour ceux dont ce sera bientôt le tour !

Table des matières

Remerciements.....	2
Table des figures et tableaux	6
Publications et communications	6
Abréviations principales	9
Données bibliographiques.....	10
I- Les vésicules extracellulaires	10
1) Les microparticules.....	11
a) Définition et formation	11
b) Composition.....	12
c) Isolement.....	12
2) Les exosomes	13
a) Définition	13
b) Formation	13
i- Formation dépendante de l'ESCRT	14
ii- Formation indépendante de l'ESCRT	15
c) Sécrétion des exosomes.....	16
i- Régulation de la sécrétion des exosomes.....	17
d) Composition.....	18
i- Protéines	19
ii- Lipides	20
iii- Acides nucléiques	20
e) Interaction des exosomes avec les cellules cibles	21
f) Biodistribution des exosomes	23
g) Isolement des exosomes	24
h) Méthodes de caractérisation.....	24
II- Implication des exosomes dans différentes pathologies	25
1) Syndrome métabolique.....	25
a) Définition	25
b) Quelques chiffres.....	26
c) Pathophysiologie du SM	27
i- Rôles de l'endothélium.....	28
ii- Le monoxyde d'azote (NO).....	29
<i>Modulation de la production de NO.....</i>	<i>30</i>
iii- Le stress oxydant	31
iv- La dysfonction endothéliale	32
v- Le rôle de la mitochondrie	34
<i>Source de ROS</i>	<i>36</i>
<i>Implication de la mitochondrie dans la dysfonction endothéliale</i>	<i>37</i>
d) Prévention et thérapies actuelles pour le SM.....	38

e) Biomarqueurs du SM.....	39
2) Rôles des exosomes dans le SM.....	40
a) Exosomes comme biomarqueurs.....	40
b) Rôles des exosomes dans le SM.....	41
3) Rôle des exosomes dans le cancer.....	42
a) Exosomes comme biomarqueurs.....	42
b) Rôle des exosomes dans les cancers.....	43
c) Rôles des exosomes dans le cancer du rein.....	44
Introduction projet 1.....	46
Discussion.....	79
Introduction projet 2.....	85
Matériel et Méthode.....	86
Résultats expérimentaux.....	88
Discussion.....	92
Conclusion générale.....	95
Bibliographie.....	96
Annexe.....	107

Table des figures et tableaux

Figure 1 : Mécanismes de production des microparticules (MP)	11
Figure 2 : Origine des vésicules extracellulaires.....	14
Figure 3 : Les différentes voie de formation des vésicules intraluminales (VIL) au niveau du CMV.	15
Figure 4 : Les différents mécanismes de sécrétion des exosomes.....	17
Figure 5 : Composition globale des exosomes.....	19
Figure 6 : Les exosomes peuvent interagir avec leur cellule cible et transmettre des informations par différents mécanismes.	22
Figure 7 : Représentation schématique des mécanismes participant au développement du SM .	28
Figure 8 : Mécanisme d'action du monoxyde d'azote (NO) en tant que vasodilatateur.....	30
Figure 9 : Régulation de l'activité de la NOS endothéliale (eNOS)	31
Figure 10 : Mécanismes à l'origine d'une dysfonction endothéliale	33
Figure 11 : Cycle de vie d'une mitochondrie	35
Figure 12 : Traitements thérapeutiques ciblant les différents facteurs du SM	38
Figure 13 : Schéma bilan de l'implication des exosomes dans la dysfonction endothéliale associée au syndrome métabolique (SM).....	80
Figure 14 : Western blot montrant l'expression de la β actine.....	80
Figure 15 : Implication des microparticules de patients SM (MetS MPS) dans la dysfonction endothéliale.....	83
Figure 16 : Evolution du taux d'exosomes circulants avant et après néphrectomie chez des témoins et des patients atteints de cancer du rein.....	89
Figure 17 : Caractérisation des exosomes circulants des patients atteints de cancer du rein, avant et après néphrectomie.....	90
Figure 18 : Expression protéique de l'anhydrase carbonique IX (CA9) associée aux exosomes des témoins et des patients atteints de cancer du rein, avant et après néphrectomie.....	91
Figure 19 : Taux circulants des MP chez les témoins et patients atteints de cancer du rein et expression de la CA9 associée aux MP.....	93
Tableau 1 : Critères pour le diagnostic du syndrome métabolique (SM), définis par la Fédération International du Diabète (IDF).....	26
Tableau 2 : Caractéristiques des témoins et patients atteints de cancer du rein présents dans notre étude.....	88

Publications et communications

Publication :

Malloci M, Perdomo L, Veerasamy M, Andriantsitohaina R, Simard G, Martínez MC. Extracellular vesicles: mechanisms in human health and disease.

(en révision dans Antioxydant & Redox Signalling)

Communications :

- *Orale* :

Malloci M, Safiedeen Z, Dubois S, Simard G, Martinez MC, Andriantsitohaina R. Implication de la mitochondrie dans les effets induits par les exosomes de patients atteints de syndrome métabolique sur la fonction endothéliale.

Journée de la SFR ICAT, Angers, 31 mai 2016. Prix de la Meilleure Présentation de Recherche Translationnelle.

Malloci M, Esnault M, Dubois S, Boursier J, Gagnadoux, Andriantsitohaina R, Simard G, Martinez M. Changes of mitochondrial dynamic evoked by circulating exosomes are associated to endothelial dysfunction in metabolic syndrome.

Congrès de l'International Society for Extracellular Vesicles (ISEV), Toronto, 18-21 mai, présentée par R. Andriantsitohaina.

- *Posters* :

Malloci M, Andriantsitohaina R, Simard G, Martinez MC. Role of exosomes in endothelial dysfunction associated with metabolic syndrome.

Journées scientifiques de l'école doctorale Biologie Santé, Nantes, 10-11 décembre 2015

Malloci M, Dubois S, Andriantsitohaina R, Simard G, Martinez MC. Exosomes from metabolic syndrome patients induce endothelial dysfunction through mitochondrial-generated oxidative stress.

Arch Cardiovasc Dis 2016;8:21, Printemps de la cardiologie, Dijon, 7-8 avril 2016

Malloci M, Esnault M, Dubois S, Boursier J, Gagnadoux, Andriantsitohaina R, Simard G, Martinez M. Changes of mitochondrial dynamic evoked by circulating exosomes are associated to endothelial dysfunction in metabolic syndrome.

Arch Cardiovasc Dis 2017; 9:187, Printemps de la cardiologie, Nantes, 6-7 avril 2017

Malloci M, Esnault M, Dubois S, Boursier J, Gagnadoux, Andriantsitohaina R, Simard G, Martinez

M. Changes of mitochondrial dynamic evoked by circulating exosomes are associated to endothelial dysfunction in metabolic syndrome.

Journée de la SFR ICAT, Angers, 30 mai 2017.

Durcin M, Mallocci M, Vergori L, Dubois S, Simard G, Hue O, Martinez MC, R. Andriantsitohaina R, Le Lay S. Quantification of the circulating vesicle-bound pools of adipocytokines reveals that MFG-E8 and MIF are conveyed by plasmatic EVs.

Journée de la SFR ICAT, Angers, 30 mai 2017.

Mallocci M, Esnault M, Dubois S, Boursier J, Gagnadoux, Andriantsitohaina R, Simard G, Martinez M. Changes of mitochondrial dynamic evoked by circulating exosomes are associated to endothelial dysfunction in metabolic syndrome

Journée scientifique de l'Université de Nantes, Nantes, 2 juin 2017. Prix du meilleur poster.

Abréviations principales

ADNm : ADN mitochondrial

ARNm : ARN messenger

CA9 : Anhydrase Carbonique IX

Ca/cam : complexe calcium/calmoduline

Cav-1 : Cavéoline 1

CE : Cellule Endothéliale

CMH : complexe majeur d'histocompatibilité

CML : Cellule Musculaire Lisse

CMV : Corps Multivésiculaire

CRP : Protéine C réactive

EGFR : Epidermal growth factor receptor

eNOS : NO Synthase endothéliale

ESCRT : Endosomal Sorting Complex Required for Transport

GMPc : Guanosine Monophosphate cyclique

IDF : International Diabetes Federation

IMC : Indice de Masse Corporelle

lncRNA : ARN long non codant

miARN : microARN

MP : Microparticule

NO : Monoxyde d'azote

NOX : NADPH Oxydase

nSMase : Sphingomyélinase neutre

NTA : Nanoparticle Tracking Analysis

OMS : Organisation Mondiale de la Santé

PS : Phosphatidylsérine

ROS : Reactive Oxygen Species (en français ERO : Espèces Réactive de l'Oxygène)

SM : Syndrome Métabolique

SNP : Nitroprussiate de Sodium

SOD : Superoxyde Dismutase

VE : Vésicule Extracellulaire

VIL : Vésicule Intraluminale

Données bibliographiques

I- Les vésicules extracellulaires

Les cellules sont capables de libérer dans l'espace intercellulaire des vésicules de différentes origines. Longtemps considérées comme de simple débris cellulaires, il existe maintenant assez de preuves indiquant que les vésicules extracellulaires (VE) participent à la communication entre les cellules et peuvent ainsi être impliquées dans de nombreux processus cellulaires. Entourées d'une bicouche phospholipidique, les VE contiennent des protéines et acides nucléiques de la cellule mère, qui sont regroupés dans la base de données Vesiclepedia (<http://www.microvesicles.org>) contenant les composants identifiés dans tous les types de VE. Ce cargo actif peut être transmis à des cellules cibles, faisant des VE de véritables vecteurs biologiques. De plus, il a été décrit que les VE pouvaient être de potentiels biomarqueurs dans de nombreuses pathologies. En effet, les VE sont facilement détectables, plus stables que certains biomarqueurs solubles et accessibles de manière non-invasive dans la plupart des fluides biologiques. A travers leur composition, les VE peuvent refléter l'état fonctionnel ou pathologique de leur cellule mère et de leurs tissus d'origine et ainsi être plus pertinents, quand les marqueurs traditionnels ne sont pas suffisants (Müller, 2012).

Bien que l'intérêt pour les VE ne fasse que s'accroître, il n'existe pas encore de nomenclature officielle pour classifier les différents types de vésicules. De nombreux termes sont utilisés, variant d'ectosomes, microparticules, microvésicules, nanovésicules, et exosomes à « exosome-like » particules et exovésicules (van Dommelen et al., 2012), ce qui rend la comparaison difficile entre les différents résultats publiés. De plus, le manque de « gold standard » pour les techniques d'isolement et de caractérisation a été souligné (Gould and Raposo, 2013). Pour répondre à ce problème, la Société Internationale pour les Vésicules Extracellulaires (ISEV) a donné des directives pour isoler et caractériser les VE le plus précisément possible. Cependant, elle reconnaît qu'il n'existe pas de protocole standard et nous invite à la prudence quant à l'interprétation des résultats (Lötvall et al., 2014).

Actuellement, les VE sont communément classifiées selon leur origine intracellulaire. Ainsi, on peut considérer qu'il existe trois principales populations de VE : les corps apoptotiques, les microparticules (MP) provenant du bourgeonnement de la membrane plasmique et les exosomes, formés à partir du compartiment endosomal. Les corps apoptotiques, dont la taille est comprise entre 1 à 4 μm et qui sont libérés par les cellules en cours d'apoptose, ne seront pas détaillés dans cette introduction.

1) Les microparticules

a) Définition et formation

Les microparticules (MP) sont définies comme de petites vésicules dont la taille varie entre 0,1 et 1µm. Elles sont libérées par bourgeonnement de la membrane plasmique, suite à une activation cellulaire ou une stimulation apoptotique. Leur formation a été décrite pour la première fois par Wolf et collaborateurs (Wolf, 1967) lors de l'activation plaquettaire et généralisée à de nombreux types cellulaires. En effet, au cours de l'activation cellulaire, par un agoniste ou différents stress, l'influx calcique qui s'ensuit induit une réorganisation du cytosquelette de manière dépendante de la calpaïne (Dachary-Prigent et al., 1995) nécessaire au bourgeonnement des MP (Figure 1). De plus, l'activité de certaines enzymes (scramblases et floppases) permet l'externalisation de la phosphatidylsérine (PS), normalement située sur le feuillet interne de la membrane plasmique. Par ailleurs, l'intégrité des radeaux lipidiques est également requise pour cette entrée calcique et pour l'exposition de PS (Kunzelmann-Marche et al., 2002).

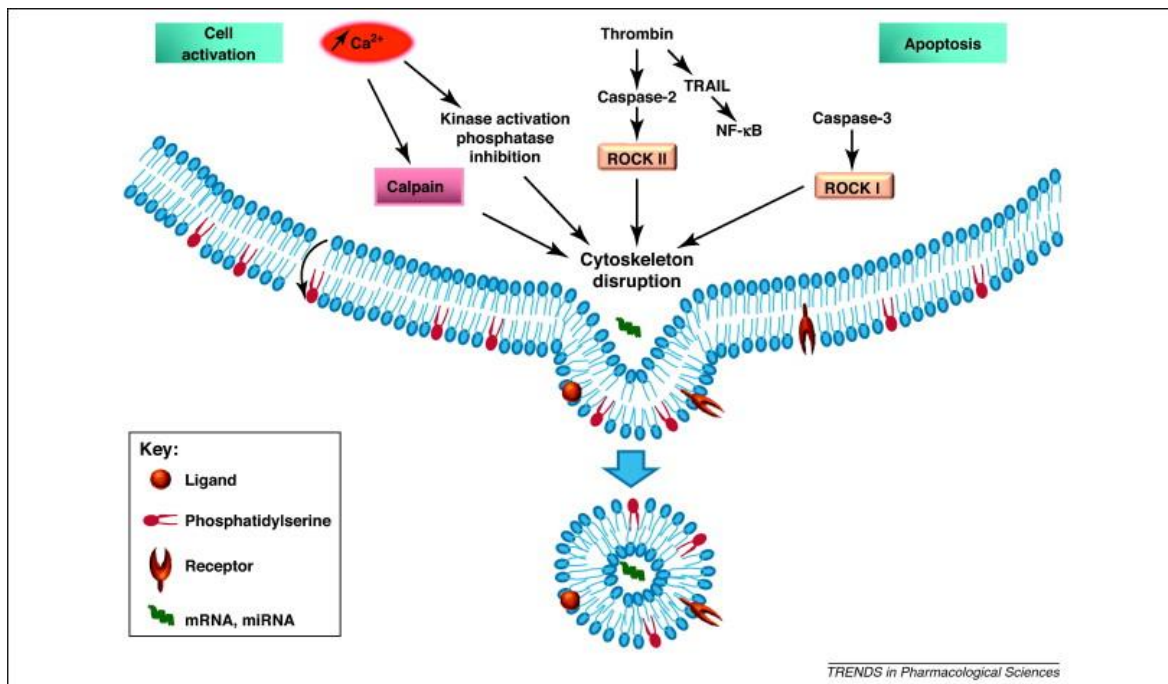


Figure 1 : Mécanismes de production des microparticules (MP) : Les MP sont formées par bourgeonnement de la membrane plasmique, à la suite d'une activation cellulaire associée à une augmentation du calcium intracellulaire, de l'activation de la voie ROCK II via la thrombine ou encore de l'apoptose et l'activation de la voie ROCK I (Martinez et al., 2011).

D'autres mécanismes moléculaires ont été décrits comme impliqués dans la production de MP, indépendamment d'une activation cellulaire et d'une augmentation calcique. Ainsi, les MP peuvent également être produites à la suite de l'apoptose. Le clivage de la caspase-3 conduit à l'activation constitutive des sérines/thréonine kinases ROCK I (Rho-associated protein kinase-1) et à la phosphorylation de la chaîne légère de la myosine, provoquant ainsi une augmentation de la

contraction cellulaire, ce qui favoriserait le bourgeonnement de MP (Sebbagh et al., 2001) (Figure 1). Tandis que le bourgeonnement des MP intervient lors des phases précoces de l'apoptose, les corps apoptotiques sont produits lors des dernières étapes du processus apoptotique (Beyer and Pisetsky, 2010). Enfin, à l'interface entre l'activation et l'apoptose, la thrombine s'est révélée être un puissant inducteur de la libération de MP par les cellules endothéliales (Sapet et al., 2006) par activation de la caspase-2 et des Rho-kinases ROCK II, indépendamment de l'apoptose (Sapet et al., 2006) (Figure 1). Ainsi, bien que n'importe quelle cellule puisse émettre potentiellement des MP, les mécanismes responsables du bourgeonnement membranaire semblent différer selon les cellules et les stimuli d'origine.

b) Composition

Contrairement aux exosomes, il n'existe pas de contenu caractéristique des MP. En effet, différentes études ont confirmé que la composition et la fonction des MP dépendent à la fois du stimulus qui a engendré leur libération et de leur origine cellulaire (Bochmann et al., 2014; Soleti et al., 2012). Concernant leur composition, les MP présentent des éléments de leur membrane d'origine comme des ligands (Agouni et al., 2011) et des récepteurs (Chen et al., 2012), des acides nucléiques comme les ARNm et miARN (Diehl et al., 2012) et des protéines provenant des compartiments mitochondriale, nucléaire et cytosolique (Dean et al., 2009). De par leur mécanisme de formation, on peut tout de même noter que les MP sont enrichies en protéines membranaires. Par ailleurs, la PS externalisée à la membrane de MP induit le recrutement de facteurs de coagulation. Ainsi, les MP dérivées des cellules monocytaires, plaquettaires ou encore endothéliales possèdent un fort potentiel procoagulant (Morel et al., 2006). D'ailleurs, chez les patients atteints du syndrome de Scott, maladie hémorragique rare liée à un déficit d'exposition de la PS (Suzuki et al., 2010), l'émission de MP procoagulantes est très faible et contribue à la tendance hémorragique (Toti et al., 1996). Néanmoins, les MP circulantes ne lient pas toutes l'annexine-V (qui se lie à la PS) et en conséquence, n'externalisent pas toutes la PS, suggérant différents modes de vésiculation des MP (Connor et al., 2010).

c) Isolement

Le protocole généralement utilisé pour isoler les MP, comme les exosomes, est basé sur une série de centrifugations différentielles, permettant de séparer les plus grosses vésicules (MP) des plus petites (exosomes) selon la vitesse de sédimentation. Ainsi, à partir de prélèvements sanguins, l'élimination des cellules sanguines est effectuée à 250xg pour récupérer le plasma pauvre en plaquettes, puis les débris cellulaires sont sédimentés à 1 500xg, et enfin l'isolement des MP est réalisé autour de 13 000xg selon les études (Théry et al., 2001).

2) Les exosomes

a) Définition

Les exosomes ont été décrits pour la première fois il y a trente ans, comme de nanovésicules d'origine endosomale, libérées à la suite de la fusion entre le corps multivésiculaire (CMV) et la membrane plasmique, dans des réticulocytes de rats (Harding et al., 1983) et de moutons (Pan et al., 1985). En effet, en étudiant par microscopie électronique le trafic intracellulaire du récepteur à la transferrine, il a été observé que ce récepteur pouvait être internalisé pour être recyclé et qu'il se retrouvait ensuite à la surface des exosomes dans le milieu extracellulaire.

Depuis, de nombreux types cellulaires ont montré leur capacité à libérer des exosomes *in vitro*, comme les cellules hématopoïétiques (les cellules B-, T-, dendritiques, les macrophages et plaquettes), épithéliales et neuronales, les adipocytes, les fibroblastes, les cellules souches et différents types cellulaires tumorales (Urbanelli et al., 2013). De plus, après la première découverte de la présence d'exosomes dans le plasma humain (Caby et al., 2005), les exosomes ont été décrits dans la plupart des fluides biologiques, comme la salive, l'urine, le fluide séminal, le liquide amniotique ou encore le lait maternel (Urbanelli et al., 2013).

Depuis lors, les multiples rôles des exosomes dans les maladies cardiovasculaires, la tumorigénèse ou encore la propagation des maladies neurodégénératives sont devenues évidents, suggérant un rôle potentiel des exosomes en tant que biomarqueurs et cibles thérapeutiques.

Les exosomes peuvent être définis par leur taille comprise entre 30-150 nm. La taille des exosomes semble dépendre de leur type cellulaire d'origine (50 nm dans les réticulocytes (Pan et al., 1985) 102 nm dans les cellules B de lymphome (Oksvold et al., 2014) ou encore de 80 à 120 nm dans les adipocytes (Durcin et al., 2017). Pour identifier les exosomes, la taille n'est pas un critère suffisant car les plus petites MP peuvent avoir des tailles de l'ordre de la centaine de nm. Ainsi, l'analyse d'au moins trois marqueurs spécifiques est conseillée pour identifier de manière plus précise une population d'exosomes (Lötvall et al., 2014). En fait, certaines protéines sont communément enrichies dans les exosomes d'origine cellulaire différente et peuvent être ainsi considérées comme des marqueurs exosomaux. Parmi elles, on retrouve les tétraspanines CD9, CD81, CD63 ; les protéines endosomales TSG101 et ALIX, ou encore Mfge8 (Lötvall et al., 2014). Cependant, la pertinence de ces marqueurs est actuellement discutée (Kowal et al., 2016).

b) Formation

Au contraire des MP, les exosomes proviennent du compartiment endosomal et sont formés comme les vésicules intraluminales (VIL) (Figure 2). Les VIL sont formées à partir de la membrane de l'endosome précoce, par bourgeonnement vers l'intérieur, séquestrant ainsi les protéines, les lipides et le cytosol, dans le but de les dégrader, recycler ou de libérer par exocytose ce contenu. L'endosome précoce mature ensuite en endosome tardif et l'accumulation d'une multitude de VIL entraîne la formation du corps multivésiculaire (CMV). Le principal devenir de CMV est de fusionner avec le lysosome, assurant ainsi la dégradation de son contenu. Cependant, le CMV

peut également fusionner avec la membrane plasmique pour libérer les VIL par exocytose, qui deviennent alors des exosomes dans l'espace extracellulaire (Juan and Fürthauer, 2015).

Différents mécanismes ont été proposés comme participant à la formation des exosomes et ils peuvent être classés en deux groupes selon l'implication de la machinerie ESCRT (Endosomal Sorting Complex Required for Transport).

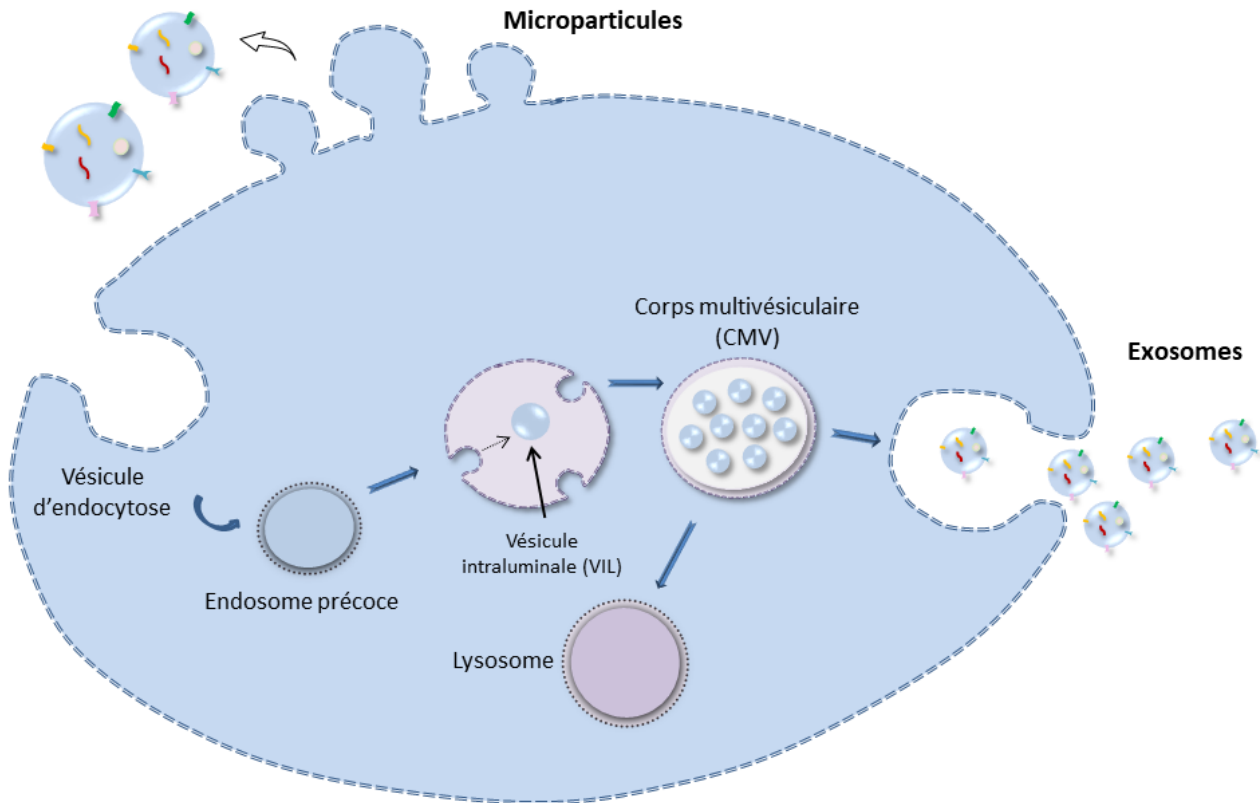


Figure 2 : Origine des vésicules extracellulaires : Les microparticules sont libérées par bourgeonnement de la membrane plasmique. Les exosomes sont formés dans le compartiment endosomal comme les vésicules intraluminales (VIL) par invagination du corps multivésiculaire (CMV). Le CMV peut alors dégrader son contenu en fusionnant avec le lysosome ou libérer les VIL comme exosomes dans l'espace extracellulaire.

i- Formation dépendante de l'ESCRT

L'implication de la machinerie ESCRT dans la biogenèse des exosomes a été initialement suggérée par des études protéomiques montrant la présence, dans des exosomes de différents types cellulaires, des protéines associées à l'ESCRT, TSG101 and ALIX (Théry et al., 2002). Le rôle du complexe ESCRT est initialement d'assurer le transport des protéines ubiquitinylées de l'endosome au lysosome, menant à leur dégradation. Ainsi, la machinerie ESCRT permet la formation du CMV en internalisant les protéines dans les VIL, par bourgeonnement de la membrane endosomale (Figure 3). Il a été suggéré que les complexes ESCRT-0, -I et -II forment un hétéro-oligomère stable qui fonctionne de concert pour reconnaître et séquestrer les protéines ubiquitinylées dans la membrane endosomale. Quand le cargo est collecté, les complexes

ESCRT-I et ESCRT-II peuvent initier le processus de bourgeonnement de la membrane endosomale. De plus, ESCRT-II induit l'assemblage du complexe ESCRT-III, qui à son tour, finalise le bourgeonnement de la membrane et sa scission pour former les VIL (Schmidt and Teis, 2012).

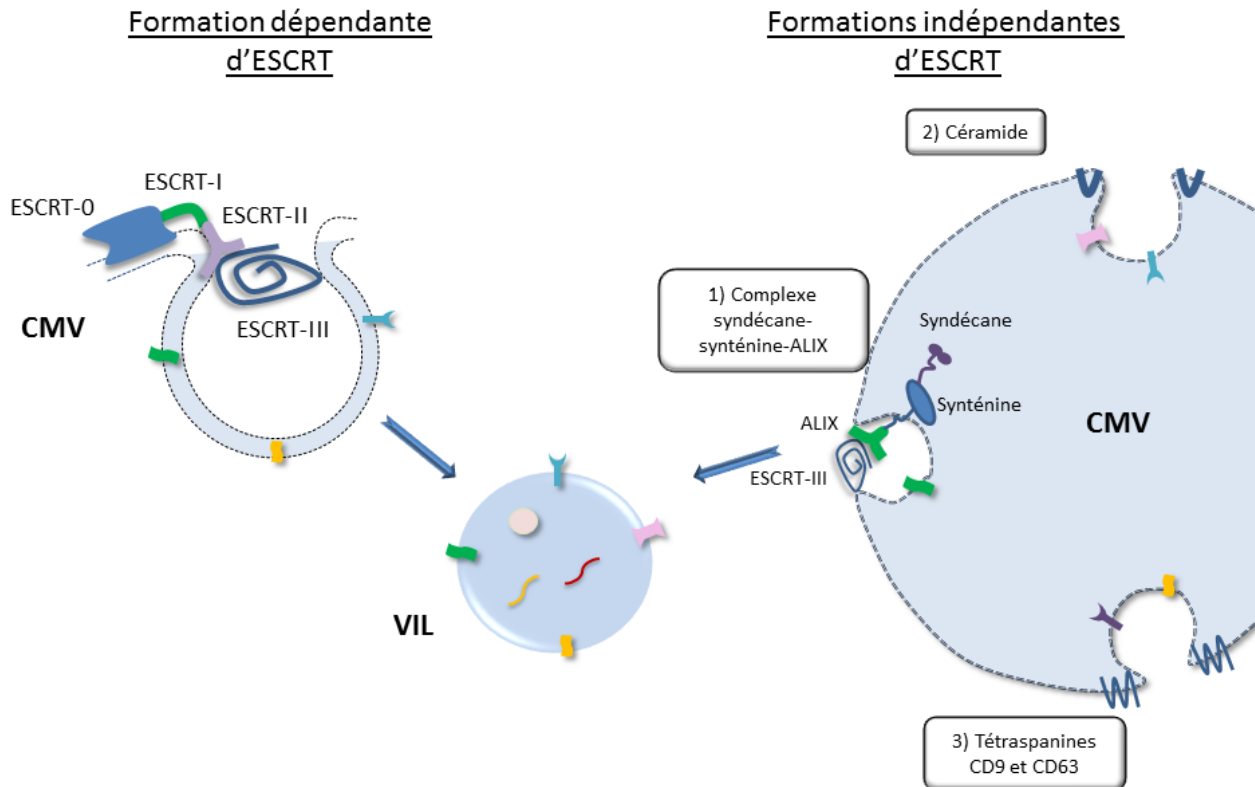


Figure 3 : Les différentes voie de formation des vésicules intraluminales (VIL) au niveau du CMV : VIL sont classiquement formées par la machinerie ESCRT : l'ESCRT-0 reconnaît les protéines à séquestrer, l'ESCRT-I et II initient le processus de bourgeonnement et l'ESCRT-III finalise la formation des VIL. Trois voies indépendantes d'ESCRT ont également été décrites : 1) les céramides facilitent l'invagination des VIL dans les cellules oligodendrogliales ; 2) le complexe syndécane-synténine-ALIX est responsable du bourgeonnement des VIL dans les cellules tumorales MCF7 ; et 3) les tétraspanines CD9 et CD63 sont impliquées dans la formation des exosomes dans les cellules dendritiques et dans les mélanosomes, respectivement.

ii- Formation indépendante de l'ESCRT

Bien que, comme décrit précédemment, la voie dépendante de l'ESCRT semble être le principal mécanisme de formation des exosomes, d'autres voies alternatives ont également été proposées (Figure 3).

Tout d'abord, il a été montré que les cellules tumorales MCF7 sécrètent des exosomes à travers une voie impliquant les protéines syndécane, synténine et ALIX. En effet, la protéoglycane à sulfate d'héparane syndécane et sa protéine de signalisation intercellulaire synténine peuvent contrôler la formation des exosomes par interaction directe avec ALIX en soutenant le

bourgeoisement intraluminal de la membrane endosomale. Ces exosomes contenant la syndécane sont également enrichis en CD63 et céramide. La suppression en aval d'ALIX, CHMP4, and VPS4 bloque leur biogenèse (Baietti et al., 2012), suggérant que ces protéines associées aux complexes ESCRT sont impliquées dans cette voie de formation, bien qu'elle soit indépendante de la voie ESCRT classique. De plus, la digestion enzymatique des chaînes sulfate de l'héparane sur les syndécanes par les héparanases, facilite le bourgeoisement de la membrane endosomale et la biogenèse des exosomes et stimule leur sécrétion (Roucourt et al., 2015). Par la suite, Ghossoub et collègues (Ghossoub et al., 2014) ont également décrit la GTPase ADP ribosylation factor 6 (ARF6) et son effecteur la phospholipase D2 comme régulateurs de cette voie de biogenèse. ARF6 et la phospholipase D2 peuvent affecter la formation des exosomes en contrôlant le bourgeoisement des VIL dans le CMV.

Dans les cellules oligodendrogiales, Trajkovic et collègues (Trajkovic et al., 2008) ont trouvé que la libération des exosomes est réduite après l'inhibition de la sphingomyélinase neutre (nSMase), une enzyme responsable de la production de céramide. Plus précisément, ce lipide peut faciliter l'invagination de la membrane des VIL à travers sa structure en forme de cône et induire la formation des exosomes. Les exosomes dérivés de cette voie sont enrichis en protéolipoprotéine, CD63, CD81 et TSG101.

De plus, les tétraspanines CD9 et CD63 ont également été décrites comme directement impliquées dans la formation des exosomes. En effet, Buschow et collègues (Buschow et al., 2009) suggèrent que les cellules dendritiques peuvent générer des exosomes portant MHC II à travers une voie dépendante de CD9, sans l'implication de la machinerie ESCRT. Dans les mélanosomes, CD63 participe directement à la sortie du domaine luminal de la protéine prémélanosome (PMEL) dans les VIL, de façon indépendante de l'ESCRT (van Niel et al., 2011). Ainsi, la formation des exosomes semble impliquer de nombreux mécanismes et reste encore à être complètement élucidée. Par ailleurs, ces différentes voies de production peuvent influencer la composition des exosomes et, ainsi, induire la formation de différentes populations. Cela peut expliquer notamment la diversité fonctionnelle et moléculaire des exosomes sécrétés par les différents types cellulaires.

c) Sécrétion des exosomes

Une multitude de mécanismes ont également été proposés pour la libération des exosomes. Tout d'abord, pour sécréter les exosomes, le CMV doit être transporté, amarré à la membrane plasmique et fusionner avec elle pour libérer les VIL dans l'espace extracellulaire. Pour cela, il a été suggéré que les protéines de la famille Rab, de petites GTPases impliquées dans le trafic des vésicules intracellulaires, leur amarrage et leur fusion avec la membrane plasmique (Stenmark, 2009), pouvaient être impliquées. En effet, l'équipe de Savina (Savina et al., 2005) a montré que, dans des cellules érythroleucémiques, Rab11 régule la sécrétion des exosomes. Plus précisément, Rab11 agit en amarrant et fusionnant le CMV, en présence de calcium (Savina et al., 2005). Par

ailleurs, dans les oligodendrocytes, l'inhibition de Rab35 empêche la sécrétion des exosomes (Hsu et al., 2010). De plus, Rab27a et Rab27b favorise la sécrétion des exosomes par les cellules HeLa. Ces deux isoformes de Rab27 sont impliquées dans l'amarrage du CMV à la membrane plasmique (Ostrowski et al., 2010). Les exosomes libérés par cette voie sont enrichis en protéines de l'endosome tardif (comme CD63, ALIX, and TSG101) (Abels and Breakefield, 2016). Enfin,

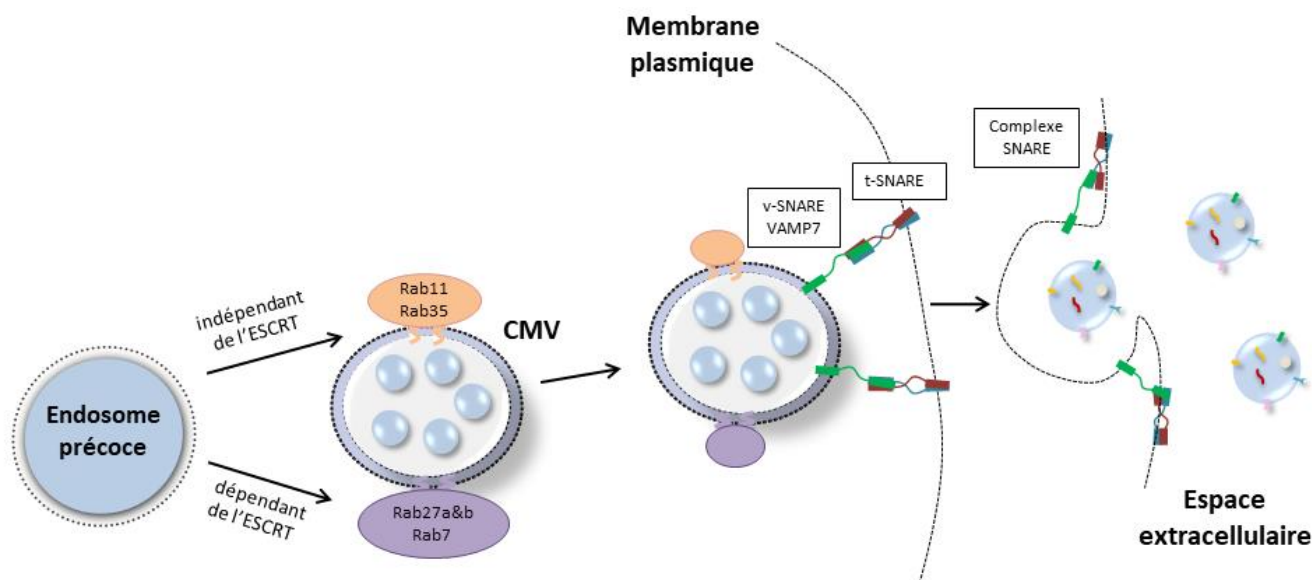


Figure 4 : Les différents mécanismes de sécrétion des exosomes : Les exosomes peuvent être libérés grâce à certaines protéines de la famille Rab : Rab11, Rab35, Rab27 a&b et Rab7, responsables du transport, de l'amarrage et de la fusion du CMV avec la membrane plasmique. La protéine v-SNARE VAMP7 peut également être impliquée dans la fusion du CMV avec la membrane plasmique.

Rab7 a aussi été décrit comme étant impliqué dans la sécrétion d'exosomes contenant Alix et synténine dans de cellules de cancer du sein (Baietti et al., 2012). Cependant, une autre équipe a montré que la suppression de Rab7 n'affecte pas la sécrétion des exosomes à partir de cellules HeLa (Ostrowski et al., 2010), suggérant que ce mécanisme pourrait être dépendant du type cellulaire.

Par ailleurs, la protéine v-SNARE, VAMP7, a été décrite comme participant à la fusion entre le CMV et la membrane plasmique pour libérer les exosomes de cellules érythroleucémiques (Fader et al., 2009).

Une fois encore, les mécanismes permettant la sécrétion des exosomes semblent être multiples, dépendants de l'origine cellulaire et ne sont pas encore complètement élucidés.

i- Régulation de la sécrétion des exosomes

La plupart des cellules sécrètent de façon constitutive des exosomes. Cependant, pour certains types cellulaires, cette sécrétion peut être modulée. Ainsi, la maturation des réticulocytes induit la sécrétion d'exosomes contenant le récepteur à la transferrine (Pan et al., 1985). De plus, la sécrétion des exosomes peut être induite durant la dégranulation des mastocytes déclenchée

par un complexe IgE-antigène (Raposo et al., 1997). Dans les lymphocytes T, l'activation TCR induit une augmentation de la sécrétion d'exosomes présentant TCR/CD3 à leur surface (Blanchard et al., 2002). L'équipe de Buschow (Buschow et al., 2009) a montré que l'interaction de cellules dendritiques avec des cellules T activées induit la sécrétion d'exosomes portant des molécules du complexe majeur d'histocompatibilité (CMH) II. De plus, la production d'exosomes par les cellules B est augmentée en présence de cellules T CD4⁺, probablement à l'engagement du BCR ou à l'activation par la protéine CD40 (Arita et al., 2008).

d) Composition

Comme les MP, les exosomes contiennent de multiples protéines et acides nucléiques, protégés par une bicouche lipidique. Cependant, leur composition est la conséquence de leur formation à travers le compartiment endosomal aussi bien que des différents mécanismes associés à leur sécrétion. Ainsi, la composition des exosomes diffère des MP et de leur cellule d'origine. Par exemple, une étude protéomique et lipidomique a comparé la composition des exosomes, des MP et de leur cellule d'origine pour trois lignées cellulaires différentes : les cellules de glioblastome U87, les cellules de carcinome hépatocellulaire Huh7 et des cellules souches mésenchymateuses humaines dérivées de la moelle osseuse (MSCs). Ils ont trouvé que le profil protéique des exosomes était plus différent de celui de leur cellule d'origine que celui des MP. De plus, les exosomes semblent enrichis en protéines de la matrice extracellulaire, de récepteurs liant l'héparine et en protéines impliquées dans la réponse immunitaire et l'adhésion cellulaire, alors que les MP sont enrichies en protéines provenant du réticulum endoplasmique, du protéasome et de la mitochondrie. Les exosomes et les MP diffèrent également dans leur composition lipidique. De plus, la composition des exosomes diffère d'une lignée cellulaire à une autre : les exosomes des Huh7 et des MSCs sont spécifiquement enrichis en cardiolipine tandis que les exosomes des U87 sont enrichis en sphingomyéline (Haraszti et al., 2016).

La composition de ces vésicules peut dépendre de leur cellule d'origine mais également des fonctions spécifiques de celle-ci. En effet, Conde-Vancells et collègues (Conde-Vancells et al., 2008) ont analysé les exosomes provenant d'hépatocytes primaires de rat et d'une lignée hépatique murine, et ont rapporté que ces exosomes contenaient des protéines pas encore décrites dans les exosomes, associées des fonctions spécifiques de ces cellules comme des apolipoprotéines ou des cytochromes P450. Dans des VE dérivées d'une lignée adipocytaire, notre laboratoire a montré que les exosomes et les MP possèdent une signature protéique spécifique et suggèrent que cette différence de contenu pourrait prédire la fonction biologique de ces VE (Durcin et al., 2017). Toutes les protéines, acides nucléiques et lipides décrits comme étant contenu dans les exosomes sont inventoriés dans base de données libre appelée Exocarta (<http://www.exocarta.org/>) qui actuellement liste plus de deux cents publications sur de multiples cellules eucaryotes. En utilisant cet outil, il est possible de déterminer quels composants sont communément exprimés dans les exosomes et ceux qui sont spécifiques à un type cellulaire ou un

organisme donné. Les exosomes présentent, en effet, un contenu en protéines, lipides et acides nucléiques qui peut être caractéristique (Figure 5).

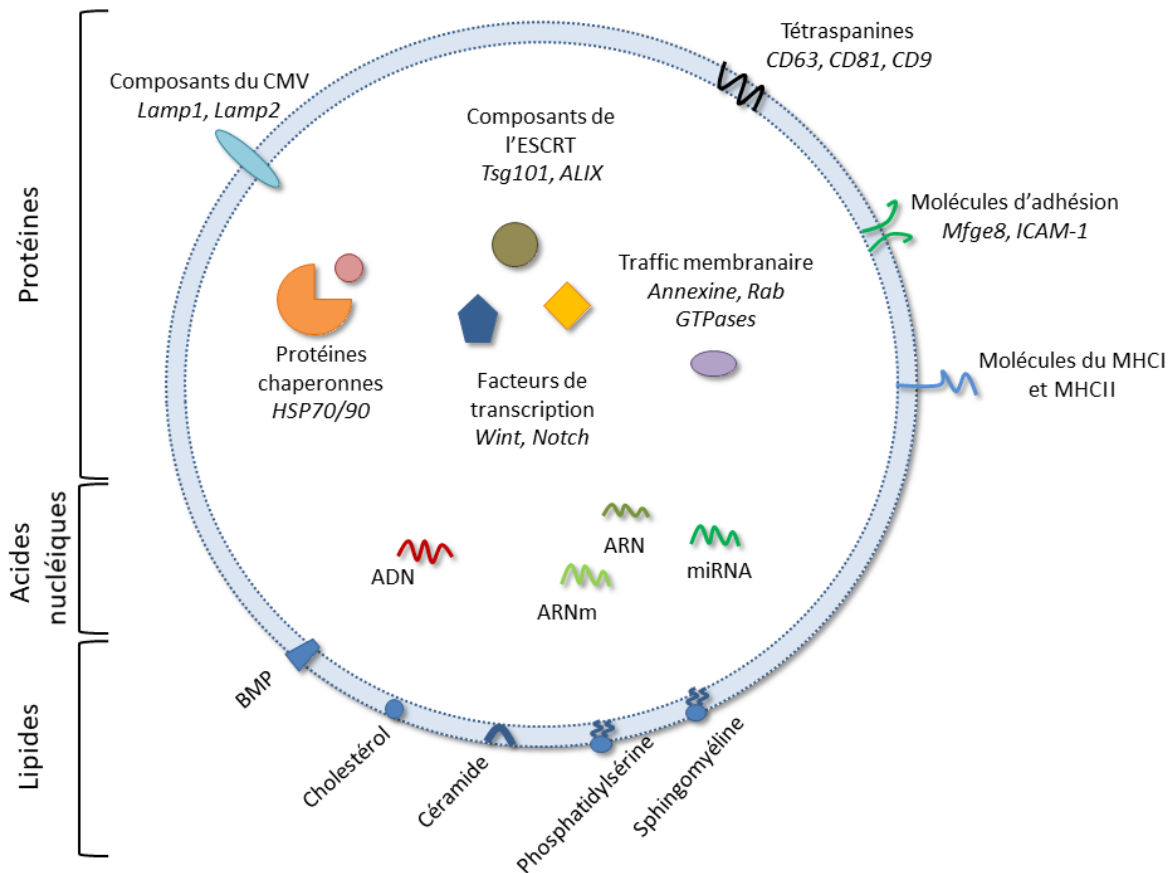


Figure 5 : Composition globale des exosomes : Représentation schématique des composants que l'on peut retrouver enrichis dans les exosomes. Parmi les protéines enrichies dans les exosomes, on peut retenir les tétraspanines, les composants associés au complexe ESCRT ou au CMV ou encore les molécules du MHC I et MHCII. De plus, ils transportent des acides nucléiques comme de l'ADN nucléaire et mitochondrial mais surtout des molécules d'ARN : principalement des microARN (miARN) mais aussi des ARN messagers (ARNm) et des ARN non codants. Enfin, au niveau lipidique, les exosomes sont composés de BMP, de cholestérol, de céramide, de phosphatidylsérine et de sphingomyéline. Cependant, cette composition globale peut varier selon l'origine cellulaire des exosomes.

Abréviations : CMV = corps multivésiculaire, ESCRT= endosomal sorting complexes required for transport, CMH = complexe majeur d'histocompatibilité, BMP = Bis(Monoacylglycero)Phosphate

i- Protéines

Les protéines communément exprimées dans les exosomes sont logiquement celles associées avec les mécanismes responsables de leur biogenèse et sécrétion, incluant les protéines associées au compartiment endosomal. Ainsi, les exosomes sont enrichis en protéines associées au complexe ESCRT (ALIX, TSG101), impliquées dans la structure du CMV (Lamp1 and Lamp2, les tétraspanines CD9, CD81, CD63), les protéines de trafic membranaire (Rab GTPases, annexines), des protéines de fusion (v-SNARES), les protéines chaperonnes heat-shock proteins

(HSP 90/70) ou encore les protéines du CMH I et II, mais aussi des protéines provenant de la membrane plasmique (flottiline 1, Mfge8, cavéoline 1 (Cav-1)) (pour revue voir Colombo et al., 2014).

En général, les protéines associées aux réticulum endoplasmique, Golgi et au noyau ne sont pas retrouvés dans les exosomes (Lötvald et al., 2014), cependant les exosomes peuvent porter des facteurs de transcription comme Notch, Wnt, qui sont normalement retrouvés dans le noyau (Abels and Breakefield, 2016).

ii- Lipides

La composition lipidique des exosomes a été décrite pour la première fois en 1989 par Vidal et al. Ils ont comparé la composition d'exosomes produits *in vitro* à partir de réticulocytes primaires de cochon d'Inde et leur cellules mères, et ont trouvé que les exosomes et les réticulocytes ont une composition similaire en cholestérol, contrairement au contenu en phosphatidyléthanolamine qui est diminué dans les exosomes comparé aux réticulocytes (Vidal et al., 1989). Depuis, de nombreuses études ont rapporté la composition lipidique d'exosomes dérivés de différents types cellulaires comme des cellules cancéreuses (Llorente et al., 2013), des mastocytes et cellules dendritiques (Laulagnier et al., 2004), des adipocytes (Durcin et al., 2017) ou des cellules souches mésenchymateuses (MSCs) (Haraszti et al., 2016). Ces comparaisons ont montré que, bien que la composition lipidique des exosomes diffère de celle de leur cellule d'origine, les exosomes affichent des caractéristiques lipidiques communes indépendamment de leur origine cellulaire. En effet, les exosomes sont principalement enrichis en cholestérol, sphingomyéline, céramide et exposent la phosphatidylsérine à leur surface. Par ailleurs, le phospholipide Bis(Monoacylglycero)Phosphate (BMP) (un phospholipide essentiellement localisé dans le CMV) pourrait être un marqueur lipidique spécifique des exosomes (Laulagnier et al., 2004).

Il est intéressant de noter que la composition lipidique des exosomes peut également servir de biomarqueurs et être impliquée dans différentes pathologies (pour revue voir Record et al., 2014). Récemment, il a été montré que le contenu lipidique des exosomes urinaires était modifié chez les patients atteints de cancer de la prostate (Skotland et al., 2017).

iii- Acides nucléiques

Les exosomes ont la capacité de transporter et transférer de l'information génétique, et d'ainsi moduler l'expression génique de cellules cibles. Dans un petit nombre de cas, de l'ADN a été retrouvé dans les exosomes, incluant de l'ADN génomique et mitochondrial. Cependant les exosomes contiennent principalement de petits ARN, dont la plupart dérivent des ARN ribosomiaux 18S et 28S et des ARN de transfert (Abels and Breakefield, 2016).

Les exosomes transportent également des ARN messagers (ARNm) et des microARN (miARN), qui ont été trouvés pour la première fois dans les exosomes de mastocytes humains et murins (Valadi et al., 2007). Depuis, différentes études ont montré la capacité des exosomes de transférer

des ARNm/miARN fonctionnels (Montecalvo et al., 2012; Skog et al., 2008). Par ailleurs, Montecalvo et al (2012) décrivent que les cellules dendritiques peuvent transférer des miARN fonctionnels via les exosomes et que différents profils de miARN ont été observés selon leur maturation, suggérant ainsi que les cellules peuvent choisir le contenu en miARN des exosomes libérés. De plus, il a été montré par l'équipe de Brianza-Padilla (Brianza-Padilla et al., 2016) que le profil en miARN exosomaux peut être modulé par certains stimuli, comme une ingestion chronique de sucrose, chez le rat. Récemment, les miARN exosomaux semblent être de bons candidats comme biomarqueurs dans différentes pathologies.

Il a également été décrit que les exosomes peuvent contenir d'autres types d'ARN comme les ARNs longs et courts non codants, les ARN piwi-interacting, les ARN vault ou encore ARN Y (Abels and Breakefield, 2016).

Il est intéressant de noter que la capture des ARN dans les exosomes les protège de la dégradation par les ARNases une fois libérés dans l'environnement extracellulaire.

Enfin, à cause de la variété des techniques d'isolation et des différents types cellulaires et conditions de culture utilisées pour analyser le contenu des exosomes, la comparaison des résultats existant sur la composition de ces vésicules nécessite une analyse critique.

e) Interaction des exosomes avec les cellules cibles

Il est devenu rapidement évident que les exosomes jouent un rôle important dans la communication intercellulaire. Une fois libérés, les exosomes peuvent interagir avec leurs cellules cibles et activer des voies de signalisation intracellulaire en transmettant un message médié par leur contenu. Cependant, il a longtemps été difficile de visualiser l'action des exosomes, à cause de la petite taille de ces vésicules. Il est actuellement possible de mieux étudier ces mécanismes en marquant les exosomes avec des sondes fluorescentes spécifiques des lipides membranaires comme le DiD (Tian et al., 2010), les PkH26/67 ou encore la Rhodamine B (Parolini et al., 2009). Ainsi, il a été montré que les exosomes pouvaient interagir avec des cellules cibles par différents mécanismes (Figure 6).

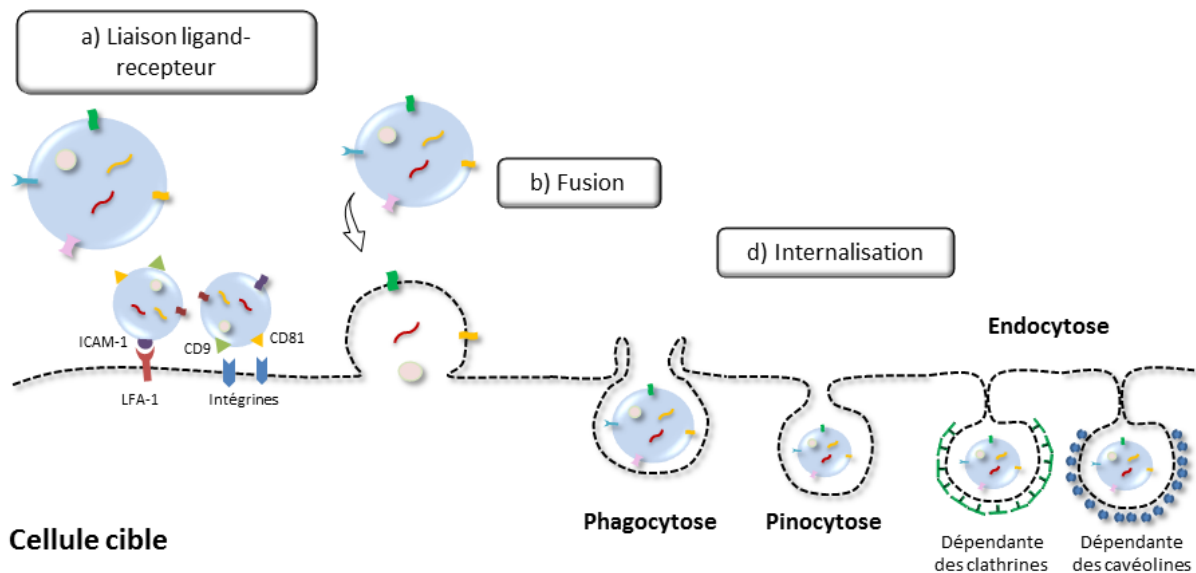


Figure 6 : Les exosomes peuvent interagir avec leur cellule cible et transmettre des informations par différents mécanismes : a) une interaction ligand-récepteur activant une signalisation intracellulaire comme ICAM-1 et son récepteur LFA1 ou les tétraspanines CD9 et CD81 liées aux intégrines dans les cellules dendritiques ; b) la fusion directe avec la membrane plasmique ; et c) l'internalisation par différents processus : phagocytose, pinocytose et endocytose indépendante ou dépendante des clathrines et des cavéolines.

Tout d'abord, la liaison des exosomes à la surface cellulaire peut se faire à travers une simple interaction avec de nombreuses protéines de contact, des récepteurs de surfaces ou des ligands de la cellule cible. Parmi tous ceux qui ont été décrits, il est possible de retenir le rôle de LFA-1 dans les cellules dendritiques dans la capture des exosomes dendritiques portant ICAM-1 (Segura et al., 2005). De plus, l'inhibition d'ICAM-1 et de son récepteur LFA-1, ou des tétraspanines CD9 et CD81 et de leurs supposés ligands, les intégrines α v et β 3, diminue la capture des exosomes dendritiques par les cellules dendritiques (Morelli et al., 2004). Ensuite, les exosomes peuvent également transmettre leur contenu par fusion directe entre les membranes exosomales et plasmique des cellules cibles. Cela a été décrit dans les cellules dendritiques (Montecalvo et al., 2012) et de mélanome (Parolini et al., 2009). Finalement, le contenu exosomal peut être internalisé par différentes voie d'endocytose : l'endocytose dépendante des caveolae dans les cellules épithéliales (Nanbo et al., 2013), dépendante de la clathrine dans les neurones (Frühbeis et al., 2013) et les cellules PC12 (Tian et al., 2014), indépendante de la clathrine mais dépendante du cholestérol et radeaux lipidiques dans les cellules endothéliales et différents types de cellules cancéreuses (Svensson et al., 2013), macropinocytose par les microglies (Fitzner et al., 2011) et cellules tumorales (Nakase et al., 2015) ou phagocytose par les macrophages (Feng et al., 2010) et les PC12 (Tian et al., 2014).

Tian et collègues (Tian et al., 2010) ont montré que la température influence l'endocytose des exosomes, puisqu'à 4°C, l'absorption des exosomes, dépendant du réseau d'actine, est inhibé. Il a également été observé dans les mélanomes que la fusion des exosomes était optimale à température physiologique (37°C) comparée à des températures plus basses (Parolini et al., 2009). Ils ont également remarqué que la fusion des exosomes avec des cellules tumorales peut être modulée par le pH : en condition acide (pH=6), la libération des exosomes par les mélanomes est augmentée et les exosomes sont mieux absorbés par les mélanomes.

Pour améliorer l'absorption des exosomes, Nakase et Futaki (Nakase and Futaki, 2015) ont proposé de combiner des lipides cationiques avec un peptide sensible au pH qui augmente la liaison des exosomes à la membrane plasmique et favorise leur endocytose.

Il est intéressant de noter que l'héparine peut bloquer la liaison à la membrane et l'absorption des exosomes (Atai et al., 2013). Bien que le mécanisme exact ne soit pas encore élucidé, les auteurs suggèrent que les exosomes contiennent des ligands qui peuvent se lier directement à l'héparine, empêchant ainsi la liaison des exosomes avec les récepteurs des cellules cible.

f) Biodistribution des exosomes

L'intérêt des exosomes comme outils thérapeutiques étant réel, il est essentiel de connaître leur devenir dans l'organisme en étudiant leur biodistribution. Des études indépendantes indiquent que la demi-vie d'exosomes purifiés après injection en intraveineuse chez la souris, est très courte, de l'ordre de deux minutes (Takahashi et al., 2013). Différentes études de biodistribution d'exosomes injectés en intraveineuse démontrent que ces vésicules s'accumulent principalement dans le foie, la rate et les poumons (Lai et al., 2014 ; Manca et al., 2016 ; Takahashi et al., 2013 ; Wiklander et al., 2015). Cependant, Manca et al (Manca et al., 2016) décrivent après 24h, que les exosomes dérivés de lait de vache sont principalement détectés dans le foie et la rate après injection intraveineuse alors qu'après administration orale, la majorité des exosomes est localisée dans les intestins. De plus, il a été montré que le profil de distribution des exosomes était modifié après injection dans la souris par trois voies différentes : intraveineuse, intrapéritonéale et subcutanée (Wiklander et al., 2015). Cette équipe a également montré que l'origine cellulaire des exosomes peut influencer leur biodistribution en comparant les différents taux d'accumulation des exosomes provenant de cinq lignées cellulaires différentes (Wiklander et al., 2015).

Ainsi, la biodistribution des exosomes semble dépendre de leur origine cellulaire, de la voie d'administration et de la capacité des différentes cellules cibles à internaliser des exosomes spécifiques.

Par ailleurs, la plus lente absorption des exosomes chez des souris présentant un système immunitaire inné diminué et une déficience du complément suggère que l'absorption des exosomes peut être régulé par le système immunitaire inné (Smyth et al., 2015).

g) Isolement des exosomes

Les exosomes sont habituellement purifiés à partir de surnageant de culture cellulaire ou de fluides biologiques par des centrifugations (les même que pour isoler les MP) et ultracentrifugations en série, réalisées à 4°C et classiquement isoler à 100,000×g durant 70 minutes. Cependant, l'intensité et la durée des différentes étapes de centrifugation peuvent varier selon le type de fluide récupéré. Pour une meilleure purification, il est possible d'effectuer l'utracentrifugation sur gradient de sucrose (2-0,25 M sucrose). Les exosomes flottent à une densité comprise entre 1,13 à 1,19 g/ml. Certaines étapes de centrifugation peuvent également être évitées en filtrant la préparation avec des filtres de 0,22 µm (Théry et al., 2001).

Il est aussi possible d'immunoprécipiter les exosomes, sans besoin d'ultracentrifuger, en utilisant des billes magnétiques couplées à des anticorps (Clayton et al., 2001) dirigés contre des protéines exposées à la membrane des exosomes. Cependant cette approche permet d'isoler uniquement une sous-population d'exosomes marquées positivement, donc l'analyse qui en découlera ne reflètera pas l'entière population des exosomes (Théry et al., 2001). Enfin, les techniques de chromatographie d'exclusion de taille, basées sur la séparation des particules selon leur taille, présenteraient l'avantage de permettre une meilleure élimination de possibles contaminants isolés avec les exosomes, tout en conservant leur intégrité et activité biologique (Blans et al., 2017). Des vésicules plus uniformes en taille et dont les composants sont retrouvés en plus grande quantité (protéines et ARN) sont observés par chromatographie *versus* centrifugation (Taylor et al., 2011). Différents kits commerciaux comme l'Exoquick, permettant l'isolement des exosome ont été commercialisés. Il a été montré que les exosomes circulants isolés par précipitation à l'ExoQuick produit de l'ARN et des protéines exosomal d'une plus grande pureté avec un rendement d'isolement supérieur aux techniques de chromatographie et d'ultracentrifugation (Taylor et al., 2011). Ainsi, les kits d'extraction semblent être une bonne alternative lorsque les quantités en échantillons biologiques d'origine sont limitées.

En résumé, de multiples méthodes d'isolement des exosomes existent. La plus adaptée dépendra de la quantité de matériel disponible et de l'objectif final dans l'utilisation de ces vésicules. Cependant, ces études comparatives ont montré que ces différentes stratégies d'isolation produisaient différentes populations de VE, d'où le besoin d'un consensus pour une méthode d'isolement standardisée.

h) Méthodes de caractérisation

Une simple purification par ultracentrifugation n'est pas suffisante pour définir les vésicules isolées comme étant des exosomes et une association de critères protéique, morphologique et physique doit être utilisée pour identifier les exosomes parmi les autres VE. Tout d'abord, la morphologie et la taille des exosomes peuvent être déterminées par microscopie électronique à transmission (MET), qui permet de visualiser directement ces vésicules. En la combinant avec un

immunomarquage aux billes d'or, on peut ajouter des informations biochimiques concernant la surface des exosomes. Ensuite, le Nanoparticle tracking analysis (NTA) permet de déterminer non seulement la taille mais aussi la concentration de particules de 30 à 1000 nm de diamètre (Balaj et al., 2012). Le logiciel NTA est alors capable de suivre chaque trajectoire des VE grâce à leur mouvement Brownien, de relier ce mouvement à une taille de particule et de calculer leur concentration totale. Enfin, les marqueurs exosomaux peuvent être identifiés et quantifiés par des méthodes classiques comme le western blot, la spectrométrie de masse ou encore la cytométrie en flux.

II- Implication des exosomes dans différentes pathologies

Il a été montré que les exosomes sont impliqués dans de nombreuses pathologies (pour revue voir Mallocci et al., 2017, en annexe). Pendant ma thèse, j'ai pu étudier leurs possibles rôles dans deux pathologies différentes : une étude chez des patients présentant des troubles métaboliques afin d'étudier l'implication des exosomes dans le syndrome métabolique, et des résultats plus préliminaires sur le rôle des exosomes comme biomarqueurs chez des patients atteints de cancer du rein.

Ainsi, je vous présenterai dans un premier temps le syndrome métabolique ainsi que l'implication des vésicules extracellulaires dans cette pathologie.

Puis dans un deuxième temps, je vous présenterai le rôle des exosomes dans le cancer, et plus particulièrement le cancer du rein.

1) Syndrome métabolique

a) Définition

Le syndrome métabolique (SM) n'est pas une entité nouvelle puisque dès les années 1950, Jean Vague avait décrit une forme d'obésité caractérisée par la répartition des graisses dans la partie supérieure du corps (obésité androïde) prédisposant au diabète et à l'athérosclérose. En 1988, GM Reaven a défini plus précisément cette pathologie en regroupant différentes anomalies cliniques et biologiques sous le terme de syndrome X ou syndrome d'insulinorésistance (Reaven, 1988). Par la suite, les termes de syndrome plurimétabolique ou dysmétabolique ont également été employés. Si ces différentes terminologies sont toutes synonymes, les premières définitions médicales de ce syndrome ne sont apparues que récemment (la première en 1999 par l'Organisation Mondiale de la Santé (OMS)), en raison de l'augmentation de la prévalence de l'obésité.

Actuellement, le SM est défini comme un ensemble de facteurs de risques pour les maladies

cardiovasculaires et le diabète de type 2. Ces facteurs comprennent l'hypertension, un taux élevé en triglycérides, une hyperglycémie, une obésité viscérale représentée par le tour de taille, et un taux diminué en cholestérol HDL. Un patient atteint de syndrome métabolique doit présenter au moins 3 de ces 5 critères (Alberti et al., 2009).

Ces critères ont été définis par plusieurs organisations, comme l'EGIR (European Group for the Study of Insulin Resistance) ou encore le NCEP:ATPIII (National Cholesterol Education Program-Third Adult Treatment Panel) (O'Neill et al., 2016). Cependant, le classement des patients de notre étude sera basé sur ceux définis par l'IDF (International Diabetes Federation). Il est à noter que dans la définition de l'IDF, le critère de l'obésité centrale proposé est celui pour les populations blanches d'origine européenne, et qu'il est modulé selon l'origine ethnique de la population.

Critères :	Homme	Femme
Tour de taille (cm)	≥94	≥80
Glycémie (g/l)	≥1	
Cholestérol HDL (g/l)	<0,40	<0,50
Tension (mmHg)	Systolique ≥ 130 et/ou diastolique ≥ 85 mm Hg	
Triglycérides (g/l)	≥1,5	

Tableau 1 : Critères pour le diagnostic du syndrome métabolique (SM), définis par la Fédération International du Diabète (IDF). Le patient défini comme SM doit présenter au moins trois de ces cinq critères.

Si le SM semble actuellement bien défini, ses origines font encore débat. Pour certains, l'insulinorésistance reste au cœur des anomalies parce qu'elle prédispose au diabète de type 2, aux dyslipidémies qui lui sont associées et à un état pro-inflammatoire, mais elle n'est cependant pas directement corrélée à l'hypertension. Pour d'autres en revanche, l'obésité représente le facteur important dans l'étiologie du syndrome métabolique, contribuant à l'hyperglycémie, l'hypertension et l'hypercholestérolémie (Junquero and Rival, 2005).

b) Quelques chiffres

Le SM présente un intérêt clinique important à cause de sa prévalence élevée (20,3% en France selon l'IDF en 2007) (Vernay et al., 2013). Cette pathologie tend à se répandre du fait de l'augmentation de la prévalence de l'obésité, qui a plus que doublé de 1980 à 2014, selon l'OMS. Une personne est considérée obèse lorsque son Indice de Masse Corporelle (IMC) est égale ou

supérieur à 30 kg/m², et l'intervalle entre 25–30 kg/m² correspond à une personne en surpoids. En 2014, 600 millions d'adultes (13% de la population mondiale) et 41 millions d'enfants âgés de moins de 5 ans ont été diagnostiqués obèses, et ce nombre est en constante augmentation. De ce fait, en 2015, la projection de l'OMS montrait qu'en 2030, plus de 57,8% de la population adulte mondiale – 3,3 milliard de personnes– pourrait être soit en surpoids soit obèse. Il a été montré que le risque de développer le SM augmente avec l'IMC et l'âge (Vernay et al., 2013). On peut alors s'attendre à ce que le SM devienne un véritable problème de santé mondiale.

En plus des problèmes de santé directement associés avec le SM, cette pathologie augmente de 5 fois le risque de développer un diabète de type 2 et de trois fois celui de développer des maladies cardio-vasculaires. Chez l'adolescent, et plus particulièrement chez l'enfant, une relation est établie entre une obésité dans l'enfance et le niveau du risque cardiovasculaire à l'âge adulte (Weiss et al., 2004) et de récentes études ont identifié les dysfonctionnements endothéliaux comme les prémices des complications à l'âge adulte (Tounian et al., 2001). Il a également été montré que le SM augmenterait le risque de développer certains cancers (Esposito et al., 2012). Ainsi, le SM contribue à réduire la qualité et l'espérance de vie des personnes qui en sont atteintes.

c) Pathophysiologie du SM

Comme expliqué précédemment, les mécanismes impliqués dans l'apparition du SM restent à ce jour encore mal connus. Il n'y aurait pas de cause unique responsable de ces anomalies. Effectivement, l'obésité viscérale et l'insulino-résistance font parties intégrante des mécanismes incriminés dans la genèse et l'évolution du SM (Delarue et al., 2006) mais également des causes environnementales (le stress par exemple) et génétiques pourraient être impliquées.

La présence d'une obésité et donc l'accumulation de graisse viscérale est donc une cause déterminante dans ce syndrome. Les patients présentant un tour de taille élevé se distinguent par des anomalies de la tolérance au glucose, une hypertriglycémie, une baisse du HDL-cholestérol et parfois une augmentation d'apolipoprotéine B, un marqueur de risque de survenue de maladie cardiovasculaire. Plus précisément, cette accumulation de graisse viscérale est associée à un excès d'acides gras libres provenant d'une lipolyse élevée dans le tissu adipeux. La recapture et le stockage d'acides gras libre au niveau hépatique entretient l'évolution du SM en favorisant la résistance périphérique à l'insuline et la néoglucogenèse. Cette dyslipidémie caractéristique du SM est caractérisée par la production accrue de lipoprotéine de basse densité de petite taille et sensibles à l'oxydation (LDL oxydées). Dans le tissu adipeux, les LDL oxydées permettent l'infiltration des monocytes circulants lors de leur adhésion aux cellules endothéliales vasculaires. Les cellules immunitaires infiltrées, en libérant des cytokines, vont alors développer un état inflammatoire. De plus, le tissu adipeux, au-delà de son rôle dans le stockage de lipides et de source d'acides gras, est un organe endocrine et paracrine qui secrète de nombreuses adipokines (le facteur de nécrose tumorale- α (TNF α), les interleukines 2 et 6 (IL2/6), l'adiponectine, la

leptine...) qui jouent un rôle majeur dans l'homéostasie énergétique et l'inflammation. L'obésité est associée à une production anormale de ces adipokines par le tissu adipeux, qui vont participer à l'inflammation et perturber la signalisation insulinique intracellulaire, favorisant ainsi l'hyperglycémie et le diabète de type II (Figure 7) (Müller, 2012).

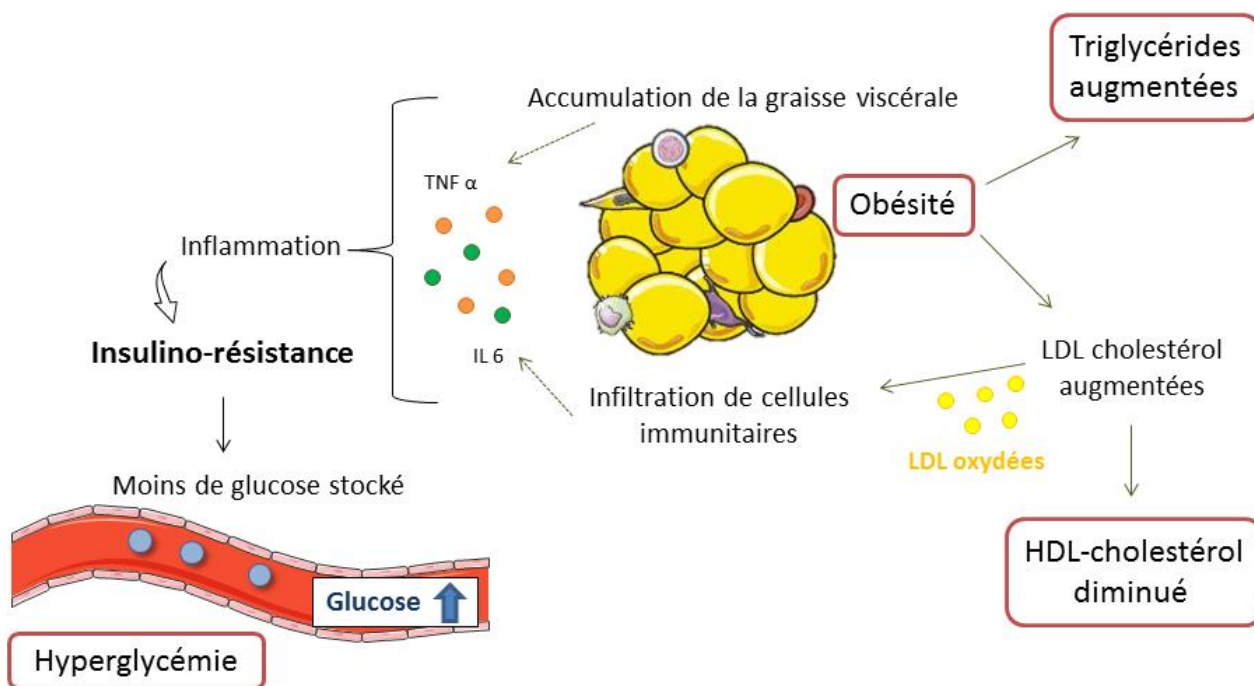


Figure 7 : Représentation schématique des mécanismes participant au développement du SM : Les patients présentant une obésité vont développer un état inflammatoire médié par des cytokines comme l'IL-6 et le TNF α . Cette inflammation va favoriser une insulino-résistance et mener à une hyperglycémie. La dyslipidémie associée à l'obésité est caractérisée par un taux de triglycérides augmenté, une augmentation des LDL-cholestérol et son oxydation, et une baisse du HDL-cholestérol.

L'ensemble de ces anomalies, souvent associées à une augmentation de la pression artérielle liée à une atteinte vasculaire, va mener au développement de l'athérogenèse et contribue à l'augmentation maladies cardio-vasculaires chez ces patients. Il semble cependant que les premiers stades de risque vasculaire correspondent à l'apparition d'une dysfonction endothéliale. En effet, il a été mis en évidence une altération de la fonction endothéliale en relation avec l'obésité, et plus particulièrement avec la graisse viscérale. Une dysfonction endothéliale est également détectée de façon très précoce associée à l'insulinorésistance avant même l'installation d'une intolérance au glucose ou d'un diabète (Ducluzeau et al., 2005).

i- Rôles de l'endothélium

Puisque l'endothélium vasculaire joue un rôle important dans le tonus et le remodelage vasculaire, sa dysfonction est une voie importante du développement des maladies vasculaires (Giannotti and Landmesser, 2007). L'endothélium est une couche monocellulaire qui tapisse la lumière de vaisseaux. Il sert de véritable barrière mais aussi d'interface active entre les tissus et la

circulation sanguine. L'endothélium régule finement la vasomotricité en produisant des agents vasodilatateurs comme le monoxyde d'azote (NO), la prostaglandine I₂ ou encore l'EDHF (facteur hyperpolarisant dérivé de l'endothélium) et des agents vasoconstricteurs comme l'endothéline et le thromboxane A₂. De plus, l'endothélium maintient une certaine fluidité sanguine en empêchant la formation de caillot par exemple, il contrôle la coagulation à travers la production de facteurs qui régule l'activité plaquettaire, la cascade de coagulation, et le système fibrinolytique (van Hinsbergh, 2012). Enfin, l'endothélium a la capacité de produire des cytokines et des molécules d'adhésion qui régule et dirige le processus inflammatoire (Widlansky et al., 2003). Ainsi, l'endothélium normal non activé favorise plutôt la vasodilatation, empêche les phénomènes de coagulation et ne facilite pas le passage d'éléments circulants vers le sous-endothélium, à l'inverse d'une dysfonction endothéliale.

ii- Le monoxyde d'azote (NO)

Le NO, principal agent vasodilatateur produit par les cellules endothéliales, joue un rôle majeur dans la fonction de ces cellules. Il peut être produit par trois différentes NO synthases (NOS) : la NOS neuronale (nNOS), la NOS endothéliale (eNOS) et la NOS inducible (iNOS) qui est spécialement transcrite en réponse à certains stimuli, comme la réponse inflammatoire. Les NOS permettent la formation de NO et de L-citrulline à partir de la L-arginine. A la suite de forces de cisaillement (« shear stress ») ou l'action de vasodilatateur comme l'acétylcholine, l'augmentation de calcium intracellulaire induit l'activation de la eNOS. Le NO alors produit diffuse jusqu'aux cellules musculaires lisses (CML) et active la guanylate cyclase, augmentant ainsi la production de guanosine monophosphate cyclique (GMPc). Le GMPc va ensuite activer la protéine kinase G qui va permettre une diminution du calcium intracellulaire et ainsi une relaxation des CML et une vasodilatation (Michel and Vanhoutte, 2010). Par ailleurs, il existe certaines molécules pharmacologiques qui libèrent du NO, comme le nitroprussiate de sodium qui permettent la relaxation des CML indépendamment de l'activation de la eNOS (Figure 8).

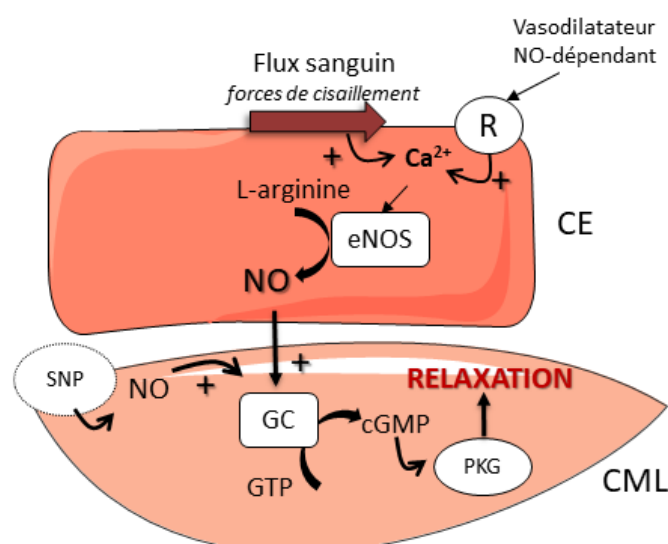


Figure 8: Mécanisme d'action du monoxyde d'azote (NO) en tant que vasodilatateur : en réponse à différents stimuli comme les forces de cisaillement ou l'action d'un agent vasodilatateur comme l'acétylcholine, l'augmentation de calcium intracellulaire va favoriser la production de NO par la NO synthase endothéliale (eNOS) et diffuser jusqu'aux cellules musculaires lisses (CML). Il va alors activer la guanylate cyclase (GC), induisant ainsi la production de GMP cyclique (GMPc). Le GMPc active la protéine kinase G (PKG) qui va induire la relaxation de la CML et une vasodilatation. Le nitroprussiate de sodium (SNP) peut libérer du NO directement à la CML et induire une vasodilatation par cette même voie.

En plus d'avoir un rôle vasodilatateur, le NO agit comme vasoprotecteur. Il inhibe l'activation des plaquettes et maintient les CML dans un état non prolifératif (Favero et al., 2014). Il permet également l'inhibition de l'adhésion leucocytaire. En effet, il a été montré que l'inhibition de l'eNOS stimule l'expression de molécules d'adhésion des leucocytes et de chimiokines qui favorisent l'infiltration de monocytes (De Caterina et al., 1995).

Modulation de la production de NO

La régulation de l'activité de l'eNOS fait intervenir plusieurs mécanismes dont l'activation de la transcription du gène de l'eNOS (par le 17β -estradiol par exemple), des modifications post-traductionnelles comme la phosphorylation et le complexe calcium-calmoduline (Ca/CaM) (Figure 9).

Dans un état inactif, l'eNOS est séquestrée dans les cavéoles à la surface des cellules, par interaction avec la Cav-1 (Frank et al., 2003). Lorsque l'acétylcholine ou la force du flux sanguin provoquent une augmentation du calcium intracellulaire, il y a formation du complexe Ca/CaM qui va entrer en compétition avec Cav-1 et induire la libération de l'eNOS (Frank et al., 2003).

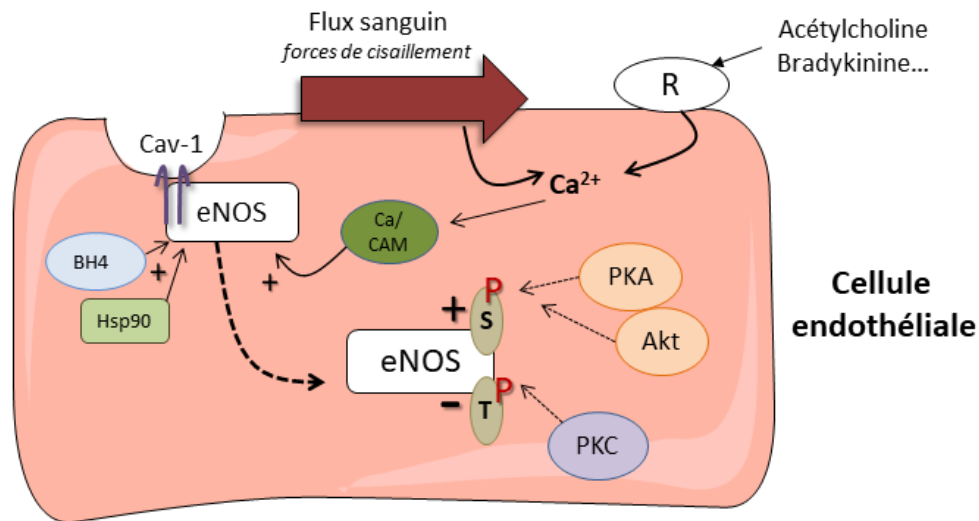


Figure 9: Régulation de l'activité de la NOS endothéliale (eNOS) : A l'état inactif, l'eNOS est séquestrée par la cavéoline 1 (Cav-1) au niveau des cavéoles. L'augmentation du calcium intracellulaire à la suite de forces de cisaillement ou d'agoniste comme l'acétylcholine, va permettre la formation du complexe calcium/calmoduline (Ca/CaM) qui va entrer en compétition avec Cav1 et induire la libération de l'eNOS. L'eNOS peut être également phosphorylée sur ses sites activateurs (Ser1177) et inhibiteur (Thr495) par différentes kinases. Son activité est également favorisée par l'Hsp90 et la tétrahydrobioptérine (BH4).

De plus, l'eNOS possède différents sites de phosphorylation, permettant son activation ou au contraire, son inhibition. Son principal site activateur est le résidu Sérine 1177 (Ser1177) qui peut être phosphorylé par la protéine kinase A (PKA) et Akt suite aux forces de cisaillement, ou encore par la calmoduline kinase II (CamKII) suite à l'action de la bradykinine (Fleming and Busse, 2003). L'activité de l'eNOS peut également être diminuée par phosphorylation au niveau de la thréonine 495 (Thr495), site inhibiteur, par la protéine kinase C (PKC) (Fleming and Busse, 2003).

Enfin, différentes protéines peuvent favoriser l'activité de l'eNOS comme Hsp90, qui augmente la production de NO dans les cellules endothéliales (Fleming and Busse, 2003) ou encore son coactivateur la tétrahydrobioptérine (BH4), qui préserve la dimérisation de l'eNOs et ainsi son activité (Yang et al., 2009).

iii- Le stress oxydant

Le stress oxydant se définit comme un déséquilibre entre la production d'espèces oxydantes (les dérivés réactifs de l'oxygène, appelés ROS (reactive oxygen species), et leur neutralisation par les systèmes de défense antioxydant, état pouvant mener à des dommages cellulaires (Sies, 1997). Les oxydants se divisent en deux catégories, les radicaux libres, ions avec un électron non apparié, et les non-radicaux libres. Le principal ROS produit par les cellules est un radical libre, l'anion superoxyde (O_2^-). Il peut être produit par différentes enzymes comme la NADPH oxydase (NOX), la xanthine oxydase et l'eNOS découplée. La NADPH oxydase permet le

transfert d'électrons provenant de la NADPH vers une molécule d'oxygène, produisant ainsi de l' O_2^- . La famille des NADPH oxydases se compose des NOX 1 à NOX 5, et des DUOX 1 et 2, présentes au niveau de la glande thyroïde. La xanthine oxydase permet également le transfert d'un électron à une molécule d'oxygène. Cet enzyme est aussi responsable de la production d'acide urique. Enfin, dans des conditions de privation de substrat qui se présentent parfois dans l'athérosclérose, le flux normal d'électron dans l'eNOS peut être perturbé et l'enzyme produit de l' O_2^- au lieu du NO (Li and Förstermann, 2013).

L' O_2^- peut se complexer avec le NO et former un oxydant non-radicalaire, le peroxy-nitrite ($ONOO^-$), qui entraîne, d'une part, la diminution de la biodisponibilité du NO, et d'autre part, la nitration des protéines qui altère leur activité. Le peroxyde d'hydrogène, non radicalaire, complète ce groupe des principales molécules oxydantes dans l'athérosclérose (Stocker and Keaney, 2005). La mitochondrie peut également être une source de production de ROS (Stocker and Keaney, 2005) qui représentent 0,1 à 2% des ROS totaux dans la cellule endothéliale (Kluge et al., 2013).

En conditions normales, les ROS sont maintenus à un niveau optimal grâce à la balance entre leur production et leur élimination par des antioxydants enzymatiques (superoxyde dismutase (SOD), glutathione, catalase, peroxydase) et non-enzymatiques (vitamines C et E). Dans un état pathologique comme le SM, une augmentation de la production de ROS associée à une diminution de la capacité anti-oxydante est à l'origine d'un environnement déséquilibré résultant en un stress oxydant (Hutcheson and Rocic, 2012).

L'augmentation du niveau de ROS lors du stress oxydant a des effets toxiques sur les cellules et les tissus à travers l'oxydation des carbohydrates, des lipides et des protéines. Toutes ces réactions favorisent également la formation de LDL oxydées.

iv- La dysfonction endothéliale

La dysfonction endothéliale peut être définie par une altération de la capacité de l'endothélium à induire une vasodilatation. De plus, lors d'une dysfonction endothéliale, les cellules endothéliales favorisent les phénomènes de coagulation et le recrutement actif de cellules inflammatoires circulantes.

Les éléments principaux responsables de cette dysfonction endothéliale semblent être une augmentation du stress oxydatif et une diminution de la biodisponibilité du NO (Palmer et al., 1987). Cette diminution de la biodisponibilité du NO peut provenir de son interaction avec l' O_2^- pour former l' $ONOO^-$ (Figure 10). Elle peut également être induite par une diminution de l'activité de l'eNOS par une phosphorylation de la thréonine 495 (Oliveira-Paula et al., 2016) et une augmentation de l'interaction de l'eNOS avec Cav-1 induisant une perte de son activité (Kraehling et al., 2016).

La diminution de la biodisponibilité du NO, comme vu précédemment, favorise l'activation et l'agrégation plaquettaire, l'infiltration des monocytes, la prolifération des CML, l'activation des cellules endothéliales et l'augmentation du stress oxydant (Figure 10).

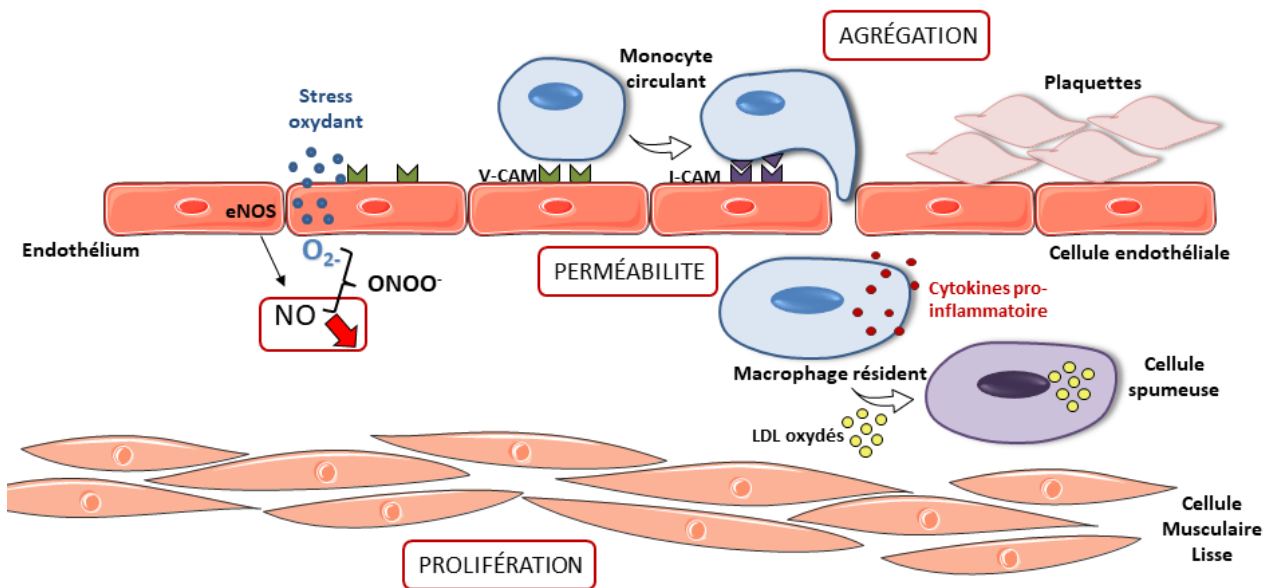


Figure 10 : Mécanismes à l'origine d'une dysfonction endothéliale : La diminution de la biodisponibilité du NO va permettre une agrégation des plaquettes et des cellules inflammatoires, une infiltration des monocytes et la formation de cellules spumeuses ainsi que la prolifération des cellules musculaires lisses. Tous ces phénomènes vont alors favoriser le développement de l'athérosclérose et, à plus long terme, de maladies cardiovasculaires.

Plus précisément, l' O_2^- produit par les cellules endothéliales, en plus de diminuer la biodisponibilité du NO, va engendrer une augmentation de l'apoptose des cellules vasculaires, l'activation de certains facteurs de transcription tels que la voie inflammatoire p65-NF κ B et induire un environnement proinflammatoire propice au développement athérosclérotique (Ellulu et al., 2016). De plus, les cellules inflammatoires vont s'accumuler au niveau des cellules endothéliales à cause de la surexpression de molécules d'adhésion (VCAM, ICAM). Ces cellules inflammatoires vont s'infiltrer au niveau de la couche sous endothéliale et s'y accumuler notamment grâce une augmentation de la perméabilité endothéliale. Parmi les cellules inflammatoires, les monocytes vont se différencier en macrophages, capable de libérer des cytokines proinflammatoires. Dans le même temps, les LDL vont également pouvoir passer la barrière endothéliale où leur oxydation sera favorisée par le stress oxydant. A ce niveau, les macrophages vont internaliser les LDL oxydées et se transformer en cellules dites spumeuses. L'accumulation de ces cellules chargées de graisse va former la strie lipidique, étape initiale de la formation d'une plaque d'athérome au sein de la paroi artérielle. La prolifération des CML va entraîner leur migration vers ce cœur lipidique et participer au développement à la formation d'une matrice fibreuse autour.

Enfin, alors que la plaque athérosclérotique se développe, un des risques majeurs est la rupture de cette plaque. Le système de coagulation peut s'activer, entraînant une agrégation plaquettaire au niveau de la zone de rupture formant un thrombus perturbant le flux sanguin.

En résumé, la dysfonction endothéliale perturbe les mécanismes de régulation de l'homéostasie vasculaire prédisposant la paroi vasculaire à la vasoconstriction, l'adhésion leucocytaire, l'activation plaquettaire, le stress oxydant, l'inflammation, la coagulation et la thrombose, menant ainsi à la pathogenèse des maladies cardiovasculaires (Favero et al., 2014).

Cet organe à part entière qu'est l'endothélium joue donc un rôle déterminant dans le maintien de l'homéostasie vasculaire. Ses dérégulations peuvent ainsi avoir de graves conséquences cardiovasculaires à long terme. La recherche de thérapies dirigées contre la dysfonction endothéliale paraît une bonne stratégie afin d'améliorer l'état des patients atteints de SM et d'empêcher le développement des maladies cardiovasculaires.

v- Le rôle de la mitochondrie

La mitochondrie est un organite principalement défini comme le centre énergétique de la cellule, mais elle régule également d'autres processus cellulaires, incluant la prolifération (Mitra et al., 2009), la réponse immunitaire (Zhou et al., 2011) ou encore l'apoptose (Kroemer et al., 2007). Les cellules comportent entre 300 à 2 000 mitochondries dont le diamètre peut varier de 0,75 à 3 µm et dont la longueur peut atteindre 10 µm.

Elles se composent de deux membranes, une membrane mitochondriale externe et une membrane mitochondriale interne, qui délimitent trois milieux : le milieu extra-mitochondrial (cytoplasme de la cellule), l'espace intermembranaire mitochondrial, et la matrice mitochondriale. La mitochondrie contient son propre génome, l'ADN mitochondrial, codant principalement pour les différents composants de la chaîne respiratoire. Ce génome est présent sous forme de 5 à 10 copies dans la mitochondrie et sa traduction est assurée par les ribosomes mitochondriaux.

Le rôle essentiel de la mitochondrie est de produire de l'énergie pour la cellule, à travers la β -oxydation, le cycle de Krebs et la chaîne respiratoire dans le cadre de la phosphorylation oxydative. La chaîne respiratoire mitochondriale, qui est composée de quatre complexes, permet le transport d'électrons aboutissant à la production d'ATP par l'ATP synthase. Comparé aux cardiomyocytes ou à d'autres types cellulaires, l'apport énergétique provenant de la mitochondrie dans l'endothélium est relativement bas, l'essentiel de la production d'ATP dans ces cellules provenant du métabolisme glycolytique anaérobie. Ainsi, le contenu en mitochondrie des cellules endothéliales est moins élevé que dans certaines cellules ayant de forts besoins en énergie. Dans des cellules endothéliales de rat par exemple, les mitochondries composent 2–6% du volume cellulaire contrairement à 28% dans les hépatocytes et 32% dans les cardiomyocytes (Kluge et al., 2013). Même si les cellules endothéliales ont une faible teneur en mitochondries, la dynamique mitochondriale agit comme un orchestre pivot de l'homéostasie cellulaire dans des

conditions normales. De ce fait, le contenu de la mitochondrie est très fortement régulé dans les cellules.

Le contenu cellulaire en mitochondrie dépend de la balance entre la biogenèse mitochondriale et la mitophagie, la dégradation des mitochondries (Figure 11). La biogenèse des mitochondries est un processus complexe et pas encore entièrement élucidé, impliquant la réplication de l'ADN mitochondrial (ADNm) et l'expression de gènes nucléaires et mitochondriaux. Elle est activée lors d'une demande d'énergie, d'une restriction calorique, ou d'un exercice physique, par exemple. La formation de nouvelles mitochondries est régulée par le coactivateur transcriptionnel PGC-1 α (peroxisome proliferation-activated receptor coactivator 1 α), qui active l'expression des facteurs respiratoires nucléaires 1 et 2 (NRF-1 et NRF-2) et les facteurs de transcriptions mitochondriaux A et B (TFAM et TFAM) qui mettent en place la biogenèse mitochondriale.

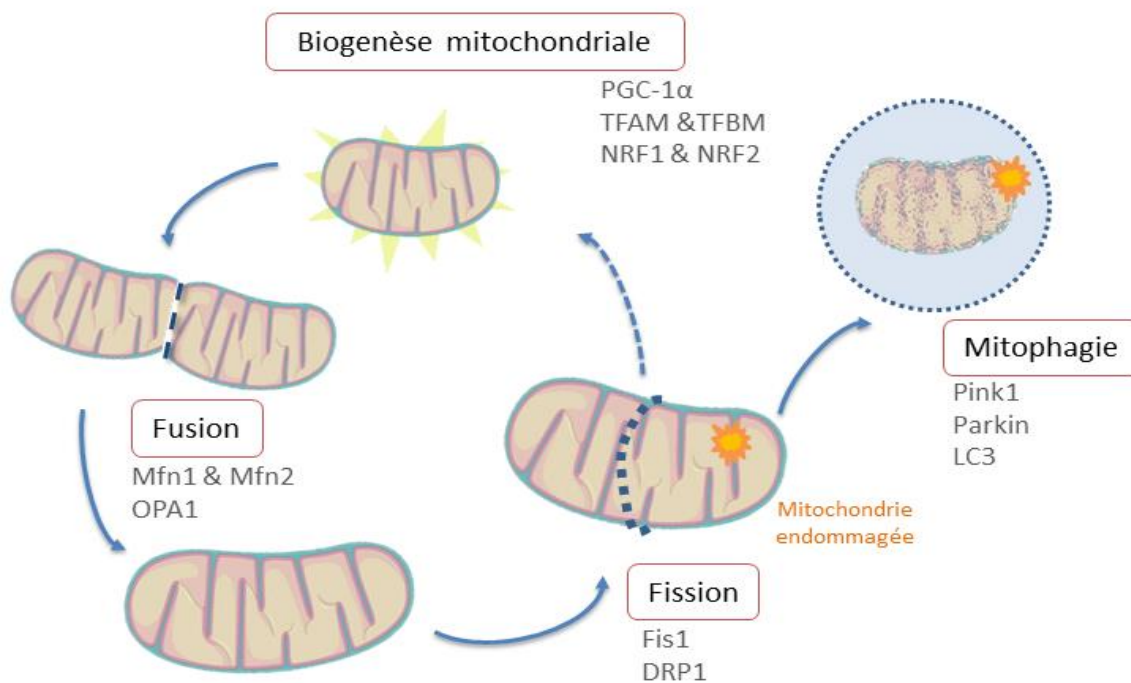


Figure 11 : Cycle de vie d'une mitochondrie : Lors d'une demande en énergie, la biogenèse de mitochondrie est initiée par le coactivateur PGC-1 α . Les mitochondries formeront alors un réseau dynamique capable de fusionner, afin de partager leur contenu, et de fission, pour éliminer leur partie endommagée et redevenir fonctionnelle. Les mitochondries endommagées ou sénescents sont ensuite dégradées par mitophagie, l'autophagie spécifique des mitochondries.

Les mitochondries forment un réseau dynamique qui fusionne et se divise (« fissionne ») constamment et peuvent ainsi créer un long réseau intracellulaire interconnecté (Figure 11). Elles sont capables de fusionner entre elles afin de faciliter la distribution des métabolites, des protéines et de l'ADNm et ainsi de répondre plus efficacement à une demande énergétique par exemple. Ce mécanisme permet également de diluer les ROS mitochondriaux. La fusion mitochondriale est assurée par trois GTPases de la famille des dynamines : la fusion des membranes externes est assurée par les Mitofusines 1 et 2 (Mfn1 et Mfn2) et la fusion de la membrane interne par OPA1

(Optic Atrophy 1). Le phénomène de fission mitochondriale permet d'éliminer une partie des mitochondries endommagées pendant un stress ou l'élimination de mitochondries sénescentes, lorsqu'elle est suivie par la mitophagie. Sur la membrane externe, le récepteur FIS1 va recruter la GTPase DRP1 (Dynamain-related Protein 1). DRP1 va alors s'assembler autour de la mitochondrie et la contraindre de manière dépendante du GTP pour assurer sa scission. DRP1 peut être régulé par phosphorylation sur ses sites activateurs et inhibiteurs (Mishra and Chan, 2016). Ces événements de fusion et fission, définis comme la dynamique mitochondriale, sont essentiels afin de générer des mitochondries de nouveau fonctionnelles et toujours assurer les besoins de la cellule. Les mitochondries fonctionnelles assurent de nouveau leur rôle dans la cellule alors que celles qui sont endommagées se dépolarisent et se font dégrader par mitophagie, l'autophagie spécifique des mitochondries. La mitophagie est principalement assurée par les protéines PINK1 (PTEN-induced putative kinase 1), située sur la membrane externe, et Parkin. PINK1 est normalement transloquée de la membrane externe vers la membrane interne pour être dégradée. Quand la mitochondrie n'est plus fonctionnelle, elle va perdre son potentiel de membrane qui empêche cette dégradation. PINK1 va alors recruter Parkin, qui assure l'ubiquitination de protéines mitochondriales situées sur la membrane externe, comme Mfn1 et 2, par exemple. L'ubiquitination des protéines de surface mitochondriales est reconnue par la protéine p62, qui va permettre le recrutement de la protéine LC3 (microtubule-associated protein 1 light chain 3), associée aux autophagosomes et ainsi permettre la mitophagie. Les mitochondries peuvent également être ciblées pour l'autophagie par l'action de Nix, un récepteur de la membrane mitochondriale qui pourrait également interagir avec LC3 (Kludge et al 2013).

Source de ROS

Lors de la respiration mitochondriale, les complexes I et III de la chaîne respiratoire peuvent produire de l' O_2^- . L' O_2^- formé par le complexe I est libéré dans la matrice mitochondriale, alors que l' O_2^- formé par le complexe III est libéré soit dans la matrice mitochondriale soit dans le cytoplasme via le transporteur mitochondrial Voltage Dependent Anion Channel (VDAC), situé sur la membrane externe mitochondriale (Zhang and Gutterman, 2007). En plus des complexes I et III, d'autres sources de ROS mitochondriaux (mROS) pourraient intervenir. En effet, la NADPH oxydase 4 (NOX4), qui est fortement exprimée dans les cellules endothéliales et qui a été localisée dans les mitochondries d'autres tissus, pourrait participer à cette production, bien que sa présence au niveau des mitochondries des cellules endothéliales ne soit pas encore confirmée (Tang et al., 2014).

Lorsque que la production de mROS devient excessive, la cellule répond à ce stress en les ciblant directement. Pour cela, la manganèse superoxide dismutase (MnSOD), la dismutase principale de la mitochondrie, est rapidement induite et inactive l' O_2^- dans la matrice mitochondriale en le transformant en H_2O_2 (Dromparis and Michelakis, 2013).

Implication de la mitochondrie dans la dysfonction endothéliale

Une des implications majeures de la mitochondrie dans la dysfonction endothéliale est l'élévation de la production de mROS. Effectivement, si la production de mROS est trop élevée et dépasse la capacité des dismutases, apparaissent alors des dommages oxydatifs, une dysfonction cellulaire ou encore la mort cellulaire. Par ailleurs, il a été montré que des souris hypertendues traitées avec un chélateur de ROS mitochondriaux, le mitoTEMPO, présentaient une diminution de l'O₂⁻ vasculaire, une augmentation de la production de NO et une amélioration de la relaxation vasculaire dépendante de l'endothélium (Dikalova et al., 2010) suggérant l'implication des mROS dans la dysfonction endothéliale.

Les mROS sont augmentés en réponse à de nombreux facteurs du SM : les LDL oxydées, les triglycérides et l'hyperglycémie (Tang et al., 2014). Par exemple, l'exposition des cellules endothéliales à l'acide palmitique augmente la mitophagie, la production de mROS et l'apoptose (Wu et al 2015). De plus, les ROS produits dans le micro-environnement vasculaire provoquent des dysfonctionnements mitochondriaux, qui à leur tour amplifient cet effet en partie en raison de sa capacité antioxydante réduite. La synthèse des protéines mitochondriales est d'ailleurs inhibée de manière dépendante de la dose par l'ONOO⁻, entraînant une diminution des taux d'ATP cellulaires et de la fonction rédox mitochondriale (Ballinger et al., 2000).

La perturbation de la dynamique mitochondriale peut également être responsable de l'élévation des mROS et participer à la dysfonction endothéliale. En conditions normales, il existe un équilibre entre la fusion et la fission mitochondriale, mais en conditions pathologiques, la fission peut primer sur la fusion, menant ainsi à une fragmentation du réseau qui n'est alors plus fonctionnel. Ainsi, chez de patients diabétiques, il a été observé que les cellules endothéliales isolées à partir de veines présentaient une fragmentation du réseau mitochondriale et une augmentation de l'expression de Fis1. De plus, *in vitro*, les cellules endothéliales aortiques humaines cultivées en présence de fortes concentrations de glucose montrent également une perte du réseau, une augmentation de l'expression de Fis1 et DRP1 et une augmentation de la production de mROS. Cette perturbation de la dynamique mitochondriale est associée à une perturbation dans l'activation de l'eNOS et la production de GMPc. Par ailleurs, la suppression de FIS1 ou DRP1 dans ces conditions maintient l'activité de l'eNOS et la biodisponibilité du NO probablement en diminuant les mROS (Shenouda et al., 2011).

Ainsi, dans des conditions normales ou les stades précoces de maladies vasculaires, la combinaison de la dynamique mitochondriale, la mitophagie et la biogenèse mitochondriale peut remplacer les mitochondries endommagées ou leurs composants et maintenir une fonction mitochondriale normale. Néanmoins, ces mécanismes de contrôle-qualité peuvent s'altérer, ce qui entraîne l'accumulation de mitochondries non fonctionnelles productrices de ROS, facilitant ainsi les maladies vasculaires.

d) Prévention et thérapies actuelles pour le SM

La plupart des patients présentant un syndrome métabolique nécessitent une thérapie médicamenteuse associée à des mesures alimentaires ; l'arsenal thérapeutique existant étant essentiellement constitué de composés ciblant un facteur de risque spécifique (Figure 12).

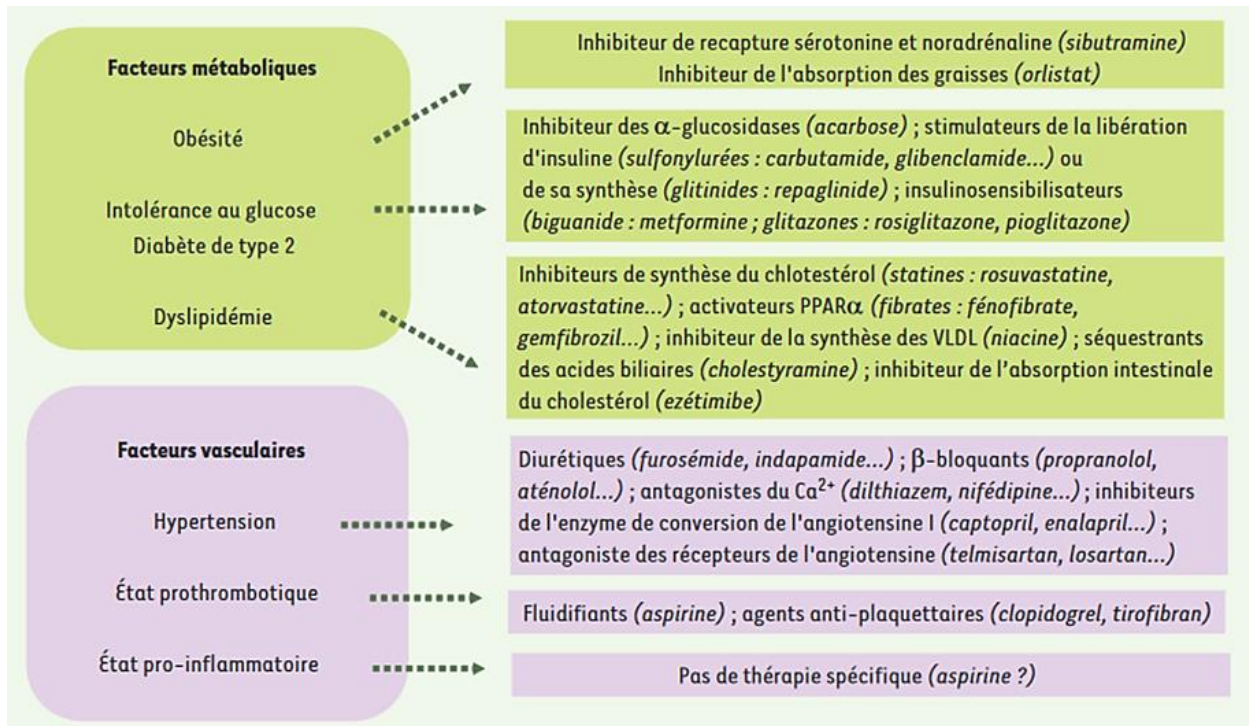


Figure 12 : Traitements thérapeutiques ciblant les différents facteurs du SM : En plus de mesures hygiéno-diététiques, il existe de nombreuses classes de médicaments traitant spécifiquement les différents facteurs du SM, qu'il est possible de combiner afin de mettre en place une thérapie multifactorielle. (Junquero 2005)

Tout d'abord une perte de poids doit être mise en place et principalement venir d'un changement dans les habitudes de vie du patient. Il est démontré que la perte de poids améliore la sensibilité à l'insuline, une baisse du taux de triglycérides et de protéine C réactive (CRP), un marqueur précoce de l'inflammation. L'alimentation recommandée est celle de type méditerranéen, ainsi que son association à de l'exercice physique. Si les modifications apportées au mode de vie ne suffisent pas, il est possible de prendre un traitement amaigrissant faisant appel aux deux molécules actuellement disponibles : la sibutramine et l'orlistat. Cependant, ces traitements sont limités du fait de leurs effets secondaires, limitant leur prescription en présence d'autres facteurs de risque du SM.

Parmi les antidiabétiques, on retrouve des sensibilisants à l'insuline comme la metformine pour aider à réduire le taux de glucose sanguin et d'insuline. Par exemple, la metformine réduit la production hépatique de glucose et cible l'insulinorésistance sans effet de prise de poids. Malgré certains effets secondaires (acidose lactique, notamment), la metformine est un incontournable

souvent proposé en première intention. Il a également été montré que les glitazones améliorent les paramètres constitutifs du SM. Cependant, ils stimulent la prise de poids au niveau de la graisse sous-cutanée et un traitement avec la rosiglitazone pouvait s'accompagner d'une détérioration du profil lipidique et d'une augmentation du risque cardiovasculaire (Junquero and Rival, 2005). Ils ont été retirés du marché en 2011.

Afin de traiter la dyslipidémie et de réduire le mauvais cholestérol (LDL), le médicament le plus prescrit reste les statines. Ces inhibiteurs de la synthèse du cholestérol représentent un traitement majeur du risque cardiovasculaire global, en permettant de réduire l'incidence des crises cardiaques et d'accidents vasculaires cérébraux (Collaboration, 2010). Plusieurs études montrent également un intérêt pour les fibrates en prévention chez les patients avec hypertriglycéridémie et HDL bas, en particulier chez ceux avec une insulino-résistance (Delarue et al., 2006).

Pour abaisser la pression artérielle, les mesures hygiéno-diététiques peuvent déjà diminuer la pression artérielle systolique jusqu'à 15 mm Hg (Junquero and Rival, 2005). Il existe également de nombreux médicaments antihypertenseurs, qui présentent des modes d'action différents. Parmi ces différentes classes de médicaments, des études cliniques soulignent l'intérêt de la modulation du système rénine-angiotensine dans le traitement du diabète. Il a effectivement été montré que le losartan (antagoniste des récepteurs à l'angiotensine) était plus efficace que l'aténolol (β -bloquant également utilisé dans le traitement de l'hypertension) en réduisant la morbidité et mortalité cardiovasculaires chez des patients présentant une hypertension et du diabète (Lindholm et al., 2002).

e) Biomarqueurs du SM

Un biomarqueur est défini comme une caractéristique qui est objectivement mesurée et évaluée comme un indicateur d'un processus biologique normal, d'un processus pathogène ou d'une réponse pharmacologique à une intervention thérapeutique. Il est un indicateur de changement et fluctue donc en fonction du temps et de l'influence biologique (Müller, 2012).

Le processus actuel de diagnostic du SM est multiforme et lourd pour le patient. Bien sûr, les nombreux tests qui contribuent actuellement au diagnostic du SM sont basés sur des marqueurs sanguins. Cependant, les tests additionnels comme la tolérance au glucose et le tour de taille sont également requis. Ainsi, la recherche de meilleurs biomarqueurs moins invasifs sont toujours en cours pour cette pathologie, afin de trouver des biomarqueurs du SM qui pourraient être tous analysés dans un seul échantillon de sang.

Ces biomarqueurs doivent de préférence permettre la surveillance pour pronostiquer l'évolution des patients, de l'efficacité d'un médicament, et aussi une opportunité d'intervention précoce pour éviter le développement de plus graves pathologies, comme le diabète de type 2 et les maladies cardiovasculaires.

De potentiels biomarqueurs pouvant répondre à ces critères ont déjà été proposés. Par exemple, le taux en adiponectine, l'adipokine antiathérogénique, antidiabétique et antihyperlipidémique, est diminué chez les patients atteints de SM. De plus, le taux d'adiponectine semble diminuer avec l'augmentation du nombre de critères du SM (Mohan et al., 2005). Par ailleurs, plusieurs polymorphismes du gène de l'adiponectine, *AdipoQ*, seraient corrélés avec la prévalence du SM (Li et al., 2015). La leptine, l'hormone responsable du sentiment de satiété, pourrait également être un biomarqueur du SM. En effet, les patients atteints de SM présentent un taux augmenté en leptine (Esteghamati et al., 2011) et la combinaison entre son augmentation et un taux diminué en adiponectine serait corrélée avec le SM (Mirza et al., 2011). D'autre part, parmi les nombreux miRNA qui ont été proposés comme impliqués dans les maladies métaboliques, on peut retenir le miR-370 qui se trouve augmenté par le SM (Fernández-Hernando et al., 2013). Enfin, les VE pourraient également servir de biomarqueurs pertinents pour cette pathologie.

2) Rôles des exosomes dans le SM

De nombreuses données de la littérature montrent que les exosomes participent au développement de maladies, soient en tant que biomarqueurs, soient en tant que vecteur de la maladie. En particulier, les exosomes pourraient participer au développement du SM.

a) Exosomes comme biomarqueurs

Il a été suggéré que les VEs pouvaient être des biomarqueurs 1) peu invasifs puisque présents dans les différents fluides biologiques, 2) spécifiques car portant un cargo représentant l'état des différentes cellules de l'organisme et 3) plus stables que certaines molécules circulantes car protégées par une bicouche lipidique.

Il a déjà été démontré que le taux de MP circulantes était plus élevé chez des patients atteints de SM par rapport à des sujets témoins (Agouni et al., 2008). Par ailleurs, il a été suggéré que les exosomes pouvaient également être des biomarqueurs pertinents pour différentes pathologies métaboliques. Plus précisément, les exosomes dérivés des adipocytes sont augmentés dans l'obésité, facteur principal du SM. En effet, il a été décrit que les souris obèses *ob/ob* présentaient un taux augmenté en exosomes exprimant l'adiponectine, marqueur spécifique des adipocytes, comparé à des souris sauvages, suggérant que les adipocytes libèrent d'importante quantité d'exosomes durant l'obésité (Deng et al., 2009). De plus, la sécrétion *in vitro* d'exosomes dérivés d'adipocytes est augmentée à la suite d'une exposition à différents stimuli biologiques reliés à l'inflammation chronique associée à l'obésité comme le TNF- α et l'acide palmitique, le principal acide gras augmenté chez les patients obèses (Durcin et al., 2017). Dans les exosomes dérivés d'adipocytes provenant de jeunes patients obèses et minces, 55 miRNAs ont été montrés différemment exprimés. TGF- β et la voie Wnt/ β -caténine apparaissent comme les voies canoniques principales ciblées par ces miARNs. Ces voies sont importantes dans le développement et la progression de l'inflammation chronique et les maladies fibrotiques (Ferrante et al., 2015).

Enfin, différents miARN exosomaux ont été proposés comme biomarqueurs pour différents troubles métaboliques. Il a été montré que les miARN sanguins et exosomaux de patients atteints de syndrome métabolique étaient corrélés à certains facteurs de risque du SM. Ainsi, les miR-197, miR-23a, et miR-509-5p seraient potentiellement liés à la dyslipidémie associée au SM et miR-130a et miR-195, pourraient être corrélés à l'hypertension. De plus, miR-27a et miR-320a pourraient être associés au SM et au diabète de type 2 (Karolina et al., 2012). Par ailleurs, Kranendonk et collègues (2014) ont montré que sur une cohorte de patients présentant des troubles métaboliques, le taux d'exosomes circulants portant la cystatine C était corrélé positivement avec la prévalence du SM, un taux élevé en CRP et un taux diminué en cholestérol HDL.

b) Rôles des exosomes dans le SM

Nous nous intéresserons plus particulièrement à l'implication des VE dans la dysfonction endothéliale. Tout d'abord, Brodsky et collègues ont montré que les VE (exosomes et MP) dérivées des cellules endothéliales rénales de rats altéraient la vasorelaxation induite par l'acétylcholine et la production de NO des anneaux d'aortes provenant de rats d'une façon dépendante de la concentration. Cet effet s'accompagne d'une augmentation de la production d'O₂⁻ par les anneaux aortiques et des cellules endothéliales co-incubées avec les VE (Brodsky et al., 2004).

De plus, il a été montré *in vitro* que le contenu en ARN et protéines des exosomes dérivés de cellules endothéliales pouvaient être modulé à la suite de stimuli mimant l'athérosclérose. Ainsi, l'hypoxie et l'activation cellulaire induite par le TNF alpha sont reflétées dans la composition en ARN et protéines des exosomes, tandis que l'exposition à une forte concentration de glucose altère la composition des exosomes de façon mineure au niveau protéique et n'affecte pas la composition en ARN (de Jong et al., 2012).

Depuis, il a été confirmé au sein de notre laboratoire que les MP circulantes d'origine non plaquettaires de patients atteints de SM pouvaient induire une dysfonction endothéliale. En effet, elles diminuent la production de NO de cellules endothéliales humaines *in vitro* et altèrent la relaxation vasculaire chez la souris (Agouni et al., 2008). Par ailleurs, il a été montré récemment que le stress du réticulum ainsi que la production de ROS par la mitochondrie pouvaient contribuer à cette dysfonction endothéliale induite par les MP. Plus précisément, cette dysfonction endothéliale serait médiée par le ligand FAS porté par les MP, se liant à son récepteur FAS et activant ainsi la sphingomyélinase neutre (SMase) (Safiedeen et al., 2017).

Ces données suggèrent que les MP et les exosomes pourraient participer à la dysfonction endothéliale associée au SM et ainsi ne pas seulement être des biomarqueurs pour les maladies métaboliques mais aussi contribuer à leur développement. Cependant, si l'implication des MP semble déjà bien décrite, le rôle des exosomes reste hypothétique et encore peu de choses sont connues sur leur participation et modes d'action.

3) Rôle des exosomes dans le cancer

Un cancer est caractérisé par une prolifération cellulaire, appelée tumeur, anormalement importante au sein d'un tissu normal de l'organisme. Toutes ces cellules dérivent d'un même clone, cellule initiatrice du cancer qui a acquis certaines caractéristiques suite à une accumulation séquentielle de mutations, lui permettant de se diviser de façon illimitée en résistant à la mort cellulaire. Par la suite, ces cellules cancéreuses vont favoriser l'angiogenèse afin d'induire la formation de nouveaux vaisseaux sanguins nécessaires pour leur fournir de l'oxygène et des nutriments. Au cours de l'évolution de la maladie, certaines de ces cellules peuvent migrer de leur lieu de production et se propager dans l'organisme, formant ainsi des métastases, ce qui aboutira finalement à la mort du patient. De ce fait, le dépistage du cancer doit être le plus précoce possible. Une description par stade reposant sur l'étendue et le volume de la tumeur est parfois utilisée. Elle distingue quatre stades :

- le stade 1 qui correspond à une tumeur unique et de petite taille,
- le stade 2 qui correspond à un volume local plus important,
- le stade 3 qui correspond à un envahissement des ganglions lymphatiques ou des tissus avoisinants,
- le stade 4 qui correspond à une extension plus large dans l'organisme sous forme de métastases.

Les cellules tumorales ainsi que les cellules du microenvironnement tumoral sont susceptibles de produire des VE, qui peuvent être impliquées à la fois dans la tumorigénèse, correspondant à toutes les étapes menant à la formation des tumeurs, et la progression du cancer. Ainsi, de nombreuses données de la littérature ont démontrées l'implication des exosomes dans différents cancers, mais également leur rôle en tant que biomarqueurs.

a) Exosomes comme biomarqueurs

Les exosomes dérivés des cellules cancéreuses peuvent contribuer à la progression du cancer à travers le transfert intercellulaire d'un cargo contenant protéines, lipides et acides nucléiques dans le microenvironnement tumoral. Le cargo exosomal peut ainsi refléter l'état de certain cancer, et faire des exosomes des biomarqueurs peu invasifs pour la détection précoce, le diagnostic et le pronostic cancéreux. En effet, les taux des exosomes présents dans les différents biofluides humains peuvent être utilisés comme biomarqueurs diagnostiques pour différents cancers, évitant ainsi des procédures chirurgicales parfois lourdes afin de récupérer des biopsies chez ces patients (pour revue Soung et al., 2017). Par exemple, les exosomes circulants de patients atteints de glioblastome présentent un taux élevé d'ARNm EGFRvIII, qui peut ainsi participer au diagnostic (Skog et al., 2008). L'EGFR localisé à la membrane des exosomes est également proposé comme possible biomarqueur pour le cancer du poumon (Yamashita et al., 2013). Plus récemment, il a

également été montré que les exosomes portant la protéine Protéoglycane glypican-1 (GP1) étaient détectés spécifiquement dans le sérum de patients atteints d'un cancer pancréatique, en comparaison avec des sujets sains et patients présentant des tumeurs bénignes (Melo et al., 2015). Par ailleurs, les miARN portés par les exosomes semblent également être de biomarqueurs intéressants dans différents cancers. Par exemple, un taux élevé en miR-21 exosomaux peut servir de biomarqueur dans la prédiction de la récurrence et du mauvais pronostic vital chez des patients atteints de cancers colorectaux de stade II, III, ou IV (Tsukamoto et al., 2017). Enfin, l'analyse de 36 espèces lipidiques dans des exosomes urinaires de patients atteints de cancer de la prostate montre que la teneur en neuf espèces lipidiques est différente comparée à celle de sujets sains. Alors que les niveaux de phosphatidylsérine et de phosphatidyléthanolamine sont diminués dans les exosomes de patients de cancer de la prostate, la phosphatidylcholine, l'hexosylcéramide et la lactosylcéramide sont augmentées (Skotland et al., 2017). Ces données suggèrent que la détection de protéines, de miARN et même de lipides spécifiquement portés par les exosomes peut être utilisée comme méthode prédictive non invasive de cancer ou de réponse au traitement.

b) Rôle des exosomes dans les cancers

Les exosomes dérivés des cellules cancéreuses et des cellules du microenvironnement sont capables de transporter et transférer, entre autre, des oncogènes et de facteurs pro-angiogéniques. Ainsi, de nombreuses études rapportent leur rôle dans la tumorigenèse en favorisant la prolifération des cellules tumorales par exemple, et dans la progression de la tumeur en favorisant l'angiogenèse, la migration des cellules tumorales et la dissémination métastatique. Tout d'abord, l'inhibition de la sécrétion d'exosomes dérivés des cellules tumorales 4T1, a montré que la croissance locale des tumeurs nécessitait la mobilisation d'une population de cellules immunitaires neutrophiles induites par une sécrétion des exosomes dépendante de Rab27a avec une combinaison spécifique de cytokines et / ou de métalloprotéinases (Bobrie et al., 2012). Par ailleurs, des études *in vitro* ont montré que les exosomes provenant de cellules leucémiques favorisent la prolifération et la migration et inhibent l'apoptose des cellules de leucémie myéloïdes aiguës. Ces effets sont associés à la diminution de l'expression de miR-34a puisque lorsque les exosomes sont enrichis en miR-34a, ces effets sont inhibés (Wang et al., 2016). Enfin, il a été montré que les exosomes dérivés de glioblastomes expriment le canal intracellulaire 1 (CLIC1), qui a été impliqué dans la croissance tumorale (Setti et al., 2015). Les exosomes contenant CLIC1 stimulent la prolifération et la migration des cellules de glioblastome *in vitro* et la croissance de la tumeur *in vivo*, et ces effets sont significativement diminués par le traitement des cellules de glioblastome avec des exosomes dérivés de cellules n'exprimant plus CLIC1 (Setti et al., 2015). Les exosomes dérivés de tumeurs présentent également des activités pro-angiogéniques, médiées par leur interaction avec les cellules endothéliales. Ainsi, les exosomes dérivés de

cellules de carcinomes peuvent contenir l'oncogène EGFR (le récepteur de l'Epidermal Growth Factor) et favoriser l'angiogenèse en induisant l'expression de VEGF par les cellules endothéliales (Al-Nedawi et al., 2009). De plus, les exosomes dérivés de cellules de glioblastome peuvent incorporer Delta-like 4, ligand du récepteur Notch, qui participe à la régulation de l'angiogenèse. Ces exosomes ont une action pro-angiogénique *in vitro* et *in vivo* en augmentant le nombre de branchement entre les cellules endothéliales (Sheldon et al., 2010). Skog et collègues ont également montré que les exosomes produits par les cellules de glioblastome de patients contiennent d'autres facteurs pro-angiogéniques comme l'IL-8, l'IL-6, l'angiogénine, le VEGF et TIMP-1 (Tissue inhibitor of metalloprotease 1) (Skog et al., 2008).

Enfin, il a été suggéré que les exosomes pouvaient participer à la diffusion métastatique des cellules tumorales. Effectivement, les exosomes dérivés de mélanomes sont capables de transformer les cellules progénitrices de la moelle osseuse vers un phénotype pro-vasculogénique et pro-métastatique, favorisant leur migration vers leurs futurs sites de métastases (Peinado et al., 2012). Les exosomes dérivés de mélanomes peuvent également conditionner les ganglions lymphatiques, qui se préparent localement (dépôt de matrice extracellulaire, prolifération vasculaire) pour recevoir les futures métastases (Hood et al., 2011). De plus, il a été montré que les exosomes sécrétés par les fibroblastes présents dans le microenvironnement tumoral favorisent la mobilité des cellules de cancer du sein via la voie Wnt/PCP (voie de la polarité planaire) (Luga et al., 2012).

Ainsi, les exosomes semblent jouer un rôle dans la tumorigenèse et la progression de nombreux types de cancer. Nous nous intéresserons maintenant plus particulièrement au cancer du rein et l'implication des exosomes dans cette pathologie.

c) Rôles des exosomes dans le cancer du rein

Le cancer du rein représente environ 3% de l'ensemble des tumeurs malignes de l'adulte et constitue le troisième cancer urologique avec un ratio de deux hommes pour une femme. Durant ces dernières années, son incidence n'a cessé de croître pour atteindre en France plus de 12000 nouveaux cas par an. Environ 10 à 20% des patients atteints de carcinome rénal présente un stade avancé de la maladie au moment du diagnostic et parmi les patients diagnostiqués, 30 % développeront une récurrence et une métastase après l'exérèse de la tumeur (Capitanio and Montorsi, 2016). Au fil des années, de gros progrès ont été accomplis dans le diagnostic et le traitement du carcinome rénal mais il reste cependant le premier cancer urologique en terme de mortalité avec une survie de 5 ans de 60% tous stades confondus et de seulement 10% au stade métastatique. Le manque de sensibilité aux radiations conventionnelles et à la chimiothérapie des cancers rénaux métastatiques est une des raisons du faible pronostic.

Récemment, une meilleure compréhension de la pathogenèse du carcinome rénal a mené au développement d'inhibiteurs aux récepteurs tyrosine kinase (RTK), comme le sunitinib, possédant

des effets anti-angiogéniques et qui reste une des options thérapeutiques pour les patients atteints de carcinome rénal avancé (Motzer et al., 2007). Cependant, 10% à 20% des patients atteints de carcinome rénaux sont réfractaires à la thérapie par sunitinib, et la plupart des patients restants développe une résistance aux médicaments et une progression de la tumeur après 6-15 mois de traitement (Molina et al., 2014). Il a été montré que la résistance au sunitinib pouvait être médié par un ARN long non codant (lncRNA), appelé lncARSR (lncRNA Activated in RCC with Sunitinib Resistance). En outre, lncARSR pourrait être incorporé dans les exosomes et être transmis aux cellules sensibles, ce qui permet de disséminer la résistance au sunitinib (Qu et al., 2016).

De plus, les exosomes dérivés des cellules de carcinome rénal favorisent la prolifération des cellules tumorales en inhibant l'expression d'hépaCAM, en partie par l'activation d'Akt. L'expression du gène hépaCAM est fréquemment perdue dans diverses cellules et tissus cancéreux humains. La ré-expression de l'hépaCAM dans le carcinome rénal et les cellules cancéreuses du sein inhibe la prolifération cellulaire (Jiang et al., 2014).

Enfin, les exosomes pourraient également être de biomarqueurs pertinents pour le cancer du rein. Il a été montré que les exosomes urinaires dérivés de cellules de carcinomes rénaux de patients (Raimondo et al., 2013) présentaient 9 protéines qui étaient surexprimées (la MMP-9, la céruloplasmine, la podocalyxine, la Dickkopf related protein 4 et la carbonic anhydrase IX) et 5 sous-exprimées (l'aquaporine-1, MMP inducer, la néprilysine, la dipeptidase 1 et la synténine-1), suggérant un contenu spécifique des exosomes chez les patients atteints de cancer du rein.

Introduction projet 1

La dysfonction endothéliale étant l'élément précurseur du développement de maladies cardiovasculaires et décrite comme précocément associée au SM, elle apparaît être une cible intéressante afin de traiter les complications de cette pathologie. Le rôle des MP dans cette perturbation ayant déjà été établi par notre laboratoire, cette étude s'intéressera aux rôles des exosomes, VE plus petites et de formation différente des MP, dans ce dysfonctionnement.

Pour cela, nous avons pu isoler les exosomes circulants de la cohorte de patients METABOL, présente au CHU d'Angers, comportant des patients non-syndrome métabolique (nSM) (présentant moins de 3 critères du SM) et de patients diagnostiqués comme atteints de SM (présentant 3 critères ou plus).

Dans un premier temps, ces exosomes ont été caractérisés afin de valider notre méthode d'isolement. Le taux d'exosomes circulants a également été évalué pour les patients nSM et SM, afin de déterminer si les exosomes pourraient être des biomarqueurs pertinents pour le SM.

Par la suite, leurs effets sur la fonction endothéliale ont été étudiés *in vitro* et *ex vivo*. Nous nous sommes principalement intéressés à la production de NO et de ROS par les cellules endothéliales, après traitement avec les exosomes de patients nSM et SM, afin de déterminer si les exosomes pourraient participer à une dysfonction endothéliale dans le cadre du SM.

Ces résultats vous seront présentés sous forme d'article.

**Changes in mitochondrial dynamic-related proteins evoked by
exosomes from metabolic syndrome patients are associated with
endothelial dysfunction**

Mallocci M¹, Esnault M¹, Safiedeen Z¹, Soleti R¹, Vergori L¹, Dubois S^{1,2}, Le Lay S¹,
Mallegol P¹, Boursier J², Chevrolier A^{2,3}, Gagnadoux F^{1,2}, Andriantsitohaina R^{1,2}, Simard
G^{1,2}, Martinez MC^{1,2}

¹INSERM UMR 1063, Stress oxydant et pathologies métaboliques, UNIV Angers,
Université Bretagne Loire

²Centre Hospitalo-Universitaire d'Angers

³Institut MITOVASC, CNRS 6015, INSERM U1083, UNIV Angers, Université Bretagne
Loire

Corresponding author: M.C. Martinez, INSERM UMR 1063, Stress Oxydant et
Pathologies Métaboliques, Institut de Biologie en Santé, 4 rue Larrey, F-49933 Angers,
France. Phone: +33 2 44 68 85 79. E-mail: carmen.martinez@univ-angers.fr

Running title: Metabolic syndrome exosomes & endothelial dysfunction

Abstract

Metabolic syndrome (MetS) is characterized by a cluster of interrelated risk factors - hyperglycemia, dyslipidemia, hypertension and obesity- leading to an increased risk of cardiovascular events. Exosomes can be considered as new biomarkers of different pathologies, and can be involved in intercellular communication. Here, we hypothesize that exosomes could be implicated in MetS-associated endothelial dysfunction. Circulating exosomes of non-MetS subjects and MetS patients were isolated from plasma and characterized. Thereafter, exosomes effects on endothelial function were analyzed by measuring nitric oxide (NO) and reactive oxygen species (ROS) production and mitochondrial dynamic proteins, on human endothelial aortic cells (HAoECs). Whereas circulating levels of exosomes positively correlated with the number of MetS criteria, their size was negatively correlated with the number of MetS criteria. Moreover, exosomes mainly were originated from leukocyte and platelet origins in both non-MetS and MetS subjects. In HAoECs, exosomes from MetS patients decreased NO production through the inhibition of the endothelial NO-synthase activity. Furthermore, exosomes from MetS patients increased Mitosox-associated fluorescence, reflecting enhanced mitochondrial ROS production, leading to increased protein tyrosine nitration. This was associated with a decrease of expression of mitochondrial fusion proteins (Mfn1 and OPA1) and an increase of Fis1 expression, without modification of mitophagy. Furthermore, MetS exosome treatment decreased mtDNA/nDNA ratio but had no effect on expression of mitochondrial biogenesis actors (PGC1, NRF1 and Tfam). Finally, injection of MetS exosomes into mice impaired endothelium-dependent relaxation induced by acetylcholine. These results provide evidence that exosomes from MetS patients could be new biomarkers for this pathology and may contribute to endothelial dysfunction in MetS, by decreasing NO production, increasing oxidative stress and disturbing mitochondrial dynamic proteins. Thus, exosomes may be a future target to prevent and treat MetS.

Introduction

Metabolic syndrome (MetS) is a worldwide public health problem, characterized by a cluster of interrelated risk factors including hyperglycemia, dyslipidemia, hypertension and obesity, leading to an increased risk of cardiovascular events. Endothelial dysfunction actively participates in the development of cardiovascular diseases associated with MetS (Tziomalos et al., 2010). Among biological markers associated with the endothelium injury, extracellular vesicles (EV) have been involved in the pathogenesis and maintenance of cardiovascular and metabolic diseases (Chistiakov et al., 2015; Huang-Doran et al., 2017). EV, that carry proteins, lipids and nucleic acids, can act as biological vectors through their interaction with their target cells. There are two main types of EV: microparticles (MP) formed by budding of the plasma membrane, and exosomes, which came from the endosomal compartment. It has been described that MP from MetS patients induced endothelial dysfunction characterized by a decrease of nitric oxide (NO) release associated with the inhibition of endothelial NO-synthase (eNOS), and an increase in oxidative and nitrative stresses and unfolding protein response in human endothelial cells (Agouni et al., 2008). Furthermore, MP from MetS induced mitochondrial ROS (mROS) production accounting for the decreased bioavailability of NO (Safiedeen et al., 2017). In this respect, mitochondria can be involved in endothelial dysfunction by the increased mROS generation in response to many factors such as oxidized LDL, triglycerides and hyperglycemia (Tang et al., 2014). For instance, exposure of endothelial cells to palmitic acid increased mitophagy, mROS production, and apoptosis (Wu et al., 2015). In addition, ROS produced in the vascular microenvironment caused mitochondrial dysfunctions which, in turn, amplified this effect in part because of its reduced antioxidant capacity (Ballinger et al., 2000). Perturbation of mitochondrial dynamics may be responsible for increasing mROS and participating in endothelial dysfunction. Under normal conditions, there is a balance between fusion and mitochondrial fission, but in pathological conditions, fission can take precedence over fusion, leading to a fragmentation of the network which is no longer functional (Ong et al., 2010). Thus, in diabetic patients, it has been observed that endothelial cells isolated from veins displayed a fragmentation of the mitochondrial network and an increase in Fis1 expression, protein involved in mitochondrial fission (Shenouda et al., 2011).

Otherwise, it has been proposed that EV could also be relevant biomarkers for several pathologies. MetS patients displayed an increased circulating MP rate compared to healthy subjects (Agouni et al., 2008). Otherwise, many studies have suggested that exosomes could be also biomarkers for metabolic diseases (Lee et al., 2016). Indeed, it

has been demonstrated that circulating cystatin c-associated exosome rate was positively correlated with (i) an increase of inflammation, (ii) a decrease of HDL cholesterol and (iii) prevalence of MetS (Kranendonk et al., 2014). In addition, in circulating exosomes of patients with metabolic troubles, a significant dysregulation of seven candidates miRNAs, which are associated with risks involved in the manifestation of MetS, has been reported when compared with exosomes from healthy subjects. Among them, miR-197, miR-23a, and miR-509-5p were correlated with body mass index (BMI) and may be considered as potential contributors of dyslipidemia in MetS (Karolina et al., 2012). However, although the role of exosomes in the development of the MetS has been suggested (Huber and Holvoet, 2015), currently, no studies show the effects of these vesicles in this pathology. Thus, the aim of the present study was to define if exosomes could be implicated in MetS-associated endothelial dysfunction and could be relevant biomarkers for the diagnostic and progression of this disease.

Materials and Methods

Metabolic syndrome patients

This study was approved by the ethics committee of the University Hospital of Angers (France) (NCT: 00997165). We included 61 patients with MetS from the Department of Endocrinology and Nutrition of the University Hospital of Angers. Patients were eligible for inclusion, according to the International Diabetes Federation (IDF), when they had at least three criteria of the five following: 1) waist circumference ≥ 94 or 80 cm for men and women, respectively; 2) high systolic and diastolic pressures $\geq 130/85$ mmHg; 3) fasting glycemia $\geq 1,0$ g/l; 4) triglycerides ≥ 1.5 g/l; and 5) high-density cholesterol lipoprotein < 0.4 g/l in men or < 0.5 g/l in women. Non-metabolic syndrome patients (nMetS) consisted of 57 patients who met less than two of the MetS criteria (Table 1). All donors accepted to participate to the follow-up study.

Exosomes Isolation

Peripheral blood (20 ml) from nMetS or MetS patients was collected in EDTA tubes (Vacutainers; Becton Dickinson, Le Pont de Claix, France) from a peripheral vein using a 21-gauge needle to minimize platelet activation and was processed within 2 hours. After a 20 minute centrifugation at $270\times g$, platelet-rich plasma was separated from whole blood. Then, platelet-rich plasma was centrifuged 20 minutes at $1500\times g$ to obtain platelet-free plasma (PFP). Remaining PFP was centrifuged at $21,000\times g$ for 45 minutes to pellet MP.

The supernatant was centrifuged at 100,000xg (Optima MAX-XP ultracentrifuge and MLA-80 rotor, Beckman Coulter, Ville, Pays) for 70 minutes to pellet exosomes, which were then washed in Phosphate-Buffered Saline (PBS) (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄, KH₂PO₄ 1.8 mM, pH=7.4). Exosomes were resuspended in 200 µl of PBS and stored at 4°C until subsequent use.

Transmission electronic microscopy

Exosome preparations were first fixed overnight at 4°C with 2.5% glutaraldehyde (LFG Distribution, Lyon, France) in 0.1 M PBS. Then, exosomes were washed two times in PBS by 100,000xg centrifugation during 70 minutes. Exosomes were resuspended in milliQ water and 20 µl were deposited on Formvar®-coated copper grids for 2 minutes. Exosomes were negatively stained with 20 µl of uranyl acetate 5% (diluted in ethanol 50 %) for 30 seconds. Grids were rinsed briefly with milliQ water and left to air dry. Grids were then observed with a Jeol JEM 1400 microscope (Jeol, Croissy sur Seine, France) operated at 120 keV.

Characterization of Exosome Phenotype

Phenotype of exosomes was analyzed by flow cytometry with MACSPlex Exosome Kit (Miltenyi Biotec, Auburn, CA), according to the manufacturer's procedure. Briefly, 10 µg of purified exosomes were incubated with antibody-coated beads against 37 surface epitopes. Then, detection reagent was added, corresponding to exosomal markers CD9-, CD63- and CD81-associated beads. Positive signals indicate the presence of the respective surface epitope within the exosome population. Samples were analyzed in MACSQuant flow cytometer using MACSQuantify software. For each bead population, results are expressed in median of fluorescence, normalized with the mean of CD9-, CD63- and CD81-median of fluorescence as indicated in the manufacturer's instructions.

Exosome size and concentration by Nanoparticle Tracking Analysis (NTA)

Exosomes samples were diluted in sterile NaCl 0.9% prior to NTA (NanoSight NS300, Malvern Instruments Ltd, Ville, UK) equipped with a 405 nm laser. Videos of 90 seconds were recorded in 5 replicates per sample with optimized set parameters. Results presented correspond to the mean of the 5 videos taken for a given biological samples. Exosome concentrations were normalized by the collected plasma volume for each patient.

Cell Culture

Human aortic endothelial cells (HAoECs) were maintained in culture in endothelial cell growth medium MV2 (Promocell, Heidelberg, Germany) supplemented with 1% streptomycin/penicillin (Lonza, Basel, Switzerland). Cells were treated for 4 or 24 hours with exosomes from MetS or nMetS patients at 10 µg/ml. To scavenge mitochondrial ROS, mitoTEMPO (25 nM, Santa Cruz Biotechnology, Dallas, TX) was added 30 minutes before treatments with exosomes.

Nitric oxide (NO) and Superoxide Anion (O₂⁻) Determination by Electronic Paramagnetic Resonance (EPR)

The detection of NO production was performed using the technique with Fe²⁺ diethyldithiocarbamate (DETC; Sigma-Aldrich, St. Quentin, Fallavier, France) as spin trap. Cells were treated with 300 µl of colloid Fe(DETC)₂ (400 µM) and incubated at 37°C for 45 minutes. The detection of O₂⁻ production was performed by EPR technique using 1-hydroxy-3-methylcarbonyl-2, 2, 5, 5 tetramethyl pyrrolidine (CMH) (Noxygen, Mainz, Germany) as spin trap. Cells medium was replaced with 500 µl of deferoxamine-chelated Krebs-Hepes solution containing CMH (500 µM), deferoxamine (25 µM, Sigma-Aldrich, Saint-Louis, MO), and sodium diethylthiocarbamate (5 µM, Sigma-Aldrich), and incubated for 45 minutes at 37°C. After spin trap incubation, cells were then scrapped and frozen in plastic tubes and analyzed in a Dewar flask by EPR spectroscopy. NO and O₂⁻ measurement were performed on a tabletop x-band spectrometer miniscope (MS200; Magnettech, Berlin, Germany). The quantitative measurement of the NO and O₂⁻ signal amplitude was reported to protein concentration of samples. Results are expressed in percentage compared to the control condition.

Western Blotting

Purified exosomes (15 µg) or proteins from lysed cells (20 µg) were separated on 4-12% NuPage gels (ThermoFisher Scientific, Rockford, IL) and transferred on nitrocellulose blotting membrane (Amersham, Little Chalfont, UK). To characterized exosomes, blots were probed with anti-CD63, CD81, CD9 and TSG101 (Santa Cruz Biotechnology, Dallas, TX) and ALIX (BioLegend, San Diego, CA). In other set of experiments, blots were probed with anti-endothelial NOS (eNOS) (BD Biosciences, San Jose, CA), phospho-eNOS Ser 1177, phospho-eNOS Thr 495 (Cell Signaling, Beverly, MA) and nitrotyrosine (US Biological, Swampscott, MA). Finally, blots were probed with anti-PGC1α, Mfn1 and Mfn2 (Abcam, Cambridge, UK), Fis1 (Santa Cruz Biotechnologies), OPA1 and DRP1 (BD

Biosciences), Pink1 and Parkin (Cell Signaling). A polyclonal mouse anti-human β -actin antibody (Sigma-Aldrich) was used for standardization of protein gel loading.

Measurement of oxidative stress by confocal microscopy

Endothelial cells were grown on glass slides (Ibidi, Martinsried, Germany), and after treatments (10 μ g/ml of exosomes during 4 hours), they were washed and then incubated with either the oxidative fluorescent dye DHE (5 μ M, Sigma-Aldrich) for 30 minutes at 37°C or MitoSox red (5 μ M, Molecular Probes, Eugene, OR) for 10 minutes at 37°C. Then, cells were fixed by 4% paraformaldehyde for 20 minutes at room temperature. DAPI was added for 5 minutes. Finally, cells were visualized with confocal microscopy (CLMS 700, Zeiss, ZEN software). All images were acquired using a x40 objective.

Vascular Reactivity

All animal studies were performed using approved institutional protocols. Swiss mice (8 to 10 weeks old) were treated *in vivo* by intravenous injection of exosomes at 10 μ g/ml into the tail vein. After 24 hours, aortic rings were obtained from animals and mounted on a wire myograph. Endothelium-dependent vasodilatation was studied by cumulative application of acetylcholine (1 nM to 10 μ M, Sigma-Aldrich) in aortas with functional endothelium precontracted with U46619 (Sigma-Aldrich). The functionality of aorta muscle cells was checked by studying aorta relaxation in response to sodium nitroprusside (1 nM to 3 μ M, Sigma-Aldrich). Parameters of vascular sensitivity were analyzed by means of $pD_2 = -\log EC_{50}$, EC_{50} being the molar concentration of the agonist that produces 50% of the maximal effect; EC_{50} values were calculated by logit-log regression.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

HAoECs were untreated (control) or treated with exosomes from nMetS and MetS patients (10 μ g/ml) during 24 hours, then frozen in liquid N₂ and used to investigate the expression of mRNA for Tfam, NRF1 and DNA mitochondrial (ND5) by RT-PCR (primer sequences in Supplementary Table 1). RT-PCR analyses were performed using a Chromo4™ (Bio-Rad, Marnes la-Coquette, France) and SYBR Green detection (Fournisseur, Ville, Pays). Quantifications were realized according to the Ct method. Results were normalized with mRNA expression of β -actin.

Citrate synthase activity

The enzyme activity was used to assess the mitochondria integrity. The reaction capacity of the citrate synthase was followed by measuring the formation of the -SH group released from CoA-SH using of the reactive Ellman reagent (5,5`-dithiobis [2-nitrobenzoic], DTNB). HAOECs were untreated (control) or treated with exosomes from nMetS and MetS patients during 24 hours, then frozen in liquid N₂. Cells were resuspended in buffer (sucrose 250 mM, Trizma 20 mM, EDTA 2mM, pH 7.2) and samples were incubated with DNTB (150 μM), oxaloacetate (500 μM), actetyl-CoA (300 μM) and 0.1% of Triton X-100, during 2 minutes at 37°C. Activity was measured by absorbance at 412 nm and normalized by the amount of protein samples.

Data Analysis

Data are represented as mean ± SEM, *n* represents the number of experiences. Statistical analyses were performed by Mann-Whitney *U* or t-test for comparison of two conditions. For more than two conditions, Kruskal Wallis and subsequent Dunn post hoc test or analysis of variance for repeated measures and subsequent Bonferroni post hoc test were performed. Correlations were analyzed by Spearman and Pearson tests. *P* <0.05 was considered to be statistically significant.

Results

Circulating rate and size of exosome are correlated with metabolic disorders

The total number of circulating exosomes was significantly increased in patients with MetS compared to nMetS patients (Figure 1A). Interestingly, circulating rate was positively correlated with the number of MetS criteria (*p*=0.0014) (Figure 1B). Furthermore, circulating rate was also positively correlated with waist circumference (*p*=0.036), BMI (*p*=0.028) and level of triglycerides (*p*=0.023) of these patients (Table 2). There was no correlation between circulating rate and blood pressure, insulin-resistance (glycemia, insulin, HOMA index) and level of cholesterol (HDL, LDL, total). Unexpectedly, size of exosomes was significantly decreased in patients with MetS compared to nMetS patients (Figure 1C, 1E) and this reduction was negatively correlated with the number of MetS criteria (*p*=0.004) (Figure 1D). Moreover, exosomes size was negatively correlated with the increase of BMI (*p*=0.013), glycemia (*p*=0.019), insulinemia (*p*=0.024), HOMA index (*p*=0.023), level of triglycerides (*p*<0.0001) and LDL cholesterol (*p*=0.037) (Table 2). There was no correlation between exosomes size and waist circumference, blood pressure, HDL

and total cholesterol. These results suggest exosome circulating rate increase with obesity and size of exosome decrease with obesity and insulin-resistance. Thus, circulating rate and size of exosomes can be correlated with metabolic disorders associated with MetS.

Characterization and phenotype of circulating exosomes

Circulating exosomes purified from patients were observed in electronic microscopy. Exosomes showed a round shape and an average size of 63 nm, which confirm result from NTA experiments (Figure 2A). Moreover, isolated exosomes were enriched in specific exosomal markers: ALIX, CD63, TSG101, CD81 and CD9, compared to MP from nMetS and MetS patients (Figure 2B). ALIX and TSG101 were ESCRT-associated proteins and CD63, CD81 and CD9 were tetraspanins specifically enriched in exosomes. Finally, we analyzed the phenotype nMetS and MetS exosomes to define what were the main exosomes populations present in blood. It was performed by labelling 37 epitopes corresponding to specific membrane markers of original cells (Supplemental Data 1). As expected, circulating exosomes are mainly from platelets (CD41⁺, CD42⁺) and leukocytes (CD29⁺). However, MetS patients presented less CD41⁺, CD29⁺, CD9⁺ exosomes and more CD81⁺ exosomes compared to nMetS patients. These results suggest that nMetS and MetS patients could present different pattern of circulating exosomes.

MetS exosomes reduce NO release in endothelial cells and impair endothelium-dependent relaxation ex vivo in aorta of the mice

Firstly, different concentrations of nMetS and MetS exosomes were incubated 24 hours with the endothelial cells HAoECS to determine if exosomes can affect NO production. We showed that MetS exosomes decreased NO production in endothelial cells, according to their concentration, compared to nMetS exosomes (Supplemental Data 2). For the following experiments, the concentration of 10 µg/ml was used. This concentration was not toxic for cells: after 4 and 24 hours, neither exosomes from nMetS nor those from MetS patients affect the viability of the endothelial cells (not shown). Then, we showed that exosomes from MetS patients reduced significantly NO release in endothelial cells by ~50% (Figure 3A). To explain this decrease, we analyzed by Western blot expression and phosphorylation of enzymes linked to NO pathway. Exosomes from both nMetS and MetS patients did not modify expression of eNOS (Figure 3B). Exosomes from MetS patients did not alter eNOS phosphorylation on its activator (Ser 1177) site but they greatly increased its phosphorylation at the inhibitor (Thr 495) site (Figure 3C, 3D) when compared either to

untreated or those treated with exosomes from nMetS patients. The intravenous injection of nMetS and MetS exosomes in mice did not modify contraction to U46619 of aortas compared to the vehicle injection (not shown). However, relaxation to acetylcholine was significantly impaired in aortas taken from mice subjected to intravenous injection of exosomes from MetS patients compared to those from mice injected with either vehicle or nMetS exosomes ($E_{max} = 53\pm3\%$, $65.5\pm3\%$, and $67.5\pm3\%$, respectively) (Figure 3E). Otherwise, pD_2 in response to acetylcholine were unchanged between aorta from control mice and mice injected with nMetS and MetS exosomes (6.8 ± 0.3 , 6.9 ± 0.4 and 6.6 ± 0.4 , respectively), suggesting that exosomes from nMetS and MetS patients did not change vascular sensitivity to acetylcholine. In order to determine the functionality of vascular smooth muscle cells (VSMC), we evaluated the relaxation of aorta to sodium nitroprusside (SNP), a molecule which directly releases NO to VSMC and allows endothelium-independent relaxation. Thus, relaxation to SNP was not different between the aortas from mice injected with nMetS and MetS exosomes and the vehicle (Figure 3F), confirming that VSMC functionality was not affected during experimental set up. These results suggest that exosomes from MetS patients induce *ex vivo* endothelial dysfunction in aortas from mice.

MetS exosomes increase mitochondrial oxidative stress and decrease NO bioavailability

MetS exosomes increased O_2^- production in endothelial cells after 4 hours of treatment compared to untreated cells (control) and cells treated with nMetS exosomes (Figure 4A), whereas there was no difference on O_2^- production after 24 hours of treatment (Figure 4B). In order to define origin of this O_2^- production, endothelial cells were incubated with DHE (targeting cytosolic ROS) or Mitosox (targeting mitochondrial ROS). Thus, MetS exosomes increased mitochondrial ROS compared to nMetS exosomes and untreated cells while there was no change in cytosolic ROS production in the presence of nMetS and MetS exosomes compared to control (Figure 4C, 4D). In addition, when endothelial cells were incubated with a mitochondrial ROS scavenger, mitoTEMPO, the increase in MetS exosome-induced O_2^- production was prevented (Figure 4E). Furthermore, in presence of mitoTEMPO, the NO release induced by MetS exosomes was also restored, suggesting that mitochondrial ROS reduced NO bioavailability (Figure 4F). Indeed, NO and O_2^- can form the complex $ONOO^-$, a strong oxidant, which could react directly with electron-rich groups. We evaluated its formation in endothelial cells by studying nitration of proteins, a subsequent step to $ONOO^-$ formation, using a specific antibody against nitrotyrosine.

Interestingly, exosomes from MetS patients, but not from nMetS patients, significantly enhanced nitration of proteins in endothelial cells (Figure 4G).

MetS exosomes decrease mtDNA/nDNA ratio without affect mitochondrial biogenesis

In order to explain the increase of mitochondrial ROS, we analyzed the effect of exosomes on mitochondria in endothelial cells. We showed that MetS exosomes decreased mitochondrial DNA/total DNA (mtDNA/nDNA) ratio (Figure 5A), suggesting a decrease of mitochondria amount. However, when we evaluated citrate synthase activity, which is an indicator of mitochondrial content, there was no difference between the enzyme activity of endothelial cells control and treated with nMetS and MetS exosomes (Figure 5B). In addition, MetS exosomes did not change the co-activator PGC1 α protein expression or the gene expressions of the transcription factors Tfam and NRF1 (Figure 5C-E), responsible to the establishment of mitochondrial biogenesis, suggesting MetS exosomes did not affect mitochondrial biogenesis.

MetS exosomes disrupt expression of proteins involved in the mitochondrial dynamics, without modifying expression of proteins responsible for mitophagy

Mitochondrial dynamic is an extremely regulated process and allows the proper functioning of the cell. Firstly, we studied the expression of proteins involved in the fusion of outer membrane of mitochondria, Mfn1 and Mfn2. MetS exosomes decreased Mfn1 expression, without change Mfn2 expression, in endothelial cells. Furthermore, MetS exosomes decreased OPA1 expression (Figure 6C), which is involved in the fusion of inner mitochondrial membrane. Then, we evaluated expression of proteins involved in the mitochondrial fission, Fis1 and DRP1. MetS exosomes increased Fis1 expression in endothelial cells (Figure 6D), without change DRP1 expression (Figure 6E). Finally, we analyzed in the expression of proteins responsible for mitophagy, the specific degradation of the mitochondria, which is a phenomenon that can follow mitochondrial fission events. However, MetS exosomes did not change expression of Pink1 (Figure 6F) and Parkin (Figure 6G).

Discussion

Here, we have shown that circulating rate and size of exosomes are correlated with both factors associated with MetS and the number of MetS criteria in patients. Furthermore, exosomes from MetS patients decrease *in vitro* NO production in endothelial cells and impair *ex vivo* endothelium-dependent relaxation. The NO reduction induced by MetS exosomes is associated with an increase of mitochondrial ROS production. Alteration in expression of proteins involved in mitochondrial dynamic could participate in the oxidative stress generation.

As shown in Table 1, there were differences between MetS and nMetS patients with respect to age. Indeed, it is known that prevalence of MetS increase with age (Hildrum et al., 2007; Park and Kim, 2015). As expected, MetS patients showed greater visceral obesity as given by waist circumference, enhanced triglyceridemia, and increased blood pressure and decreased HDL cholesterol. Insulinemia, fasting glycemia, and calculated HOMA were increased arguing for insulin resistance in MetS patients. Thus, patients defined as MetS patients clearly present the criteria necessary for their diagnosis. They also displayed higher glycated hemoglobin level (HbA1c), a parameter that gives a good estimate of how well diabetes is being managed throughout the last 2 or 3 months.

Thereafter, we have shown that circulating rate of exosomes was increased in MetS patients and positively correlated with factors associated with obesity (waist circumference, BMI and level of triglycerides) (Table 2). In agreement with Arraud and colleagues (2014), the main exosome population was derived from platelets (Figure 2C), but other cells may contribute to exosomes production. It has been already reported that levels of adipocyte-derived exosomes were increased with obesity and were principally involved in insulin resistance signaling (Deng et al., 2009; Hubal et al., 2017). However, increase of whole circulating exosomes associated with obesity is poorly described, only in obese mice *ob/ob* (Phoonsawat et al., 2014). Further experiments are needed in order to establish the increased specific exosome population in MetS patients.

We have also shown that size of exosomes was negatively correlated with obesity (BMI), insulin-resistance (glycemia, insulinemia, HOMA) and dyslipidemia (level of triglycerides and LDL cholesterol). At our knowledge, this is the first time that size of exosomes was proposed to serve as a putative biomarker. Whether the size difference is a result of the cargo or of the activation of different mechanism controlling the formation of exosomes remains to be investigated. For example, it has been shown that inhibiting Rab27b, one of GTPase involved in exosome formation (Blanc and Vidal, 2017), induced a decrease of

CMV size, suggesting that the diameter of later-produced exosomes could also be reduced (Ostrowski et al., 2010).

Otherwise, we reported MetS are older than nMets patients, but age was not correlated with the circulating rate and size of exosomes (Table 2), suggesting that this parameter did not affect our results. Finally, the increase of circulating rate and decrease of exosomes size were correlated with the number of MetS criteria, suggesting that exosomes could be biomarker of the severity of this pathology.

Endothelial dysfunction is the primary event leading to the failure of vasoactive, anti-coagulant, and anti-inflammatory effects of healthy endothelium. The most important mechanism for endothelial dysfunction is the decrease in NO availability (Favero et al., 2014). Endothelial production of NO was initially considered to be dependent on the increases in intracellular calcium and binding of calcium/calmodulin to eNOS. However, eNOS regulation is a process determined by a cascade of events, including changes in eNOS mRNA and protein levels and phosphorylation of Ser, Thr, and tyrosine residues (Fleming and Busse, 2003). Here, we have shown that MetS exosomes decreased NO production by increasing phosphorylation on the inhibitor site (Thr 495) of eNOS. Endothelial dysfunction is also characterized by an oxidative stress, which appears when the antioxidant capacity of the cell is exceeded. Increasing ROS level has toxic effects on cells and tissues through the oxidation of carbohydrates, lipids and proteins (Higashi et al., 2009). This excess of the main ROS, the anion superoxide O_2^- , is associated with the elevation of protein nitration, due to the peroxynitrite complex ($ONOO^-$) formation, which alters protein activity and become deleterious for cells (Beckman and Koppenol, 1996). Thus, we have shown that MetS exosomes can induce an increase of mROS, leading to $ONOO^-$ formation, which further reduced NO availability. This excess of ROS produced by the mitochondria did not originate from a defect in the phenomena of mitochondrial biogenesis and mitophagy but could come from a modification of the expression of proteins involved in the mitochondrial dynamics. Effectively, it has been shown that dynamic changes of mitochondrial morphology, for example in high glucose conditions, contributes to ROS overproduction (Yu et al., 2006).

In addition, disruption of fusion and fission factors is also recognized impact the maintenance of mitochondrial DNA (Chen et al., 2010; Tam et al., 2015) and may probably explain the reduced content in DNA mitochondrial observed in MetS patients. Finally, we showed that MetS exosomes impaired endothelium-dependent relaxation in aorta of mice, demonstrating the effects of these vesicles on endothelial dysfunction. Indeed, MetS exosomes reduced relaxation in response to acetylcholine, without change vascular

sensitivity. All, similar results obtained with MP from MetS patients. Thus, it has been shown that endothelial dysfunction induced by MetS MP was mediated by the FAS/FAS ligand interaction, activating the neutral sphingomyelinase (nSMase) (Safiedeen et al., 2017). However, at difference of results obtained with MP, the effects induced by MetS exosomes on O_2^- production were insensitive to the nSMase inhibitor, GW4869, (data not shown) suggesting that different molecular pathways are involved on the enhancement of oxidative stress induced by either MP or exosomes from MetS. It is possible that the different mode of formation of MP and exosomes resulting in a different content may account for different molecular pathways activated in endothelial cells. Indeed, it has already been shown that MP and exosomes exhibit specific protein signature when derived from adipocytes (Durcin et al., 2017), for example. We showed that endothelial cells were able to internalize exosomes of nMetS and MetS patients (Supplemental data 3). Thus, effects induced by exosomes of MetS patients may be mediated by the exosomal cargo. Exosome contain also functional mRNA and miRNA (Valadi et al., 2007) and it has been suggested that some miRNA could be involved in endothelial dysfunction (Santulli, 2016). Thus, MetS exosomes could induce endothelial dysfunction by transferring specific miRNA to target cells.

Conclusion

These data reveals that exosomes can be involved in the development of endothelial dysfunction in MetS, promoting vascular damage and cardiovascular diseases in these patients. In addition, exosomes rate and size seem to be interesting biomarkers for the diagnosis and the severity of this pathology.

Acknowledgments

We thank SCAHU staff (Université d'Angers) for taking care of animals, G. Mabillean from SCIAM (Angers) for electronic microscopy observation and the staff of Centre Hospitalo-Universitaire d'Angers for analysis of clinical data of METABOL cohort. This work was supported by Institut National de la Santé et de la Recherche Médicale, Université d'Angers and Centre Hospitalo-Universitaire d'Angers. MM is recipient of a doctoral fellowship from INSERM-Pays de La Loire.

List of Abbreviations

BMI: Body Mass Index
eNOS: endothelial NO-synthase
EV: Extracellular Vesicle
HAoECs: Human Aortic Endothelial Cells
HOMA: Homeostatic Model Assessment
MetS: Metabolic Syndrome
MP: Microparticle
mROS: mitochondrial ROS
nMetS: non-Metabolic Syndrome
NO: Nitric oxide
nSMase: neutral Sphingomyelinase
NTA: Nanoparticle Tracking Analysis
SNP: Sodium Nitroprussiate
VSMC: Vascular Smooth Muscle Cell

References

- Agouni, A., Lagrue-Lak-Hal, A.H., Ducluzeau, P.H., Mostefai, H.A., Draunet-Busson, C., Leftheriotis, G., Heymes, C., Martinez, M.C., and Andriantsitohaina, R. (2008).** Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am. J. Pathol.* *173*, 1210–1219.
- Arraud, N., Linares, R., Tan, S., Gounou, C., Pasquet, J.-M., Mornet, S., and Brisson, A.R. (2014).** Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J. Thromb. Haemost.* *12*, 614–627.
- Ballinger, S.W., Patterson, C., Yan, C.N., Doan, R., Burow, D.L., Young, C.G., Yakes, F.M., Van Houten, B., Ballinger, C.A., Freeman, B.A., et al. (2000).** Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ. Res.* *86*, 960–966.
- Beckman, J.S., and Koppenol, W.H. (1996).** Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* *271*, C1424-1437.
- Blanc, L., and Vidal, M. (2017).** New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases* 1–12.
- Chen, H., Vermulst, M., Wang, Y.E., Chomyn, A., Prolla, T.A., McCaffery, J.M., and Chan, D.C. (2010).** Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* *141*, 280–289.
- Chistiakov, D.A., Orekhov, A.N., and Bobryshev, Y.V. (2015).** Extracellular vesicles and atherosclerotic disease. *Cell. Mol. Life Sci. CMLS* *72*, 2697–2708.

Deng, Z., Poliakov, A., Hardy, R.W., Clements, R., Liu, C., Liu, Y., Wang, J., Xiang, X., Zhang, S., Zhuang, X., et al. (2009). Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes* 58, 2498–2505.

Durcin, M., Fleury, A., Taillebois, E., Hilairet, G., Krupova, Z., Henry, C., Truchet, S., Trötz Müller, M., Köfeler, H., Mabileau, G., et al. (2017). Characterisation of adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and small extracellular vesicles. *J. Extracell. Vesicles* 6.

Favero, G., Paganelli, C., Buffoli, B., Rodella, L.F., and Rezzani, R. (2014). Endothelium and Its Alterations in Cardiovascular Diseases: Life Style Intervention. *BioMed Res. Int.* 2014.

Fleming, I., and Busse, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R1-12.

Higashi, Y., Noma, K., Yoshizumi, M., and Kihara, Y. (2009). Endothelial function and oxidative stress in cardiovascular diseases. *Circ. J. Off. J. Jpn. Circ. Soc.* 73, 411–418.

Hildrum, B., Mykletun, A., Hole, T., Midthjell, K., and Dahl, A.A. (2007). Age-specific prevalence of the metabolic syndrome defined by the International Diabetes Federation and the National Cholesterol Education Program: the Norwegian HUNT 2 study. *BMC Public Health* 7, 220.

Huang-Doran, I., Zhang, C.-Y., and Vidal-Puig, A. (2017). Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease. *Trends Endocrinol. Metab.* 28, 3–18.

Hubal, M.J., Nadler, E.P., Ferrante, S.C., Barberio, M.D., Suh, J.-H., Wang, J., Dohm, G.L., Pories, W.J., Mietus-Snyder, M., and Freishtat, R.J. (2017). Circulating adipocyte-derived exosomal MicroRNAs associated with decreased insulin resistance after gastric bypass. *Obes. Silver Spring Md* 25, 102–110.

Huber, H.J., and Holvoet, P. (2015). Exosomes: emerging roles in communication between blood cells and vascular tissues during atherosclerosis. *Curr. Opin. Lipidol.* 26, 412–419.

Kranendonk, M.E., de Kleijn, D.P., Kalkhoven, E., Kanhai, D.A., Uiterwaal, C.S., van der Graaf, Y., Pasterkamp, G., and Visseren, F.L. (2014). Extracellular vesicle markers in relation to obesity and metabolic complications in patients with manifest cardiovascular disease. *Cardiovasc. Diabetol.* 13, 37.

Lee, M.-J., Park, D.-H., and Kang, J.-H. (2016). Exosomes as the source of biomarkers of metabolic diseases. *Ann. Pediatr. Endocrinol. Metab.* 21, 119–125.

Ong, S.-B., Subrayan, S., Lim, S.Y., Yellon, D.M., Davidson, S.M., and Hausenloy, D.J. (2010). Inhibiting Mitochondrial Fission Protects the Heart Against Ischemia/Reperfusion Injury. *Circulation* 121, 2012–2022.

Ostrowski, M., Carmo, N.B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C.F., Schauer, K., Hume, A.N., Freitas, R.P., et al. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* 12, 19–30.

- Park, E., and Kim, J. (2015).** Gender- and age-specific prevalence of metabolic syndrome among Korean adults: analysis of the fifth Korean National Health and Nutrition Examination Survey. *J. Cardiovasc. Nurs.* 30, 256–266.
- Phoonsawat, W., Aoki-Yoshida, A., Tsuruta, T., and Sonoyama, K. (2014).** Adiponectin is partially associated with exosomes in mouse serum. *Biochem. Biophys. Res. Commun.* 448, 261–266.
- Safiedeen, Z., Rodríguez-Gómez, I., Vergori, L., Soleti, R., Vaithilingam, D., Douma, I., Agouni, A., Leiber, D., Dubois, S., Simard, G., et al. (2017).** Temporal Cross Talk Between Endoplasmic Reticulum and Mitochondria Regulates Oxidative Stress and Mediates Microparticle-Induced Endothelial Dysfunction. *Antioxid. Redox Signal.* 26, 15–27.
- Santulli, G. (2016).** MicroRNAs and Endothelial (Dys) Function. *J. Cell. Physiol.* 231, 1638–1644.
- Shenouda, S.M., Widlansky, M.E., Chen, K., Xu, G., Holbrook, M., Tabit, C.E., Hamburg, N.M., Frame, A.A., Caiano, T.L., Kluge, M.A., et al. (2011).** Altered Mitochondrial Dynamics Contributes to Endothelial Dysfunction in Diabetes Mellitus. *Circulation* 124, 444–453.
- Tam, Z.Y., Gruber, J., Halliwell, B., and Gunawan, R. (2015).** Context-Dependent Role of Mitochondrial Fusion-Fission in Clonal Expansion of mtDNA Mutations. *PLoS Comput. Biol.* 11.
- Tang, X., Luo, Y.-X., Chen, H.-Z., and Liu, D.-P. (2014).** Mitochondria, endothelial cell function, and vascular diseases. *Front. Physiol.* 5.
- Tziomalos, K., Athyros, V.G., Karagiannis, A., and Mikhailidis, D.P. (2010).** Endothelial dysfunction in metabolic syndrome: prevalence, pathogenesis and management. *Nutr. Metab. Cardiovasc. Dis. NMCD* 20, 140–146.
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., and Lötvall, J.O. (2007).** Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659.
- Wu, W., Xu, H., Wang, Z., Mao, Y., Yuan, L., Luo, W., Cui, Z., Cui, T., Wang, X.L., and Shen, Y.H. (2015).** PINK1-Parkin-Mediated Mitophagy Protects Mitochondrial Integrity and Prevents Metabolic Stress-Induced Endothelial Injury. *PLoS ONE* 10.
- Yu, T., Robotham, J.L., and Yoon, Y. (2006).** Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc. Natl. Acad. Sci. U. S. A.* 103, 2653–2658.

Table 1: Baseline characteristics of metabolic syndrome patients (MetS) compared to non-metabolic syndrome subjects (nMetS). The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance (HOMA = glycemia x insulinemia/22.5).

	nMetS	MetS
Number	57	61
Mean age (years)	48.18 ± 1.930	55.57 ± 1.205**
Sex ratio (male/female)	25/32	32/29
BMI (kg/m ²)	27.01 ± 0.635	32.52 ± 0.684 ***
Waist circumference	91.64 ± 1.891	109.5 ± 1.544 ***
Systolic blood pressure (mmHg)	120.8 ± 1.429	127.9 ± 1.584 **
Diastolic blood pressure (mmHg)	75.25 ± 1.533	79.47 ± 1.301 *
Glycemia (g/l)	0.9437 ± 0.0190	1.237 ± 0.035 ***
Insulinemia (μU/l)	8.166 ± 0.708	17.33 ± 1.667 ***
HbA1c (%)	5.692 ± 0.084	6.280 ± 0.137 **
HOMA	1.952 ± 0.198	5.357 ± 0.602 ***
Total cholesterol	2.057 ± 0.078	1.960 ± 0.074
HDL	0.5386 ± 0.017	0.4435 ± 0.016 ***
LDL	1.247 ± 0.057	1.168 ± 0.046
Triglycerides (g/l)	0.9123 ± 0.046	1.725 ± 0.139 ***
Treatments (%)		
Oral antidiabetic	12	51
Insulin	0	8
Anti hypertensive	18	56
Statin	16	23

*P <0.05; **P <0.01; ***P<0.001.

BMI: Body Mass Index, HbA1c: glycated hemoglobin A1c, HOMA: homeostatic model assessment.

Table 2: Correlations between circulating level and size of exosomes from nMetS and Mets patients with factors associated with metabolic syndrome.

	Unit	Circulating rate p value	Size value	p
Age	(years)	0.2510 n=46	0.076 n=49	
Waist circumference	(cm)	0.036* n=44	0.055 n=46	
Body Mass Index	(kg/m ²)	0.028* n=43	0.013* n=46	
Systolic blood pressure	(mmHg)	0.829 n=43	0.722 n=46	
Diastolic blood pressure	(mmHg)	0.250 n=42	0.425 n=45	
Glycemia	(g/l)	0.194 n=41	0.019* n=44	
Insulin	(μU/ml)	0.163 n=38	0.024* n=41	
HOMA		0.203 n=42	0.023* n=44	
Triglycerides	(g/l)	0.023* n=42	< 0.0001*** n=45	
HDL cholesterol	(g/l)	0.114 n=42	0.430 n=45	
LDL cholesterol	(g/l)	0.3447 n=39	0.0347* n=41	
Total cholesterol	(g/l)	0.739 n=34	0.9336 n=36	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

HOMA: homeostatic model assessment

Supplementary Table 1: Primer sequences of genes analyzed in Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene	Primer sequences	Tm (°C)
Tfam	CCGAGGTGGTTTTTCATCTGT	60
	CAGGAAGTTCCCTCCAACGC	64
β-actin	CGACATGGAGAAAATCTGGC	60
	AGGTCCAGACGCAGGATGG	62
NRF1	CTCGCCTTCTTCTCCCGAGG	60
	GGACAATAGCTTGCTGTCCC	62
ND5	CCTCCTAGACCTAACCTGAC	62
	GATTATGGGCGTTGATTAGTAG	62

Tfam: Transcription factor A mitochondrial, NRF1: nuclear respiratory factor 1, ND5: NADH Dehydrogenase 5, Tm: melting temperature.

Figure Legends

Figure 1: Circulating rate and size of exosomes are correlated with metabolic disorders. Circulating rate and size of exosomes from metabolic syndrome (MetS) and non-metabolic syndrome patients (nMetS) were measured by Nanosight Tracking Analysis. (A) Exosome circulating rate of nMets and Mets patients (n=23-24). (B) Correlation of exosomes circulating rate with number of MetS criteria (n=48). (C) Exosome size of nMetS and MetS patients (n=22-26). (D) Correlation of exosomes size with number of MetS criteria (n=47). (E) Example of curves obtained by NTA, corresponding to sample of a MetS patient (filled curve) and a nMetS patient (dotted curve). The graph represents concentration of exosomes (events/ml) according to the size (nm). * $P < 0.05$.

Figure 2: Characterization and phenotype of circulating exosomes. (A) Electron-microscopic observation of isolated exosomes from a MetS patient showing specific round shape and average size of 63 nm vesicles. (B) Western blotting using antibodies against ALIX, CD63, TSG101, CD81 and CD9 in circulating microparticles (MP) and exosomes both from nMets and MetS patients. (C) Phenotype of exosomes from nMetS and MetS patients by flow cytometry using antibodies-coupled beads against platelet marker (CD41), leukocyte marker (CD29) and exosomal markers (CD81, CD63, CD9) (n=20-22). * $P < 0.05$; ** $P < 0.001$.

Figure 3: Exosomes from MetS patients decrease *in vitro* NO production in endothelial cells and impair *ex vivo* endothelium-dependent relaxation. During 24 hours, HAOECs were untreated (control) or incubated with nMets or MetS exosomes (10 $\mu\text{g/ml}$). NO production was measured by electronic paramagnetic resonance. (A) NO production of control cells or cells treated with nMetS and MetS exosomes (n=6-7). (B-D) Western blotting using antibodies against eNOS (n=5) (B), phospho-eNOS Serine 1177 (n=5) (C) or phospho-eNOS Threonine 495 (n=5). Mice were injected with vehicle (PBS), nMetS or MetS exosomes (10 $\mu\text{g/ml}$) by intravenous injection for 24 hours, then aorta ring contraction and relaxation were analyzed by myography. (E) Relaxation to acetylcholine of aorta rings from mice treated with vehicle, nMetS or MetS exosomes (n=5). Results are expressed as a percentage of relaxation of U6619-induced precontraction. (G) Relaxation to sodium nitroprusside (SNP) of aorta rings from mice treated with vehicle, nMets or MetS exosomes (n=5). * $P < 0.05$.

Figure 4: Exosomes from MetS patients increase mitochondrial oxidative stress in endothelial cells. HAOECs were untreated (control) or incubated with 10 µg/ml of exosomes from nMetS and MetS patients during 4 or 24 hours. (A-B) O₂⁻ production measured by electronic paramagnetic resonance at 4 hours (n=5-7) (A) or 24 hours (n=4-7) (B). (C) Cytosolic ROS production measured by DHE staining at 4 hours (n=9). Scale bar represents 20 µm. (D) Mitochondrial ROS production measured by MitoSox labelling at 4 hours (n=7-8). Scale bar represents 20 µm. (E) O₂⁻ production measured by electronic paramagnetic resonance in HAOECs incubated for 4 hours with MetS exosomes in absence or presence of mitoTEMPO (MT) (n=6). (F) NO production measured by electronic paramagnetic resonance in HAOECs incubated with MetS exosomes in absence or presence of MT (n=6). (G) The protein nitration was evaluated by Western blotting using antibody against nitrotyrosine-modified protein (n=5). *P <0.05, ** P<0.01.

Figure 5: Exosomes from MetS patients decrease mtDNA/nDNA ratio without affecting mitochondrial biogenesis. HAOECs were untreated (control) or incubated with nMetS or MetS exosomes (10 µg/ml), during 24 hours. (A) Quantitative real-time RT-PCR analysis of normalized relative quantity of mitochondrial DNA (ND5) (n=4). (B) Citrate synthase activity, marker of mitochondrial biogenesis, normalized by total protein (n=6). (C) Western-blotting using antibody against PGC1α (n=6). (D-E) Quantitative real-time RT-PCR analysis of normalized relative quantity of Tfam (n=6) and NRF1 (n=5) mRNA expressions. *P<0.05.

Figure 6: Exosomes from MetS patients disrupt the expression of proteins involved in the mitochondrial dynamics, without modifying the expression of proteins responsible for mitophagy. (A-C) Western blotting using antibodies against proteins involved in mitochondrial fusion: Mfn1 (n=6) (A), Mfn2 (n=6) (B) and OPA1 (n=5) (C). (D-E) Western blot using antibodies against proteins involved in mitochondrial fission: Fis1 (n=6) (D) and DRP1 (n=7) (E). (F-G) Western blot using antibodies against proteins involved in mitophagy: PINK1 (n=6) (F) and Parkin (n=4) (G). *P<0.05, **P<0.01.

Supplemental methods

Exosome internalization

Isolated exosomes were stained with PKH67dye (Sigma Aldrich) in agreement with the manufacturer's procedure. Briefly, exosomes were incubated with 2 mM PKH67dye in PBS for 2 minutes at room temperature. An equal volume of fetal bovine serum was added to stop staining. Then, exosomes were centrifuged at 100,000xg for 70 minutes to remove the supernatant. The exosome pellet was resuspended in PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄, KH₂PO₄ 1.8 mM, pH=7.4). PKH67-labeled exosomes were incubated with HAoECs for 4 or 24 hours at 37°C. At the end of incubation, cells were fixed with 4% paraformaldehyde for 20 minutes and then were stained with tetramethylrhodamine isothiocyanate-phalloidin (50 mg/ml, Sigma-Aldrich) for 90 minutes at room temperature. After washing with PBS, nuclei were stained with DAPI for 5 minutes. Finally, cells were mounted and visualized with confocal microscopy (CLMS700, Zeiss, ZENsoftware). All images were acquired using a x63 objective.

Supplemental Figures

Figure 1: Phenotype of exosomes from nMetS and MetS patients by flow cytometry with MACplex Exosome Kit. Exosomes are labelled with antibody-associated beads against 37 proteins (n=20-22).

Figure 2: NO production measured by electronic paramagnetic resonance. Endothelial cells were incubated during 24 hours with different concentrations of nMetS and Mets exosomes (5, 10, 20 µg/ml) (n=1).

Figure 3: Internalization of exosomes from nMetS and MetS patients by endothelial cells. Observation with confocal microscopy of exosomes labelled with PKH67 (green), nucleus with DAPI (blue) and actin with phalloidin (red). Scale bar represents 10 µm.

Figure 1

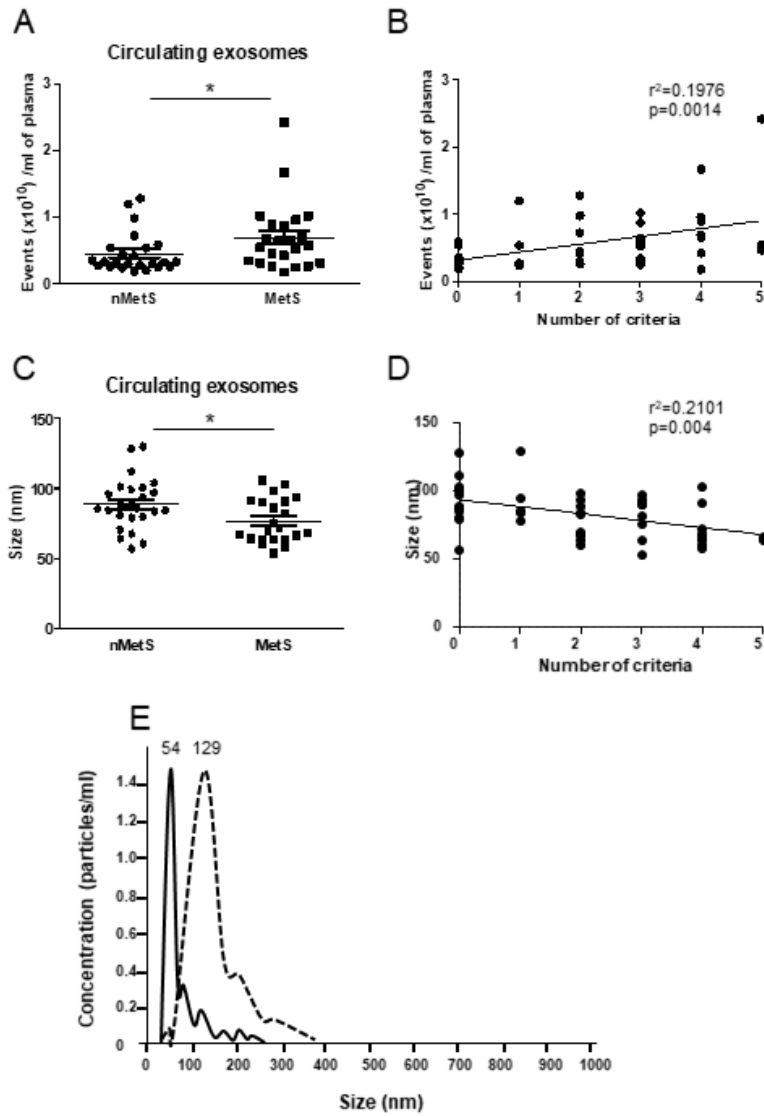


Figure 2

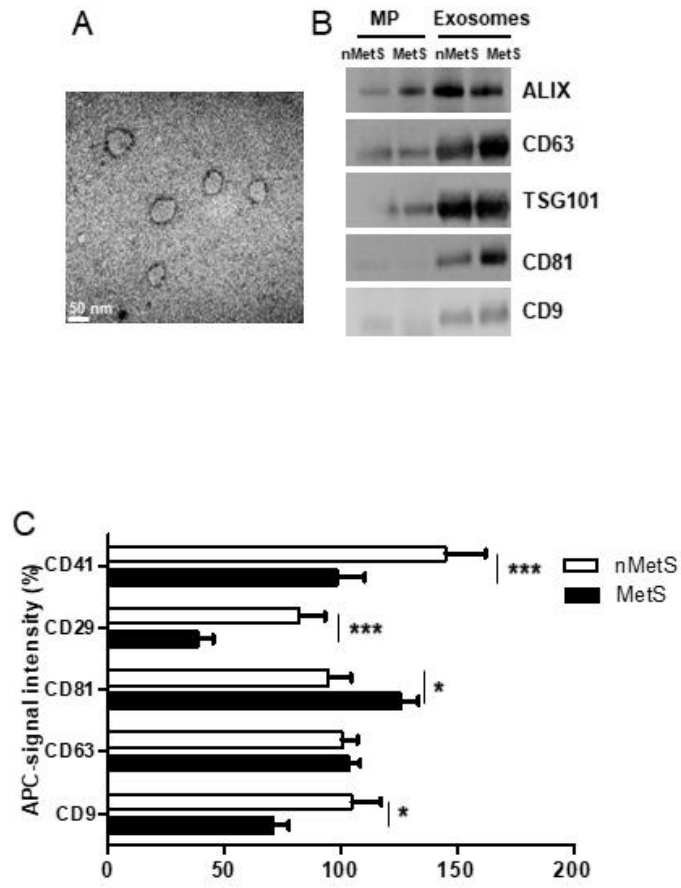


Figure 3

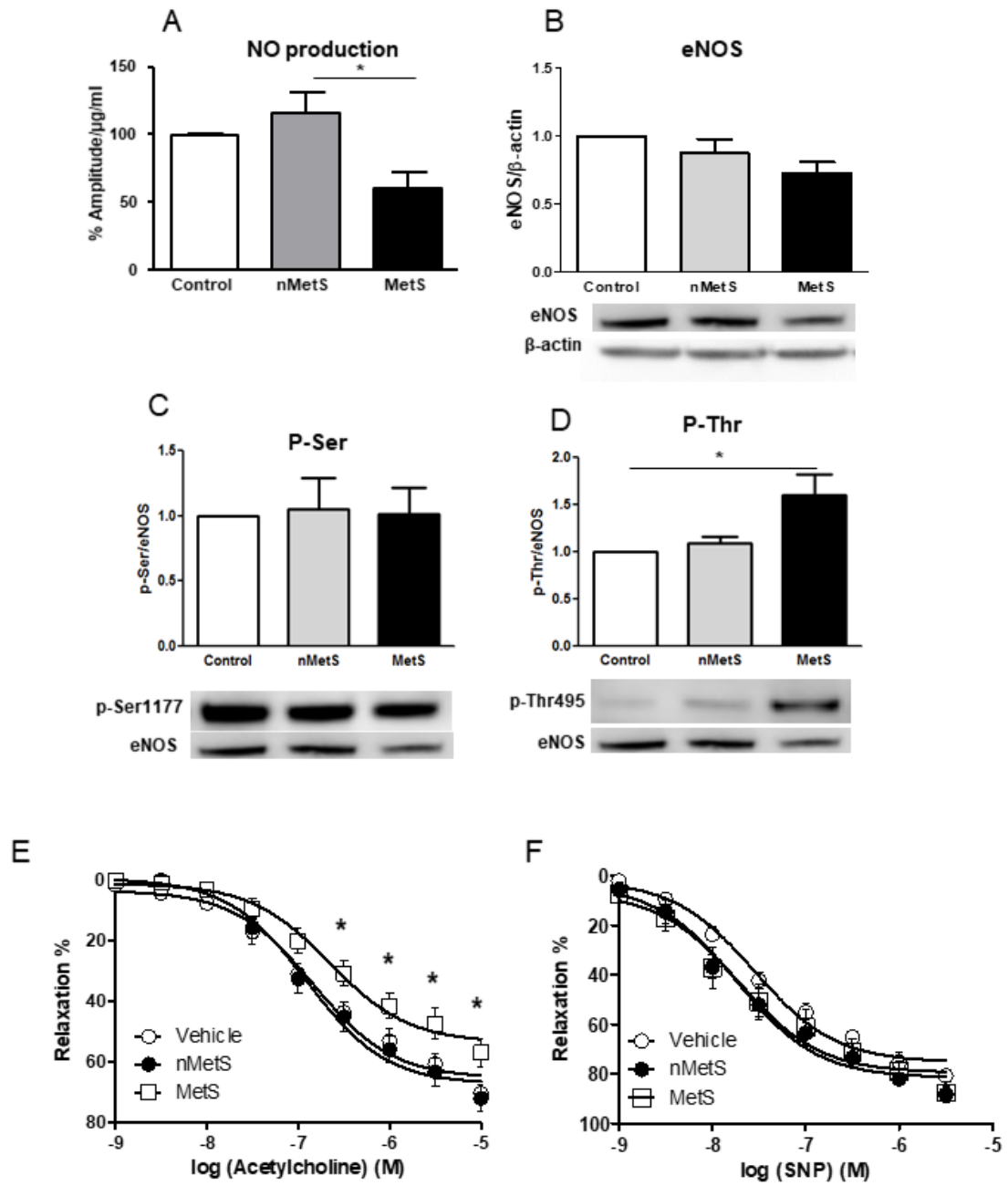


Figure 4

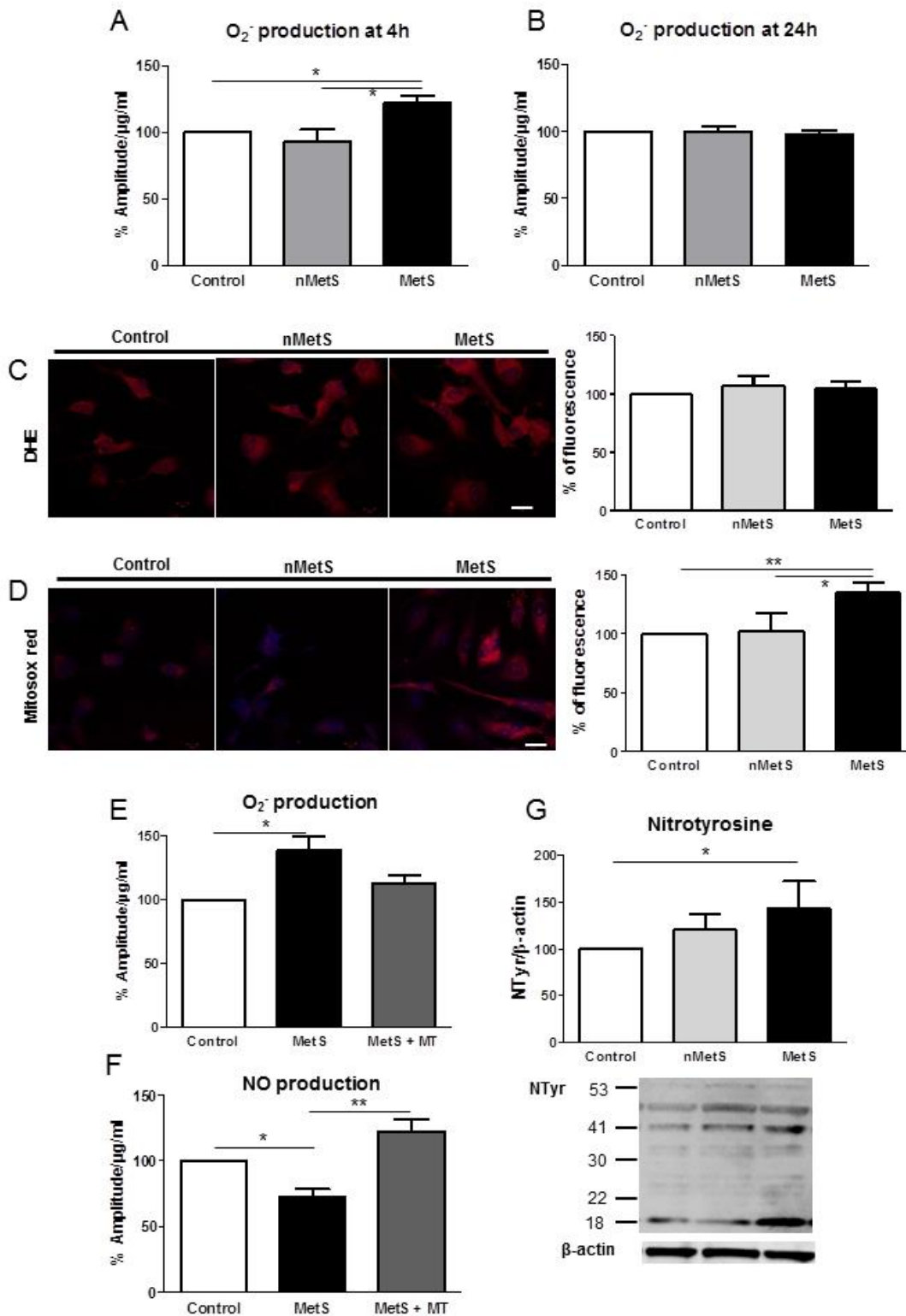


Figure 5

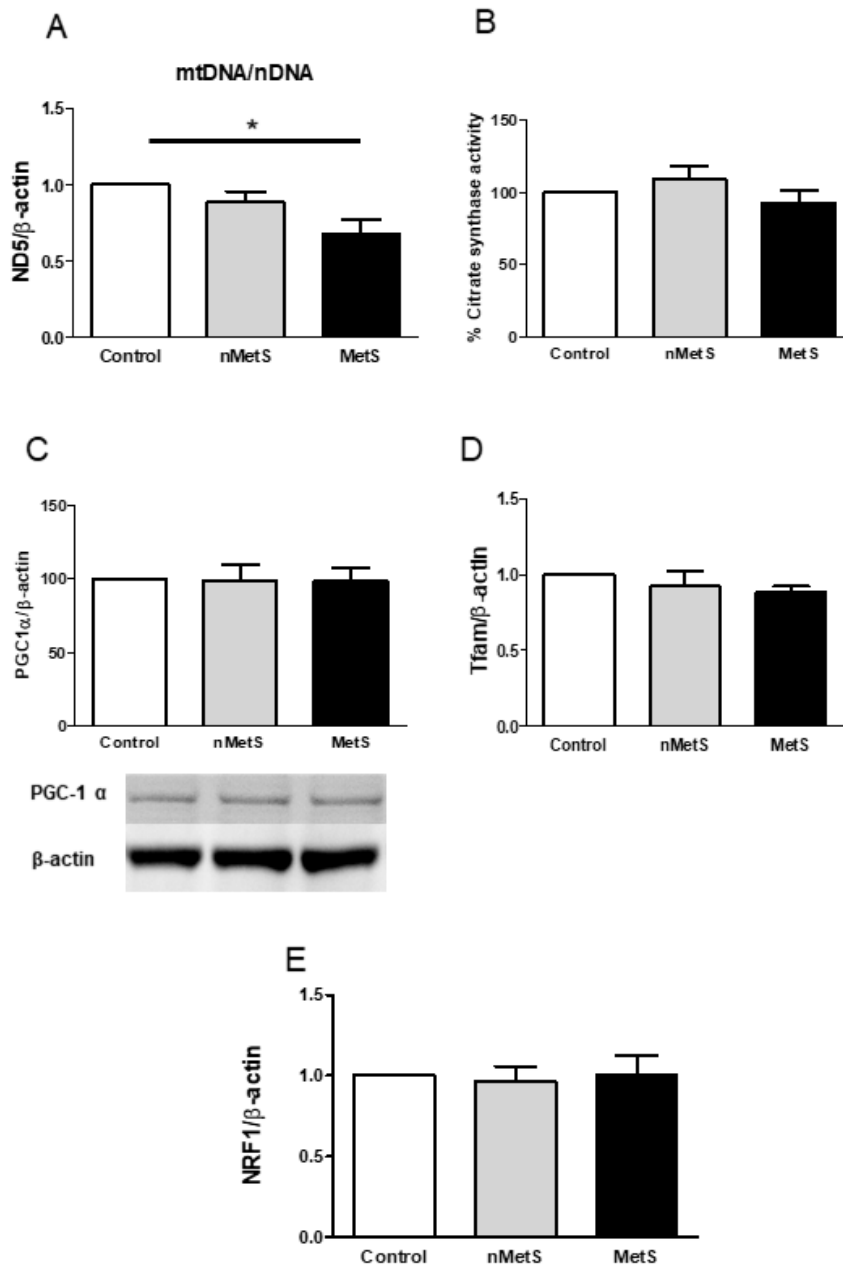
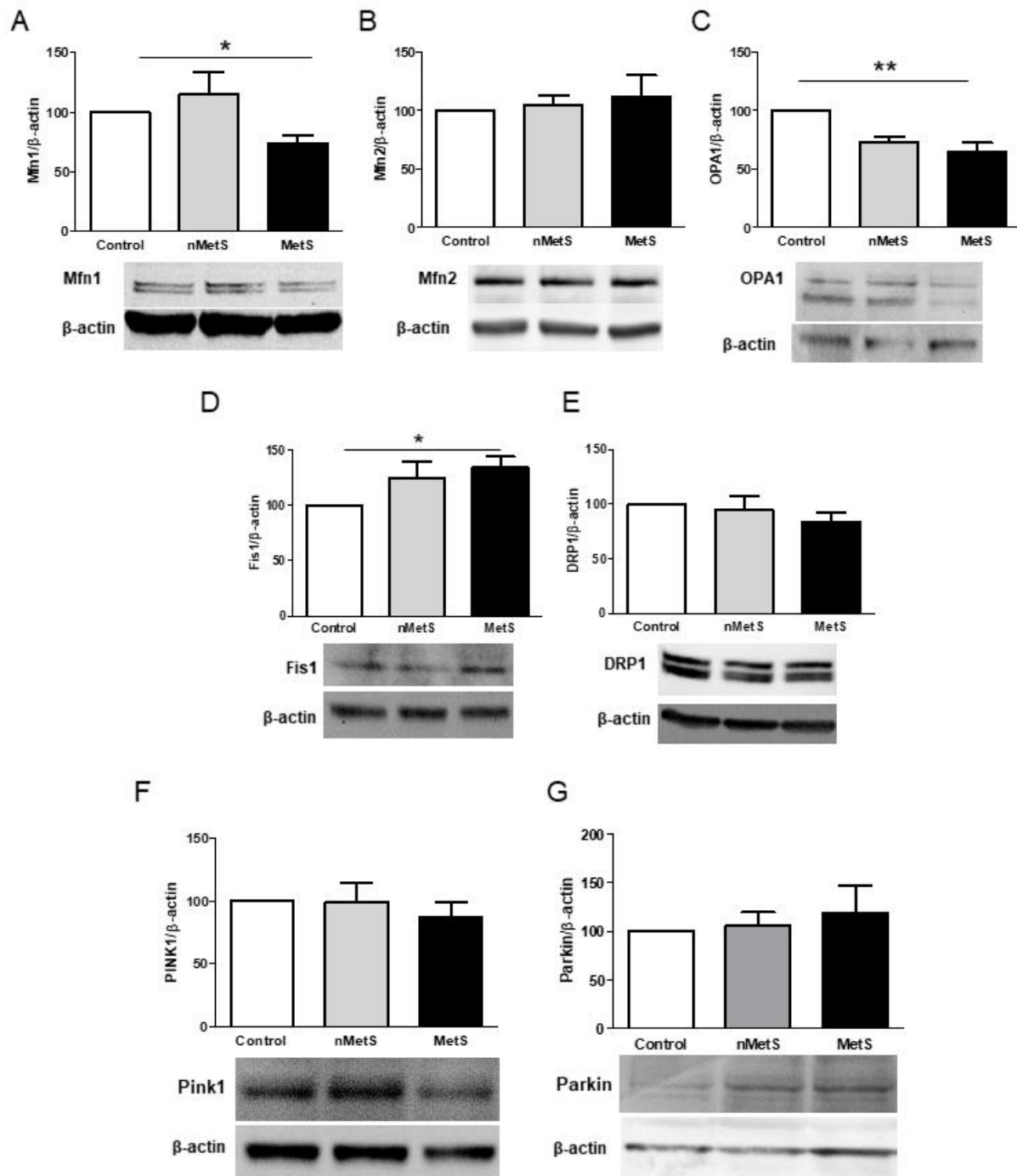


Figure 6



Supplemental data

Figure 1

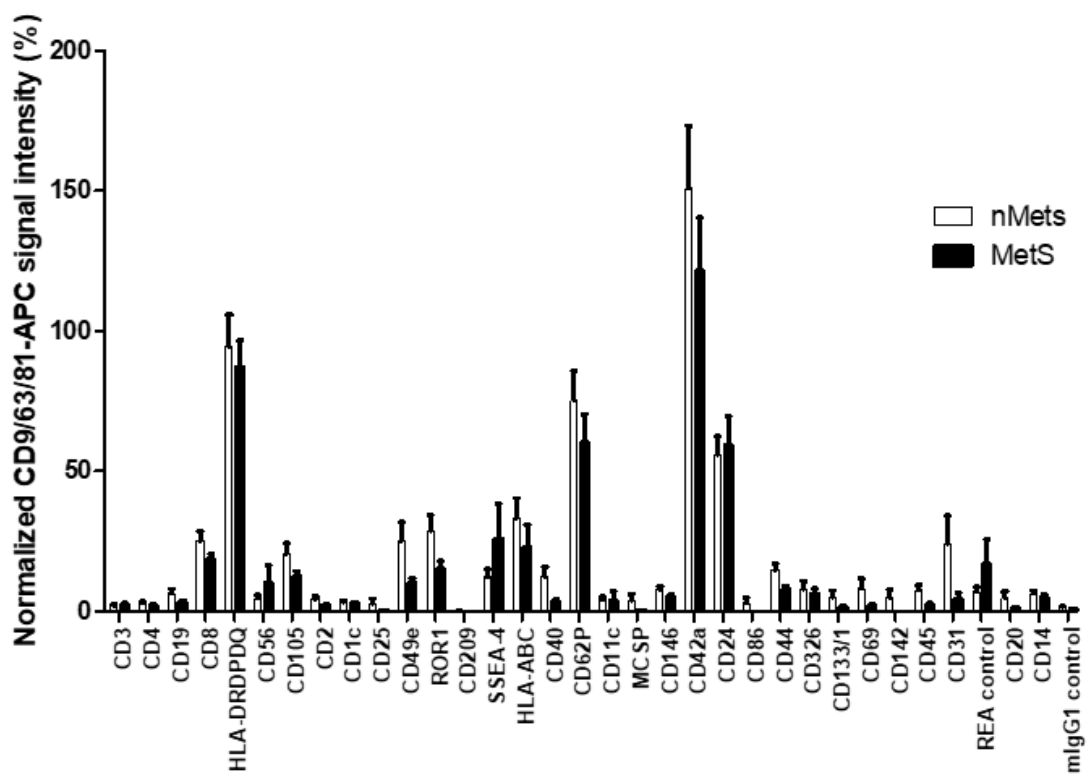


Figure 2

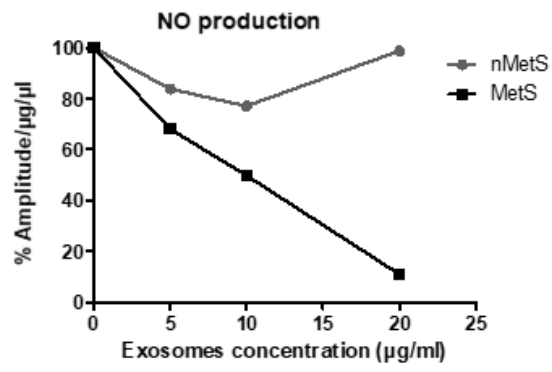
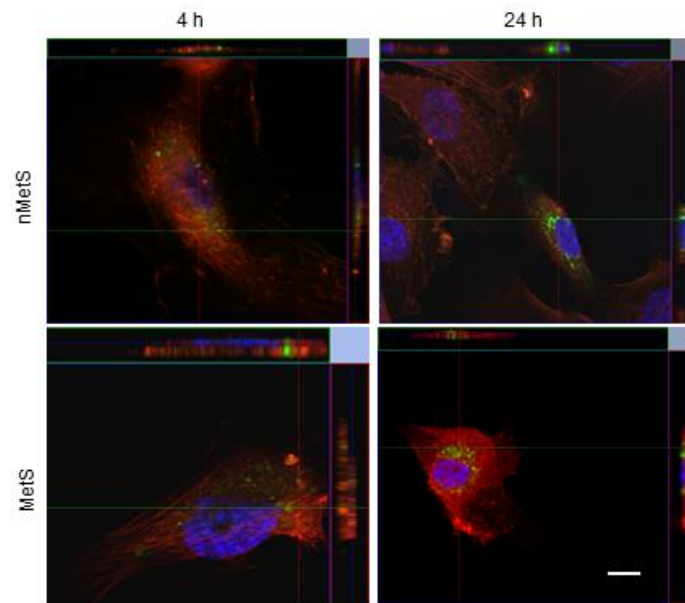


Figure 3



Discussion

Lors de cette étude, nous avons montré que les patients atteints de syndrome métabolique (SM) présentaient des exosomes circulants dont le taux est augmenté et la taille diminuée, par rapport à des patients non-syndrome métabolique (nSM). *In vitro*, les exosomes de patients SM diminuent la production de NO par les cellules endothéliales aortiques humaines (HAoECs). Cette diminution peut s'expliquer par une augmentation de la phosphorylation de la eNOS sur son site inhibiteur (la thréonine 495) et une augmentation de la production d' O_2^- mitochondrial, qui va réagir avec le NO et former de l' $ONOO^-$. Cet excès de ROS mitochondriaux peut provenir d'une perturbation dans l'expression de protéines impliquées dans la dynamique mitochondriale : les expressions de Mfn1 et d'OPA1, impliquées dans la fusion, sont diminuées alors que celle de Fis1, responsable de la fission, est augmentée, lorsque les cellules endothéliales sont traitées avec les exosomes des patients SM. *In vivo*, des souris traitées avec des exosomes de patients SM présentent une diminution du relâchement dépendant de l'endothélium, en réponse à l'acétylcholine (Figure 14). Ces résultats montrent l'intérêt des exosomes comme biomarqueurs pour le SM et leur implication dans la dysfonction endothéliale associée à cette pathologie.

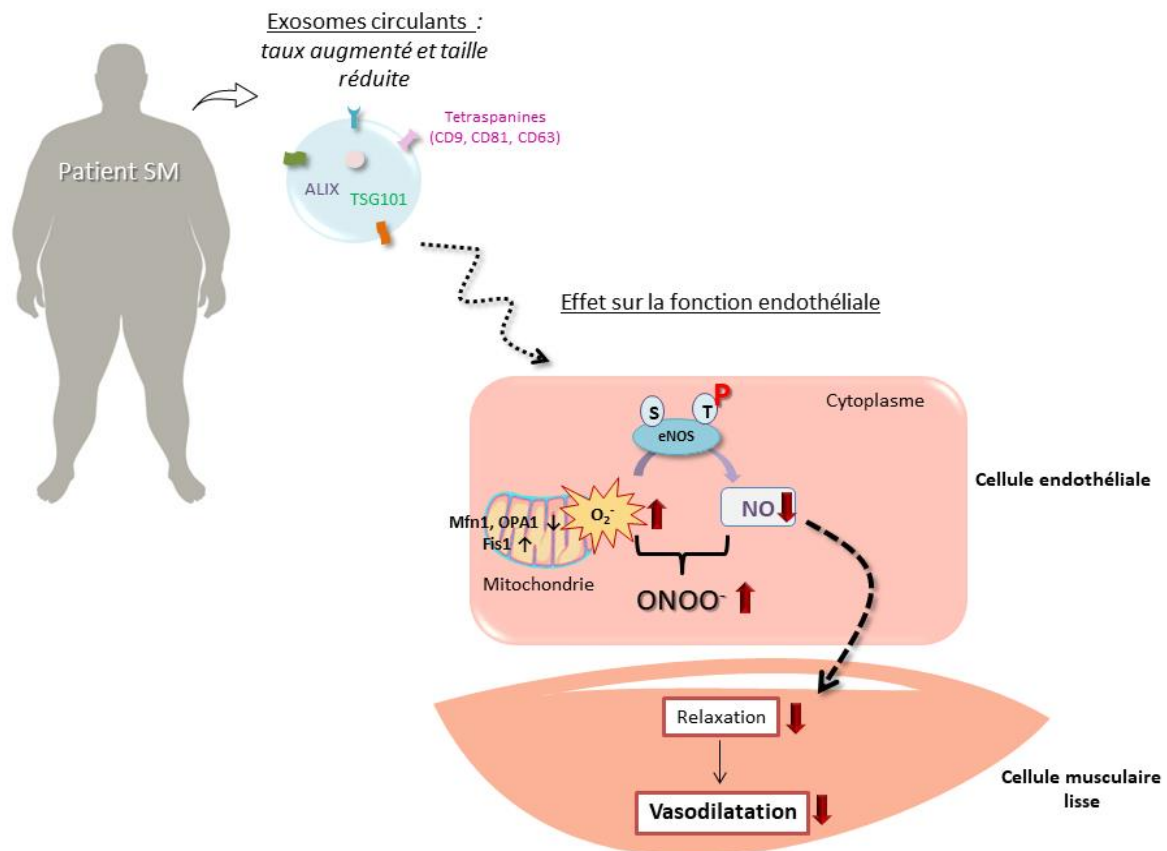


Figure 13 : Schéma bilan de l'implication des exosomes dans la dysfonction endothéliale associée au syndrome métabolique (SM). Les patients atteints de SM présentent un taux augmenté en exosomes circulants, dont la taille est réduite. Ces exosomes vont agir sur la cellule endothéliale en diminuant la libération de NO et ainsi altérer le relâchement dépendant de l'endothélium. Ces effets s'associent à une hausse de la production d'O₂⁻ mitochondriaux, pouvant provenir d'une perturbation de l'expression de protéines impliquées dans la dynamique mitochondriale.

Pour cette étude, les patients de la cohorte METABOL ont été divisé en deux groupes : ceux qui présentaient deux critères ou moins parmi les cinq critères du SM, faisaient partie des patients nSM et ceux qui présentaient trois critères ou plus, correspondaient aux patients SM. Les patients SM présentaient un tour de taille, une glycémie, un taux en triglycérides et une pression artérielle significativement plus élevés et un taux de cholestérol HDL significativement diminué par rapport aux patients nSM, paramètres correspondant aux critères du SM. Cependant, les patients SM étaient significativement plus âgés, probablement, en raison de l'augmentation de la prévalence du SM liée à l'âge.

Dans un premier temps, il était nécessaire de caractériser les exosomes purifiés à partir des prélèvements sanguins de ces patients, afin de vérifier que notre protocole expérimental permettait un isolement spécifique des exosomes. Ainsi, la taille (entre 30 et 100 nm de diamètre) et les marqueurs protéiques analysés (TSG101, ALIX, CD81, CD9 et CD63) correspondaient à ceux décrits dans la littérature pour les exosomes. D'après nos résultats, parmi les marqueurs analysés, le CD81 serait le plus spécifique car il est peu retrouvé dans les MP issues de mêmes patients. Cette observation est confirmée par les travaux de Kowal et al (2016) montrant que CD81 est fortement exprimé par les exosomes des cellules dendritiques. Cependant, certains de nos résultats divergent par rapport à ces travaux. En effet, cette équipe montre que TSG101 est retrouvé spécifiquement dans les exosomes alors que nous le retrouvons également exprimé dans les MP. De plus, la β -actine qui est présente dans les MP circulantes des patients METABOL, n'était pas retrouvée dans les exosomes, contrairement à Kowal et al (2016) (Figure 15). Ainsi, la spécificité des marqueurs exosomaux reste encore à définir. Leurs expressions peuvent dépendre de la méthode d'isolement des exosomes ou du type des cellules d'origine.

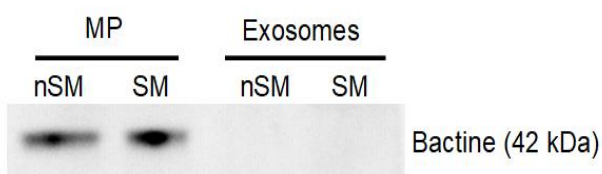


Figure 14 : Western blot montrant l'expression de la β actine. La β actine est présente dans les microparticules (MP) mais pas dans les exosomes de patients nSM et SM.

Par la suite, il semblait important de connaître l'origine cellulaire de ces exosomes. Comme décrit en introduction, ce sont plutôt les MP qui sont enrichies en protéines membranaires spécifiques des cellules d'origine, mais il est également possible de caractériser les exosomes avec les

mêmes protéines (par exemple, CD45 pour les leucocytes, CD146 pour les cellules endothéliales...). Il a été montré dans la littérature que les vésicules circulantes étaient majoritairement d'origine plaquettaire (Arraud et al., 2014). Effectivement, les exosomes que nous avons analysés sont principalement d'origine plaquettaire (CD41, CD42 et CD62P) ou leucocytaire (CD29, HLA-DRPDQ). Cependant, nous avons observé que les populations d'exosomes plaquettaires (CD41) et leucocytaires (CD29) diminuent chez ces patients SM alors que leur taux circulant total d'exosomes est augmenté. Plusieurs hypothèses peuvent être avancées pour expliquer ces données. En effet, il est possible que les exosomes ne portent pas tous des marqueurs de surface cellulaire puisque ils ne sont pas riches en protéines membranaires. La population d'exosomes augmentée pourrait aussi provenir d'un type cellulaire autre que les plaquettes ou les leucocytes. Dans ce contexte, nous nous sommes intéressés à la population d'exosomes dérivés des adipocytes, décrite comme augmentée dans l'obésité. Les exosomes dérivés des adipocytes portent spécifiquement l'adiponectine (Phoonsawat et al., 2014). Ainsi, nous avons analysé par cytométrie la présence de ce marqueur sur les exosomes de patients mais il n'est porté que par ~1% des exosomes présents dans la circulation (résultats non montrés, en collaboration avec le Dr Le Lay). De plus, aucune différence d'expression d'adiponectine n'a été observée entre les exosomes de patients nSM et SM. Cependant, c'est une protéine dont la production varie : elle est décrite comme diminuée chez les patients atteints de SM, et peut être ainsi répartie différemment dans les exosomes de ces patients. Ainsi, ce n'est peut-être pas un marqueur pertinent afin de quantifier une variation de la population d'exosomes dérivés des adipocytes. Cette population reste donc difficile à identifier et à quantifier.

Par la suite, nous avons montré que chez les patients METABOL, le taux circulant d'exosomes était corrélé positivement avec le tour de taille, l'IMC et le taux de triglycérides. Ces résultats suggèrent que la production d'exosomes semble donc augmenter avec l'obésité. De plus, ce taux augmente en fonction du nombre de critères du SM. Si beaucoup d'études montrent l'augmentation des exosomes dérivés des adipocytes lors de l'obésité (voir introduction), peu de données existent sur l'élévation du taux d'exosomes circulant, qui est seulement montré augmenté chez les souris obèses *ob/ob* (Phoonsawat et al., 2014).

De façon inattendue, nous avons également montré que la taille des exosomes diminuait avec l'augmentation de l'IMC, la glycémie, l'insulinémie, l'indice d'insulino-résistance HOMA, les triglycérides et le LDL cholestérol. La taille des exosomes semblerait donc diminuer avec l'obésité, la dyslipidémie et le diabète/la résistance à l'insuline. De plus, la diminution de la taille est corrélée avec le nombre de critères du SM. La population que nous avons caractérisée au NTA présente une taille comprise entre 60-70 nm ce qui est inférieure à celle des exosomes dérivés de plaquettes (~ 90 nm) (Goetzl et al., 2016). Les exosomes de lymphocytes B, qui présentent un diamètre de 60-80 nm (Théry et al., 2002), pourraient correspondre mais cette population ne semble pas augmentée chez les patients SM car l'expression des marqueurs des lymphocytes B (CD19, CD69, CD20, CD40 et CD86) n'est pas modifiée. Il n'existe pas encore de données de la

littérature sur l'étude de la taille des exosomes comme biomarqueur de pathologie. Cependant, il a été montré que les CMV formés dans des cellules Hela sous-exprimant Rab27b ont une taille moindre que les cellules contrôles. Rab27b contrôle la formation d'exosomes en permettant le transfert de membranes du réseau trans-golgien vers les endosomes (Ostrowski et al., 2010). Il serait plausible que la réduction de la taille des CMV dans les cellules Rab27b KO, entraîne également une diminution de la taille des exosomes. Par ailleurs, Rab27 mais également d'autres protéines de la famille Rab GTPases peuvent réguler la formation et sécrétion d'exosomes (Blanc and Vidal, 2017). D'autre part, les exosomes dérivés de cellules cancéreuses et formés via la voie dépendante des céramides, présentent un taille d'environ 60 nm (Willms et al., 2016). Néanmoins, il reste encore à identifier le(s) facteur(s) du SM qui perturbe(nt) la formation et la taille des exosomes. Il a été montré que la composition lipidique pourrait affecter la taille des exosomes (Yang et al., 2017). En effet, les exosomes isolés à partir de l'urine de patients atteints de cancer de prostate ont une taille de ~107 nm tandis que celles de témoins est de ~58 nm. Cette différence de taille est associée à un enrichissement des exosomes en certains lipides tels que les phosphatidylglycerols et les triacylglycerols (Yang et al., 2017).

Nous avons vu précédemment que les patients SM étaient plus âgés que les patients nSM. Cependant, il n'y a pas de corrélation entre l'âge des patients, la quantité et la taille des exosomes ce qui suggère que les différences observées au niveau de la concentration et de la taille des exosomes ne sont pas dues à la différence d'âge.

Ainsi, le taux circulant en exosomes augmente et leur taille diminue en fonction du nombre de critères du SM chez ces patients : ces deux paramètres pourraient servir de biomarqueurs pour la sévérité de la maladie.

Au niveau fonctionnel, les exosomes provenant des patients SM augmentent, dans les cellules endothéliales, la production de ROS mitochondriaux à 4 heures et diminuent la production de NO à 24 heures. Pour expliquer cette hausse de la production de ROS mitochondriaux, nous avons évalué si la fonction mitochondriale était affectée en présence des exosomes de patients SM. Bien que la quantité d'ADN mitochondrial semble diminuée, la biogenèse mitochondriale n'est pas modifiée. En effet, les expressions géniques de TFAM et NRF1, et l'expression protéique de PGC1 α ne sont pas modifiées, ainsi que l'activité de la citrate synthase, utilisée comme marqueur de la biogenèse mitochondriale. De plus, par microscopie confocale, les mitochondries apparaissaient fonctionnelles, lorsque le potentiel de membrane mitochondriale est détecté avec le marqueur TMRM. Après un traitement de 24 heures, nous avons observé une différence dans l'expression des protéines de fusion et fission, Mfn1 et OPA1 étant diminuées et FIS1 augmentée. En revanche, l'expression de Mfn2 et DRP1, deux protéines régulant la dynamique mitochondriale, n'ont pas été modifiées. Cependant, nous n'avons pas pu mettre en évidence une modification du réseau mitochondrial après 4 heures de traitement avec les exosomes de patients SM par rapport à des cellules non traitées ou traitées avec les exosomes nSM. Nous pouvons émettre l'hypothèse selon laquelle une compensation de l'activité des protéines impliquées dans la dynamique

mitochondriale pourrait assurer la fonctionnalité du réseau. Toutefois, cette perturbation pourrait entraîner un excès de ROS mitochondriaux qui dépasse le pouvoir antioxydant de la cellule.

Pour l'instant, il reste à déterminer les voies moléculaires par lesquelles les exosomes de patients SM agissent. Toutefois, nous avons montré que les cellules endothéliales internalisaient les exosomes de patients nSM et SM, suggérant ainsi que le cargo exosomal (protéines, acides nucléiques et lipides) pourrait être responsable de ces effets.

Dans notre laboratoire, il a déjà été montré que les MP pouvaient induire une dysfonction endothéliale dans le cadre du SM. Ces effets étaient médiés par la voie FAS/Fas ligand, activant la sphingomyélinase neutre, et induisant un stress du réticulum et la production de ROS mitochondriaux et cytosoliques (Figure 16) (Safiedeen et al., 2017). Afin d'évaluer si un tel mécanisme pouvait être responsable des effets induits par les exosomes de patients SM, nous

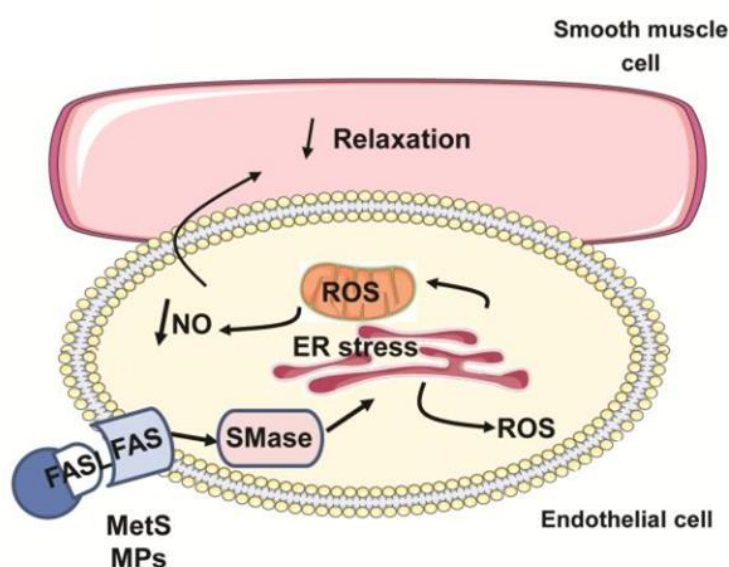


Figure 15 : Implication des microparticules de patients SM (MetS MPS) dans la dysfonction

endothéliale. Les MetS MPS agissent sur la cellule endothéliale via la voie FAS/FAS ligand, activant ainsi la sphingomyélinase neutre (SMase). La SMase va alors induire un stress du réticulum endoplasmique et une production de ROS cytoplasmique et mitochondriaux, entraînant une diminution du NO et l'altération de la vasorelaxation (Safiedeen et al 2017).

avons incubé les cellules endothéliales HAoECs avec les exosomes de patients SM en présence d'un inhibiteur de la SMase, le GW 4869, puis mesuré la production de ROS. Cependant, l'augmentation des ROS induite par les exosomes n'a pas été inhibée, montrant que le mécanisme d'action des exosomes des patients SM met en jeu une voie différente de celle utilisée par les MP. Il serait intéressant d'évaluer si ces effets sont médiés par des miARN portés spécifiquement par les exosomes de patients SM. De nombreux miARN ont été montrés comme impliqués dans la dysfonction endothéliale (pour revues Santulli, 2016 ; Sun et al., 2013). Parmi eux, miR-630 a été identifié comme associé à la dysfonction endothéliale d'enfants atteints de syndrome d'apnée du sommeil (Khalyfa et al., 2016) et pourrait être un bon candidat

Par ailleurs, il faut également noter que la concentration utilisée pour nos expériences (10 µg/ml) n'est pas la concentration circulante retrouvée chez les patients (plutôt de l'ordre de 30-40 µg/ml). Cette concentration a été choisie après la réalisation d'une gamme de concentration avec les exosomes de patients nSM et SM sur la production de NO, car elle diminuait déjà de 50 % la quantité de NO dans les cellules endothéliales. De ce fait, la véritable concentration circulante des exosomes des patients SM pourrait être encore plus délétère pour l'organisme.

Ainsi, les VEs dans le cadre du SM semblent être un reflet de la cellule malade mais elles participent aussi au développement de la maladie. Malgré des mécanismes de formation et des contenus différents, les MP et les exosomes sont tous les deux impliqués dans la dysfonction endothéliale. Il serait d'ailleurs intéressant d'étudier si leurs effets se potentialisent, si l'on traite des cellules endothéliales avec ces deux types de vésicules en même temps.

Enfin, les exosomes circulants de patients SM pourraient jouer un rôle dans d'autres mécanismes que la dysfonction endothéliale. Par exemple, il a été montré chez des patients atteints de syndrome d'apnée du sommeil que les exosomes circulants participaient au développement d'une résistance à l'insuline au niveau des adipocytes (Khalyfa et al., 2016).

Pour aller plus loin et dans une perspective thérapeutique, il serait nécessaire d'identifier et caractériser la(es) population(s) d'exosomes responsable(s) (leur formation, sécrétion...) dans le but de réduire leur libération ou leur internalisation, et ainsi d'inhiber leur effet. Cette stratégie est prometteuse car, par exemple, il a été montré qu'en bloquant Rab27a, responsable de la libération d'exosomes dérivés de cellules cancéreuses de carcinome, la taille et la dissémination de la tumeur diminuaient (Bobrie et al., 2012). Il reste maintenant à identifier leur mécanisme d'action dans le but de bloquer la voie de signalisation responsable.

Introduction projet 2

Le cancer du rein est souvent diagnostiqué dans un état avancé et quand le pronostic vital est déjà engagé. Il n'existe aujourd'hui aucun biomarqueur diagnostique ou pronostic utilisé en pratique clinique et qui permettrait de discriminer une tumeur bénigne d'une tumeur maligne ou encore de déterminer quels patients présentent une tumeur agressive à risque d'évolution rapide après chirurgie. Il a été montré que l'anhydrase carbonique IX (CA9) pourrait être un bon candidat comme biomarqueur du pronostic du cancer du rein (Pantuck et al., 2008). De plus, ce marqueur est porté par les exosomes (Dorai, 2009). Il a également été évoqué l'utilisation des exosomes isolés dans les urines comme marqueurs potentiels du diagnostic et l'expression de la CA9 associée aux exosomes urinaires a été montrée comme augmentée (Raimondo et al., 2013). En revanche, à ce jour, aucune étude n'a évalué l'intérêt des exosomes et MP circulants comme facteur pronostic ou diagnostique du cancer du rein.

Objectifs du travail

L'objectif de ce projet de recherche est d'évaluer si les exosomes circulants pourraient être de biomarqueurs pertinents dans le cancer du rein en tant que marqueurs du pronostic et/ou du diagnostic. Pour cela, nous chercherons d'abord à caractériser les exosomes circulants de patients atteints de cancer du rein avant la néphrectomie (J0), c'est-à-dire l'ablation de la tumeur présente au niveau du rein, le lendemain (J1) et un mois après (M1), en les comparant aux exosomes circulants provenant de patients subissant également une néphrectomie pour une autre cause qu'un cancer. De plus, nous essaierons de déterminer si l'expression de la CA9 par les exosomes pourrait servir de biomarqueurs pour cette pathologie.

Matériel et Méthode

Cohorte de patients

Les témoins et les patients atteints de cancer du rein participant à cette étude proviennent du Service d'Urologie du CHU d'Angers. Les patients atteints de cancer du rein subissent une ablation partielle ou totale du rein (une néphrectomie), afin d'enlever la tumeur. Les témoins présents dans cette étude n'ont pas de cancer et subissent également une néphrectomie. Ces patients subissent différents prélèvements sanguins à J0 : avant la néphrectomie, J1 : un jour après la néphrectomie et M1 : un mois après la néphrectomie. Cette étude comporte 5 témoins et 17 patients atteints de cancer du rein. Parmi ces 17 patients, 4 ont été prélevés à M1. Tous les donneurs ont donné leur accord pour participer à cette étude.

Isolation des vésicules extracellulaires (VE)

A partir des prélèvements sanguins des patients, les VE sont isolées en effectuant tout d'abord différentes centrifugations : à 250xg pendant 20 minutes pour enlever les cellules sanguines, puis le surnageant est récupéré et centrifugé à 1500xg pendant 20 minutes pour enlever les débris cellulaires et les plaquettes. Le plasma pauvre en plaquette (PPP) est récupéré et centrifugé à 17000xg pendant 45 minutes. Le culot ainsi récupéré correspond aux MP qui seront ensuite lavées et resuspendues dans du chlorure de sodium 0,9% (NaCl). Le PPP est alors centrifugé à 100 000xg pendant 70 minutes, pour isoler les exosomes, qui sont ensuite lavés dans du PBS et subissent une deuxième ultracentrifugation (100 000xg, 70 minutes). Ils sont finalement resuspendus dans 200 µL de PBS (NaCl 137 mM, KCl 2,7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1,8 mM, pH=7,4). Un dosage protéique des exosomes par la méthode de Lowry (kit Biorad) est effectué afin de déterminer le taux circulant de protéines exosomales pour chaque patient, normalisé par le volume de plasma. Les résultats sont exprimés en µg de protéines par ml de plasma (µg/ml). Les exosomes sont conservés à 4°C jusqu'à utilisation.

Taux circulant et taille des exosomes par Nanoparticle tracking analysis (NTA)

Les exosomes isolés sont centrifugés à 100 000xg pendant 70 minutes pour être repris dans du NaCl 0,9% injectable, afin d'éviter au maximum les poussières et ainsi le bruit de fond lors du passage en NTA, qui est capable de déterminer la taille de particules entre 30 et 1000 nm. Le NTA est effectué en utilisant l'appareil NanoSight NS300 (Malvern Instruments Ltd, Worcestershire, UK) équipé d'un laser de 405 nm. Pour chaque échantillon, cinq vidéos de 90 secondes sont enregistrées. Le logiciel est alors capable de suivre chaque trajectoire individuelle des exosomes en fonction de leur mouvement Brownien, relier ce mouvement à une taille de particule et de calculer la concentration totale, pour chaque échantillon. L'enregistrement des données et l'analyse sont effectuées avec le logiciel NTA version 3.1. Les résultats présentés correspondent à la moyenne des 5 vidéos prises pour un échantillon.

Caractérisation du phénotype des exosomes

Les origines cellulaires des exosomes circulants ont été déterminées par le kit MACSPlex Exosome (Miltenyi Biotec, Auburn, CA) en accord avec la procédure du fabricant. En bref, 10 µg d'exosomes ont été ajoutés à des billes couplées à des anticorps conjugués au fluorochrome APC contre 37 épitopes différents. Ensuite, les exosomes liés aux billes sont reconnus par les réactifs de détection : des billes associées aux anticorps anti-CD63, -CD81 et -CD9, les marqueurs spécifiques des exosomes. Les échantillons sont alors analysés par cytométrie MACSQuant (Miltenyi Biotec) à l'aide du logiciel MACSQuantify. Un marquage positif indique la présence de l'épitope de surface dans la population d'exosomes. Les résultats ont été exprimés en médiane de fluorescence, normalisée par la moyenne de la médiane de fluorescence de CD63, CD81 et CD9.

Western Blot

Les exosomes de patients (15 µg) sont préparés avec un tampon composé de LDS Sample Buffer et de Reducing Agent (NuPAGE, Invitrogen) Les échantillons sont ensuite chargés dans des gels d'acrylamide NuPAGE 4-12% (ThermoFisher Scientific). La migration des échantillons s'effectue à 150V pendant 90 minutes. Les gels sont ensuite montés en sandwich, et transférés ainsi sur une membrane de nitrocellulose (Amersham) à 110V pendant 1h30 à 4°C. Les membranes sont ensuite recouvertes de rouge ponceau afin de vérifier le transfert et de visualiser la quantité totale des protéines. Les membranes sont alors saturées dans de la BSA 5% (diluées dans du Tris-buffered saline-Tween (TBST) 1X) pendant 1h. Par la suite, les membranes sont incubées pendant 90 minutes avec l'anticorps primaire dirigé contre la protéine d'intérêt. Après 3 lavages de 10 minutes au TBS-T 1x, l'anticorps secondaire dirigé contre l'espèce de l'anticorps primaire et conjugué à la HRP (Horseradish peroxydase), est ajouté pendant 90 minutes. A nouveau, 3 lavages de 10 min sont effectués. La révélation des protéines se fait par chimiluminescence, avec un imageur Bio-Rad. Elle est effectuée en déposant le révélateur Luminol (Santa Cruz Biotechnologies, Dallas, TX) sur la membrane, qui va réagir avec la peroxydase et libérer un signal lumineux. Si l'intensité est trop faible pour être analysée, la révélation peut être faite en utilisant du Femto (Thermo Scientific). La quantité de protéine d'intérêt peut être normalisée par la quantité totale de protéines, les exosomes ne portant pas la β -actine, le gène de ménage le plus couramment utilisé. La quantification se fait par le logiciel Image J.

Les anticorps utilisés sont dirigés contre CD63, CD9 et TSG101 (Santa Cruz Biotechnologies) et CA9 (Cell Signaling, Beverly, MA).

Résultats expérimentaux

	<i>Témoins</i>	<i>Patients</i>
<i>Nombre</i>	5	17
<i>Ratio H/F</i>	3/2	14/3
<i>Age</i>	65 ± 14	52 ± 10 *
<i>Diabète (%)</i>	0	35
<i>HTA (%)</i>	40	65
<i>Type de néphrectomie (%) :</i>		
<i>Elargie</i>	100	80
<i>Partielle</i>	-	20
<i>Type de cancer (%) :</i>		
<i>CRCC</i>	-	71
<i>TPII</i>	-	12
<i>Autre type</i>	-	17
<i>Grade Furhmann (%) :</i>		
<i>I</i>	-	6
<i>II</i>	-	35
<i>III</i>	-	24
<i>IV</i>	-	18

Tableau 2 : Caractéristiques des témoins et patients atteints de cancer du rein présents dans notre étude. Les grades Furhman correspondent à la gravité de la tumeur. *P<0.05. Pour les analyses statistiques, un Mann-Whitney a été réalisé.

Abréviations : H/F : homme/femme, HTA : hypertension artérielle, CRCC : carcinome à cellules claires, TPII : carcinome tubulo-papillaire type II.

Comme montré en Table 2, cette étude comporte plus de patients atteints de cancer du rein que de témoins. Parmi les patients cancéreux, il y a plus d'hommes que de femmes. Ces patients sont également plus jeunes que les témoins ($p=0,0311$). Parmi ces témoins, personne ne présente de diabète mais 40% présentent une hypertension artérielle (HTA), tandis que chez les patients atteints de cancer du rein 35% présentent un diabète et 65% une HTA. La plupart de ces patients subissent une néphrectomie élargie (100% chez les témoins et 80% chez les patients) par rapport à une néphrectomie partielle (20% chez les patients cancéreux). Le cancer du rein le plus représenté chez ces patients est le carcinome à cellules claires (71%). 12% cependant présentent un carcinome tubulo-papillaire type II. Les autres types de cancer correspondent au sarcome et à l'adénome oncocytaire (17%). Les cancers du rein peuvent être classés en fonction du grade histopronostic de Furhman qui détermine la gravité de la tumeur en fonction du degré de différenciation des cellules prélevées lors de la biopsie ou de la néphrectomie. Les patients de notre étude présentent des tumeurs de grade I, II, III et IV pour 6, 35, 24 et 18 % respectivement.

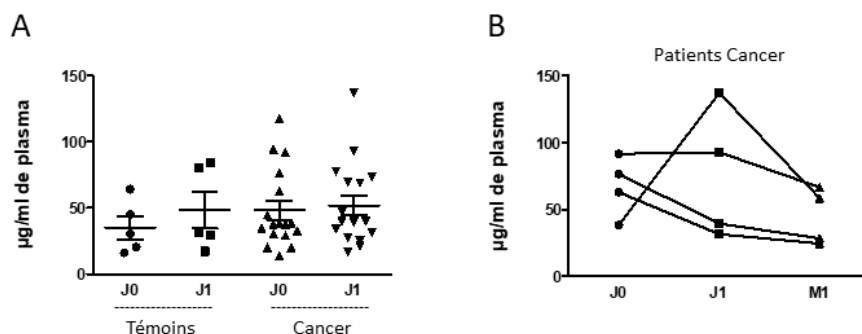


Figure 16 : Evolution du taux d'exosomes circulants avant et après néphrectomie chez des témoins et des patients atteints de cancer du rein.

Le taux circulant exosomal correspond à la quantité de protéines exosomales, déterminée par dosage protéique. J0 représente le jour de la néphrectomie et le prélèvement a lieu avant l'opération ; J1, un jour après la néphrectomie et M1, un mois après la néphrectomie.

(A) Taux circulants exosomal des témoins et patients atteints de cancer du rein à J0 et à J1 (n=5-17).

(B) Taux d'exosomes circulants des patients atteints de cancer du rein à J0, J1 et M1 (n=4).

Nous avons ensuite déterminé le taux d'exosomes circulants pour chaque patient. Ainsi, on observe qu'un jour après la néphrectomie (J1), le taux d'exosomes circulants chez les témoins et les patients cancéreux ont tendance à augmenter (Figure 16A). De plus, à J0, les patients atteints de cancer du rein ont tendance à avoir un taux d'exosomes circulant augmenté par rapport aux témoins (Figure 16A). Par ailleurs, un mois après néphrectomie, les patients atteints de cancer du rein présentent principalement un taux d'exosomes circulant qui a une tendance à diminuer (Figure 16B).

Par la suite, les exosomes isolés ont été caractérisés par Western blot, pour déterminer la présence de marqueurs spécifiques des exosomes. Effectivement, les exosomes provenant des patients atteints de cancer du rein à J0 et J1 présentent bien les marqueurs CD63, TSG101 et CD9 (Figure 17A). De plus, les exosomes circulants de patients cancéreux présentent une taille similaire à J0, J1 et M1 (Figure 17B), qui n'est pas différente de celles des exosomes isolés à partir des témoins à J0 et J1 (Figure 17C). Cependant, lorsqu'on étudie l'origine cellulaire des exosomes de patients cancéreux à J0, J1 et M1, nous observons que les exosomes d'origine plaquettaire (CD42a) diminuent significativement entre J0 et M1 et J1 et M1, ainsi que les exosomes d'origine leucocytaire (CD29) entre J1 et M1 (Figure 17D).

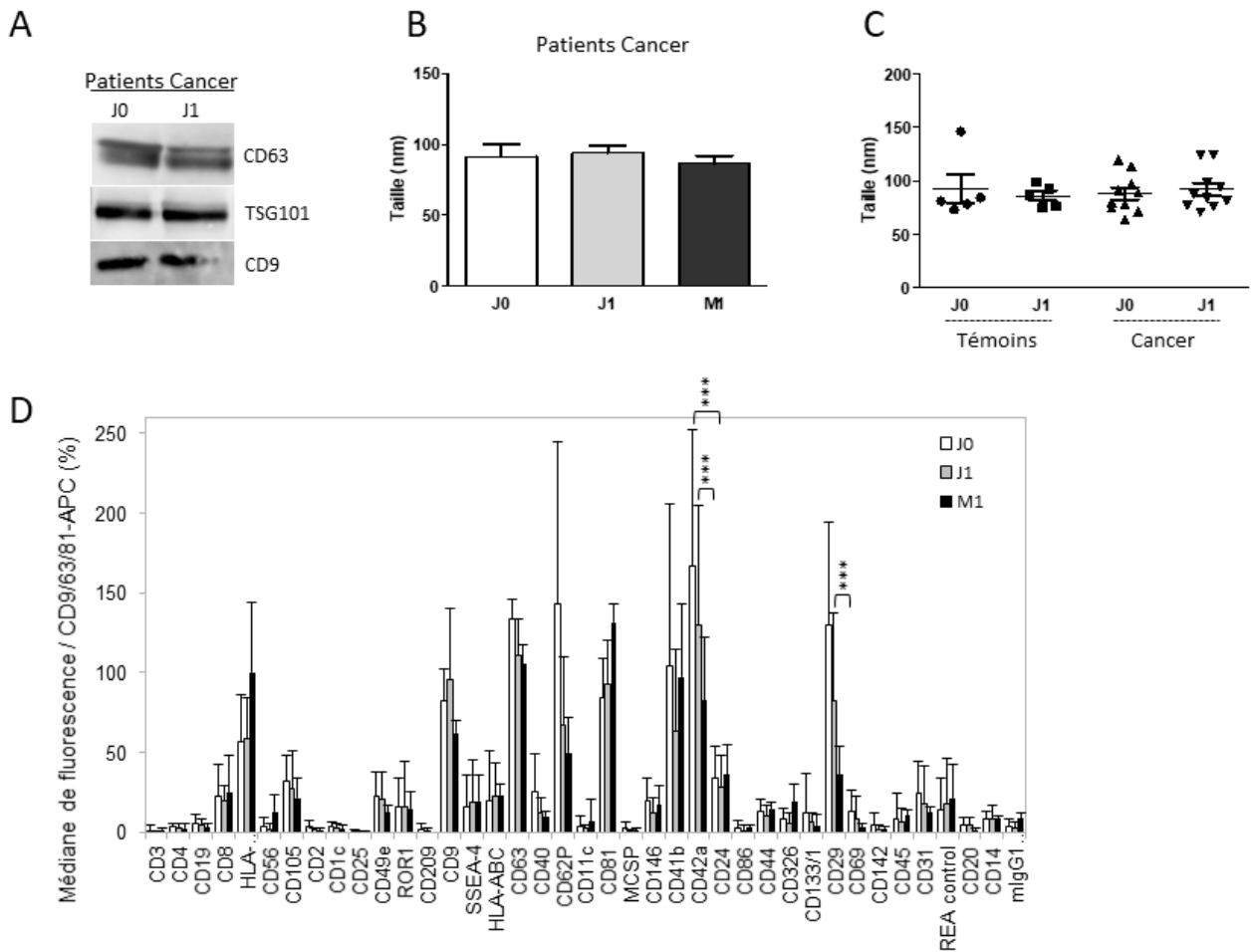


Figure 17 : Caractérisation des exosomes circulants des patients atteints de cancer du rein, avant et après néphrectomie.

(A) Western blot illustrant les marqueurs exosomaux CD63, TSG101 et CD9. (B) Taille des exosomes des patients atteints de cancer du rein à J0, J1 et M1 (n=4). (C) Taille des exosomes circulants de témoins et des patients atteints de cancer du rein à J0 et J1 (n=5-10). (D) Phénotype des exosomes des patients atteints de cancer du rein à J0, J1 et M1 (n=4-9).

***P<0,001. Pour les analyses statistiques, un ANOVA suivi du test post-hoc Bonferroni ont été utilisés.

Enfin, nous nous sommes intéressés à l'expression de l'anhydrase carbonique IX (CA9) associée aux exosomes, par western blot. Chez les patients atteints de cancer du rein, une tendance à la diminution de l'expression de la CA9 est observée à M1, par rapport à J0 et J1 (Figure 18A). Cependant, nous avons observé deux évolutions différentes de l'expression de la CA9 en fonction des patients. Effectivement, l'expression de la CA9 des patients présentant une tumeur de grade II (les deux courbes hautes, figure 18A) augmente à J1 pour redevenir proche de leur expression à J0 en M1, alors que celle des patients présentant une tumeur de grade IV (les deux courbes basses, figure 18A) diminue à J1 avec une expression qui reste similaire à J1 en M1. Par ailleurs, en comparant l'expression de la CA9 plus spécifiquement à J0, on observe que les patients présentant une tumeur de grade IV ont une expression de la CA9 associée aux exosomes qui semble augmentée par rapport à ceux de grade II (Figure 18B). Toutefois, lorsque l'on compare les témoins et patients cancéreux à J0 et J1, l'expression de la CA9 portée par les exosomes ne semble pas être modifiée (Figure 18C).

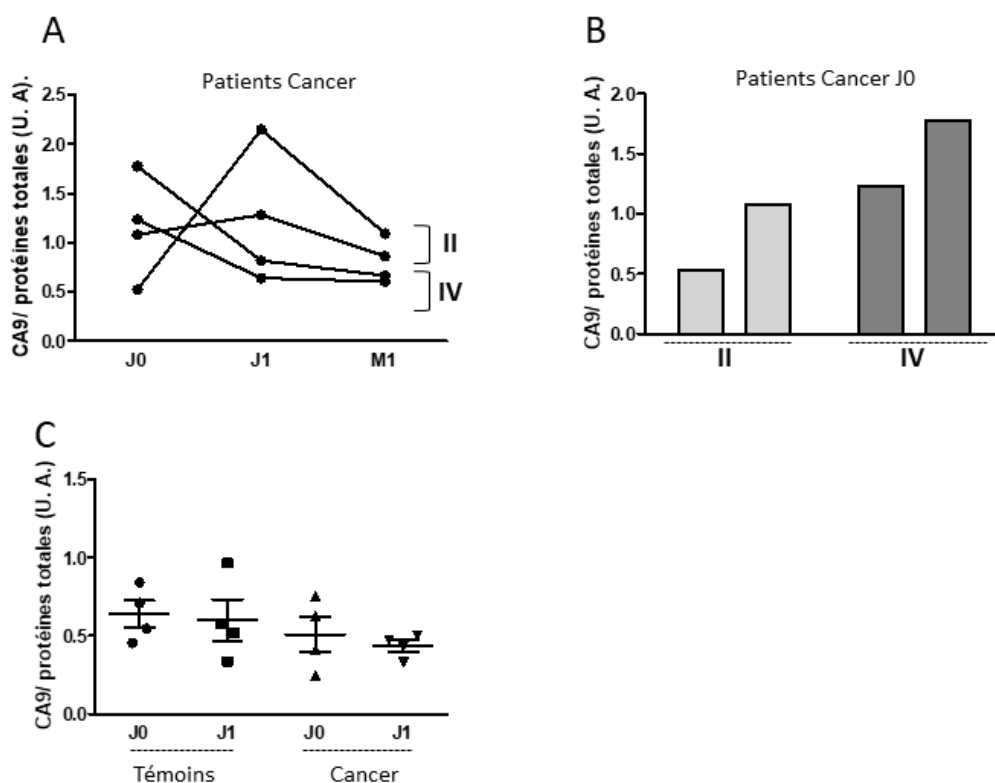


Figure 18 : Expression protéique de l'anhydrase carbonique IX (CA9) associée aux exosomes des témoins et des patients atteints de cancer du rein, avant et après néphrectomie.

L'expression de la CA9 est déterminée par western blot et normalisée par la quantité totale de protéines pour un échantillon, révélée au rouge ponceau.

(A) Expression de la CA9 présente dans les exosomes de patients atteints de cancer du rein à J0, J1 et M1 (n=4). (B) Expression de la CA9 en fonction du stade tumorale (Fuhrman) des patients atteints de cancer du rein à J0 (n=2). (C) Expression de la CA9 présente dans les exosomes de témoins et de patients atteints de cancer du rein à J0 et à J1 (n=4).

Discussion

Ces résultats préliminaires montrent que chez les patients atteints de cancer du rein, le taux d'exosomes circulants semble augmenter par rapport à celui des témoins, et que ce taux semble diminuer un mois après l'ablation de la tumeur. Par ailleurs, les témoins et les patients atteints de cancer du rein présentent des exosomes circulants d'une taille similaire et provenant des mêmes populations cellulaires. De plus, même si l'expression de la CA9 semble décroître après l'ablation de la tumeur et pourrait être corrélée positivement avec la gravité de la tumeur, nos données suggèrent que les patients atteints de cancer du rein et les témoins présentent une expression similaire de ce marqueur.

Tout d'abord, il est nécessaire d'augmenter le nombre de patients pour cette étude, afin d'en confirmer les résultats. Effectivement, elle ne comporte pour l'instant que 5 témoins et 4 patients prélevés à M1.

Les caractéristiques de ces patients (Table 2) montrent qu'il y a plus d'hommes que de femmes atteints de cancer du rein, ce qui semble cohérent avec le fait que le cancer du rein est deux fois plus présent chez les hommes. Les patients de notre cohorte présentent principalement un carcinome à cellules claires, qui est décrit comme le cancer du rein le plus fréquent (80 à 90%). Les témoins et les patients cancéreux sont hypertendus tandis que 35% des patients atteints de cancer sont également diabétiques. Nous avons montré dans le projet 1, que ces deux pathologies n'étaient pas corrélées avec une augmentation du taux circulant d'exosomes, mais que le diabète (à travers la glycémie et l'insulino-résistance) pouvaient être corrélé avec la taille des exosomes. Cependant, ces patients présentent des exosomes de taille similaire à celle des témoins, non-diabétiques, suggérant qu'ils n'ont pas encore développé l'élément modifiant la taille des exosomes.

Nous avons montré dans un premier temps que le taux d'exosomes circulant semble être augmenté chez les patients atteints de cancer du rein. Effectivement, il a été montré dans certains cancers que la production d'exosomes était augmentée (dans le cancer des ovaires par exemple (Taylor and Gercel-Taylor, 2008)), ce qui semble cohérent avec le fait que les cellules tumorales sont capables de libérer ces vésicules. Cependant, à J1, le taux d'exosomes de patients et témoins semble augmenter puis diminuer à M1. L'augmentation des exosomes à J1 pourrait provenir de l'acte chirurgical en lui-même puisque cette augmentation est retrouvée chez les deux types de patients. Lorsque l'on étudie la provenance cellulaire des exosomes à J0, J1 et M1, nous avons observé qu'il y a significativement moins d'exosomes circulants plaquettaires (CD42a) et leucocytaires (CD29). Ainsi, la diminution observée pourrait être due à la diminution des exosomes provenant initialement des cellules de la tumeur (qui est enlevée suite à la néphrectomie) mais également d'autres types cellulaires dont la sécrétion serait augmentée lors de l'établissement de la tumeur.

Par la suite, nous avons montré que les exosomes des témoins et des patients atteints de cancer du rein étaient bien associés à la CA9 (Figure 18). Cependant, bien que l'expression de la CA9 semble diminuer chez les patients cancéreux après la néphrectomie, suggérant ainsi qu'elle pourrait provenir des exosomes dérivés de la tumeur, les témoins et les patients cancéreux présentent une expression similaire de cette protéine. Ces résultats sont contradictoires avec la littérature puisque la CA9 portée par les exosomes urinaires a été montrée comme augmentée chez les patients atteints de carcinome rénal (Raimondo et al., 2013). Outre le fait de notre petit nombre de patients, notre technique utilisée peut être également discutée. En effet, le Western blot ne permet pas de faire beaucoup d'échantillons en même temps, demande plus de matériel et peut être moins reproductible que la cytométrie en flux par exemple. Par ailleurs, les exosomes circulants et urinaires présentent peut-être des contenus protéiques différents. En parallèle, la caractérisation des MP circulantes des patients atteints de cancer du rein a été réalisée au sein du laboratoire (travaux de L. Vergori, Figure 19).

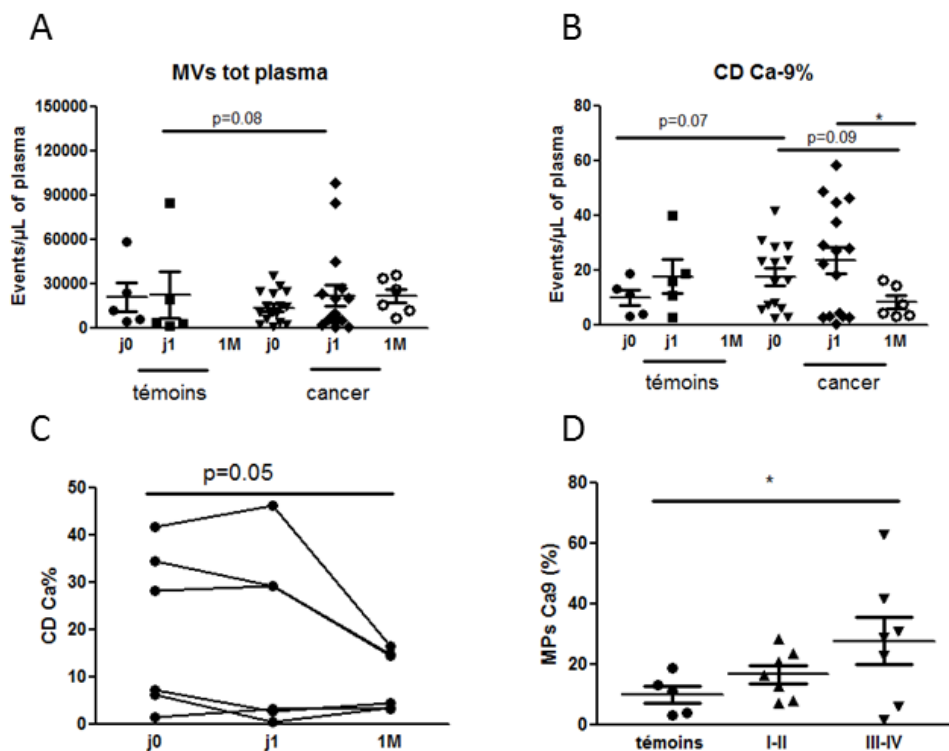


Figure 19 : Taux circulants des MP chez les témoins et patients atteints de cancer du rein et expression de la CA9 associée aux MP. (Travaux non publiés de L. Vergori, SOPAM)

Le taux circulants et l'expression de la CA9 a été déterminée par cytométrie en flux.

(A) Taux circulants des MP chez les témoins et patients atteints de cancer du rein à J0, J1 et M1.

(B) Pourcentage de MP positives pour la CA9 chez les témoins et les patients atteints de cancer du rein à J0, J1 et M1.

(C) Evolution du pourcentage de MP positives pour la CA9 chez les patients atteints de cancer du rein à J0, J1 et M1.

(D) Pourcentage de MP positives pour la CA9 en fonction du grade de Fuhrman chez les témoins et patients atteints de cancer du rein à J0.

Ainsi, il a également été montré que le taux de MP circulantes était augmenté chez les patients atteints de cancer du rein par rapport aux témoins et semble augmenter à J1 (Figure 19A). De plus, l'expression de CA9 associée aux MP est augmentée chez les patients atteints de cancer du rein par rapport aux témoins et est diminuée à M1 par rapport à J0 et J1 (Figure 19B-C). Enfin, les patients de grade III-IV présentent plus de CA9 par rapport à ceux de grade I-II et témoins (significatif par rapport aux témoins) (Figure 19D). Ainsi, les MP et les exosomes semblent de la même façon être augmentés chez les patients atteints de cancer du rein, leur production augmentant à J1 et diminuant à M1 et l'expression de la CA9 associée à ces vésicules pourraient être un marqueur de la gravité de cette pathologie.

Il sera nécessaire de poursuivre cette étude pour confirmer si les VE ainsi que le marqueur CA9 associé seraient des biomarqueurs pertinents pour le cancer du rein. Il existe également d'autres protéines qui ont été décrites comme augmentées dans les exosomes de patients atteints de cancer du rein (la MMP-9, la céruloplasmine, la podocalyxine et la Dickkopf related protein 4, (Raimondo et al., 2013)) qu'il serait possible d'étudier. De plus, il pourrait être intéressant de déterminer le rôle fonctionnel de ces exosomes puisqu'il a été montré que les exosomes dérivés de cellules tumorales pouvaient être impliqués dans le développement et à l'agressivité de la tumeur (Bobrie et al., 2012 ; Setti et al., 2015 ; Wang et al., 2016).

Conclusion générale

Dans un premier temps, nous avons montré que les patients atteints de SM présentaient un taux d'exosomes circulants augmenté dont la taille est diminuée. De plus, ces deux paramètres sont corrélés avec le nombre de critère du SM chez ces patients, et associés avec certains facteurs du SM. Par ailleurs, ces vésicules pourraient participer à la dysfonction endothéliale associée au SM en diminuant la production de NO et le relâchement dépendant de l'endothélium, en réponse à l'acétylcholine.

Dans un deuxième temps, le taux d'exosomes circulants semble également augmenté chez les patients atteints de cancer du rein mais le marqueur CA9 associé à ces vésicules ne semble pas enrichi dans les exosomes de ces patients par rapport aux témoins.

Ces deux études suggèrent que les exosomes sont des cibles intéressantes à la fois comme biomarqueurs et vecteurs de pathologie. Ces vésicules peuvent en effet contenir un cargo reflétant l'état de leur cellule d'origine et il a déjà été montré que certaines protéines/ lipides/ miARN spécifiquement enrichis pouvaient servir de biomarqueur.

Ce travail a également permis de comparer deux types de VE : les exosomes et les MP, pour les deux pathologies. Ces vésicules sont toutes les deux des reflets de l'état de leur cellule d'origine et peuvent ainsi participer aux mêmes événements physiopathologiques. Elles peuvent également servir toutes les deux de biomarqueur dans la même pathologie. Ainsi dans le SM, les exosomes, comme les MP, pourraient participer à la dysfonction endothéliale. Les effets de ces deux populations pourraient d'ailleurs se potentialiser, mais ne sont pas induits par la même voie.

Par ailleurs, ces deux types de vésicules peuvent également présenter des rôles distincts du fait de la différence de leur contenu, induite par des voies de formation différentes. Durcin et al ont montré que les adipocytes libéraient plus d'exosomes que de MP, et que leur différence de contenu suggérait des rôles différents pour ces deux populations. On peut donc également envisager que l'effet d'une population peut primer sur l'autre, dans certaines situations.

De plus, les marqueurs spécifiques pour chaque population de VE sont encore discutés donc l'identification des différents types de VE et leurs effets associés peut rester controversée. Il est également nécessaire de déterminer une terminologie spécifique pour chaque population de VE, utilisée par tout le monde. La recherche bibliographique est en effet à faire avec un œil critique, en vérifiant bien la méthodologie afin de ne pas confondre les populations de VE et de pouvoir comparer les résultats.

Enfin, nous avons montré que les exosomes pouvaient participer au développement de pathologie mais il est également important de noter l'intérêt croissant pour ces vésicules en tant qu'outil thérapeutiques (pour revue voir Mallocci et al., 2017). La nécessité de mieux connaître ces vésicules et de les caractériser apparaît d'autant plus essentielle.

Bibliographie

- Abels, E.R., and Breakefield, X.O. (2016). Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell. Mol. Neurobiol.* **36**, 301–312.
- Agouni, A., Lagrue-Lak-Hal, A.H., Ducluzeau, P.H., Mostefai, H.A., Draunet-Busson, C., Leftheriotis, G., Heymes, C., Martinez, M.C., and Andriantsitohaina, R. (2008). Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am. J. Pathol.* **173**, 1210–1219.
- Agouni, A., Ducluzeau, P.-H., Benameur, T., Faure, S., Sladkova, M., Duluc, L., Leftheriotis, G., Pechanova, O., Delibegovic, M., Martinez, M.C., et al. (2011). Microparticles from patients with metabolic syndrome induce vascular hypo-reactivity via Fas/Fas-ligand pathway in mice. *PloS One* **6**, e27809.
- Alberti, K.G.M.M., Eckel, R.H., Grundy, S.M., Zimmet, P.Z., Cleeman, J.I., Donato, K.A., Fruchart, J.-C., James, W.P.T., Loria, C.M., Smith, S.C., et al. (2009). Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* **120**, 1640–1645.
- Al-Nedawi, K., Meehan, B., Kerbel, R.S., Allison, A.C., and Rak, J. (2009). Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 3794–3799.
- Arita, S., Baba, E., Shibata, Y., Niuro, H., Shimoda, S., Isobe, T., Kusaba, H., Nakano, S., and Harada, M. (2008). B cell activation regulates exosomal HLA production. *Eur. J. Immunol.* **38**, 1423–1434.
- Arraud, N., Linares, R., Tan, S., Gounou, C., Pasquet, J.-M., Mornet, S., and Brisson, A.R. (2014). Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J. Thromb. Haemost.* **12**, 614–627.
- Atai, N.A., Balaj, L., Veen, H. van, Breakefield, X.O., Jarzyna, P.A., Noorden, C.J.F.V., Skog, J., and Maguire, C.A. (2013). Heparin blocks transfer of extracellular vesicles between donor and recipient cells. *J. Neurooncol.* **115**, 343–351.
- Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., et al. (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* **14**, 677–685.
- Balaj, L., Tigges, J., Toxavidis, V., Ivanov, A.R., Skog, J., Momen Heravi, F., Alian, S., Ericsson, M., Distel, R., and Kuo, W.P. (2012). Alternative Methods for Characterization of Extracellular Vesicles.
- Ballinger, S.W., Patterson, C., Yan, C.N., Doan, R., Burow, D.L., Young, C.G., Yakes, F.M., Van Houten, B., Ballinger, C.A., Freeman, B.A., et al. (2000). Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ. Res.* **86**, 960–966.
- Beyer, C., and Pisetsky, D.S. (2010). The role of microparticles in the pathogenesis of rheumatic diseases. *Nat. Rev. Rheumatol.* **6**, 21–29.
- Blanc, L., and Vidal, M. (2017). New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases* **1–12**.

- Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., and Hivroz, C. (2002). TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/ ζ Complex. *J. Immunol.* *168*, 3235–3241.
- Blans, K., Hansen, M.S., Sørensen, L.V., Hvam, M.L., Howard, K.A., Möller, A., Wiking, L., Larsen, L.B., and Rasmussen, J.T. (2017). Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography. *J. Extracell. Vesicles* *6*.
- Bobrie, A., Krumeich, S., Reyal, F., Recchi, C., Moita, L.F., Seabra, M.C., Ostrowski, M., and Théry, C. (2012). Rab27a Supports Exosome-Dependent and -Independent Mechanisms That Modify the Tumor Microenvironment and Can Promote Tumor Progression. *Cancer Res.* *72*, 4920–4930.
- Bochmann, I., Ebstein, F., Lehmann, A., Wohlschlaeger, J., Sixt, S.U., Kloetzel, P.-M., and Dahlmann, B. (2014). T lymphocytes export proteasomes by way of microparticles: a possible mechanism for generation of extracellular proteasomes. *J. Cell. Mol. Med.* *18*, 59–68.
- Brianza-Padilla, M., Carbó, R., Arana, J.C., Vázquez-Palacios, G., Ballinas-Verdugo, M.A., Cardoso-Saldaña, G.C., Palacio, A.G., Juárez-Vicuña, Y., Sánchez, F., Martínez-Martínez, E., et al. (2016). Inflammation Related MicroRNAs Are Modulated in Total Plasma and in Extracellular Vesicles from Rats with Chronic Ingestion of Sucrose. *BioMed Res. Int.* *2016*.
- Brodsky, S.V., Zhang, F., Nasjletti, A., and Goligorsky, M.S. (2004). Endothelium-derived microparticles impair endothelial function in vitro. *Am. J. Physiol. - Heart Circ. Physiol.* *286*, H1910–H1915.
- Buschow, S.I., Nolte-’t Hoen, E.N.M., Van Niel, G., Pols, M.S., Ten Broeke, T., Lauwen, M., Ossendorp, F., Melief, C.J.M., Raposo, G., Wubbolts, R., et al. (2009). MHC II in Dendritic Cells is Targeted to Lysosomes or T Cell-Induced Exosomes Via Distinct Multivesicular Body Pathways. *Traffic* *10*, 1528–1542.
- Caby, M.-P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., and Bonnerot, C. (2005). Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* *17*, 879–887.
- Capitani, U., and Montorsi, F. (2016). Renal cancer. *Lancet Lond. Engl.* *387*, 894–906.
- Chen, P., Douglas, S.D., Meshki, J., and Tuluc, F. (2012). Neurokinin 1 Receptor Mediates Membrane Blebbing and Sheer Stress-Induced Microparticle Formation in HEK293 Cells. *PLoS ONE* *7*.
- Clayton, A., Court, J., Navabi, H., Adams, M., Mason, M.D., Hobot, J.A., Newman, G.R., and Jasani, B. (2001). Analysis of antigen presenting cell derived exosomes, based on immunomagnetic isolation and flow cytometry. *J. Immunol. Methods* *247*, 163–174.
- Collaboration, C.T.T. (CTT) (2010). Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *The Lancet* *376*, 1670–1681.
- Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annu. Rev. Cell Dev. Biol.* *30*, 255–289.
- Conde-Vancells, J., Rodriguez-Suarez, E., Embade, N., Gil, D., Matthiesen, R., Valle, M., Elortza, F., Lu, S.C., Mato, J.M., and Falcon-Perez, J.M. (2008). Characterization and Comprehensive Proteome Profiling of Exosomes Secreted by Hepatocytes. *J. Proteome Res.* *7*, 5157.
- Connor, D.E., Exner, T., Ma, D.D.F., and Joseph, J.E. (2010). The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. *Thromb. Haemost.* *103*, 1044–1052.

- Dachary-Prigent, J., Pasquet, J.M., Freyssinet, J.M., and Nurden, A.T. (1995). Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca²⁺-ATPase inhibitors. *Biochemistry (Mosc.)* *34*, 11625–11634.
- De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A., Shin, W.S., and Liao, J.K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* *96*, 60–68.
- Dean, W.L., Lee, M.J., Cummins, T.D., Schultz, D.J., and Powell, D.W. (2009). Proteomic and functional characterisation of platelet microparticle size classes. *Thromb. Haemost.* *102*, 711–718.
- Delarue, J., Allain, G., and Guillermin, S. (2006). Le syndrome métabolique. *Nutr. Clin. Métabolisme* *20*, 114–117.
- Deng, Z., Poliakov, A., Hardy, R.W., Clements, R., Liu, C., Liu, Y., Wang, J., Xiang, X., Zhang, S., Zhuang, X., et al. (2009). Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes* *58*, 2498–2505.
- Dikalova, A.E., Bikineyeva, A.T., Budzyn, K., Nazarewicz, R.R., McCann, L., Lewis, W., Harrison, D.G., and Dikalov, S.I. (2010). Therapeutic Targeting of Mitochondrial Superoxide in Hypertension. *Circ. Res.* *107*, 106–116.
- van Dommelen, S.M., Vader, P., Lakhal, S., Kooijmans, S.A.A., van Solinge, W.W., Wood, M.J.A., and Schiffelers, R.M. (2012). Microvesicles and exosomes: Opportunities for cell-derived membrane vesicles in drug delivery. *J. Controlled Release* *161*, 635–644.
- Dorai, T. (2009). Carbonic Anhydrase IX Shedding Through Exosomes in Renal Carcinoma Cells. *Glycobiol. Insights* *2009*, 3–12.
- Dromparis, P., and Michelakis, E.D. (2013). Mitochondria in vascular health and disease. *Annu. Rev. Physiol.* *75*, 95–126.
- Ducluzeau, P.-H., Demiot, C., and Custaud, M.-A. (2005). Syndrome métabolique et dysfonction endothéliale. *Sang Thromb. Vaiss.* *17*, 83–92.
- Durcin, M., Fleury, A., Taillebois, E., Hilairet, G., Krupova, Z., Henry, C., Truchet, S., Trötz Müller, M., Köfeler, H., Mabileau, G., et al. (2017). Characterisation of adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and small extracellular vesicles. *J. Extracell. Vesicles* *6*.
- Ellulu, M.S., Patimah, I., Khaza'ai, H., Rahmat, A., Abed, Y., and Ali, F. (2016). Atherosclerotic cardiovascular disease: a review of initiators and protective factors. *Inflammopharmacology* *24*, 1–10.
- Esposito, K., Chiodini, P., Colao, A., Lenzi, A., and Giugliano, D. (2012). Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. *Diabetes Care* *35*, 2402–2411.
- Esteghamati, A., Noshad, S., Khalilzadeh, O., Morteza, A., Nazeri, A., Meysamie, A., Esteghamati, A., and Nakhjavani, M. (2011). Contribution of serum leptin to metabolic syndrome in obese and nonobese subjects. *Arch. Med. Res.* *42*, 244–251.
- Fader, C.M., Sánchez, D.G., Mestre, M.B., and Colombo, M.I. (2009). TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. *Biochim. Biophys. Acta* *1793*, 1901–1916.
- Favero, G., Paganelli, C., Buffoli, B., Rodella, L.F., and Rezzani, R. (2014). Endothelium and Its

- Alterations in Cardiovascular Diseases: Life Style Intervention. *BioMed Res. Int.* 2014.
- Feng, D., Zhao, W.-L., Ye, Y.-Y., Bai, X.-C., Liu, R.-Q., Chang, L.-F., Zhou, Q., and Sui, S.-F. (2010). Cellular Internalization of Exosomes Occurs Through Phagocytosis. *Traffic* 11, 675–687.
- Fernández-Hernando, C., Ramírez, C.M., Goedeke, L., and Suárez, Y. (2013). MicroRNAs in metabolic disease. *Arterioscler. Thromb. Vasc. Biol.* 33, 178–185.
- Ferrante, S.C., Nadler, E.P., Pillai, D.K., Hubal, M.J., Wang, Z., Wang, J.M., Gordish-Dressman, H., Koeck, E., Sevilla, S., Wiles, A.A., et al. (2015). Adipocyte-derived exosomal miRNAs: a novel mechanism for obesity-related disease. *Pediatr. Res.* 77, 447–454.
- Fitzner, D., Schnaars, M., Rossum, D. van, Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hanisch, U.-K., and Simons, M. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *J Cell Sci* 124, 447–458.
- Fleming, I., and Busse, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R1-12.
- Frank, P.G., Woodman, S.E., Park, D.S., and Lisanti, M.P. (2003). Caveolin, caveolae, and endothelial cell function. *Arterioscler. Thromb. Vasc. Biol.* 23, 1161–1168.
- Frühbeis, C., Fröhlich, D., Kuo, W.P., Amphornrat, J., Thilemann, S., Saab, A.S., Kirchhoff, F., Möbius, W., Goebbels, S., Nave, K.-A., et al. (2013). Neurotransmitter-Triggered Transfer of Exosomes Mediates Oligodendrocyte–Neuron Communication. *PLoS Biol.* 11.
- Ghossoub, R., Lembo, F., Rubio, A., Gaillard, C.B., Bouchet, J., Vitale, N., Slavík, J., Machala, M., and Zimmermann, P. (2014). Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nat. Commun.* 5, 3477.
- Giannotti, G., and Landmesser, U. (2007). Endothelial dysfunction as an early sign of atherosclerosis. *Herz* 32, 568–572.
- Goetzl, E.J., Goetzl, L., Karliner, J.S., Tang, N., and Pulliam, L. (2016). Human plasma platelet-derived exosomes: effects of aspirin. *FASEB J.* 30, 2058–2063.
- Gould, S.J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J. Extracell. Vesicles* 2.
- Haraszti, R.A., Didiot, M.-C., Sapp, E., Leszyk, J., Shaffer, S.A., Rockwell, H.E., Gao, F., Narain, N.R., DiFiglia, M., Kiebish, M.A., et al. (2016). High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J. Extracell. Vesicles* 5.
- Harding, C., Heuser, J., and Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97, 329–339.
- van Hinsbergh, V.W.M. (2012). Endothelium—role in regulation of coagulation and inflammation. *Semin. Immunopathol.* 34, 93–106.
- Hood, J.L., San, R.S., and Wickline, S.A. (2011). Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis. *Cancer Res.* 71, 3792–3801.
- Hsu, C., Morohashi, Y., Yoshimura, S.-I., Manrique-Hoyos, N., Jung, S., Lauterbach, M.A., Bakhti, M., Grønborg, M., Möbius, W., Rhee, J., et al. (2010). Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J. Cell Biol.* 189, 223–232.
- Hutcheson, R., and Rocic, P. (2012). The Metabolic Syndrome, Oxidative Stress, Environment, and

Cardiovascular Disease: The Great Exploration. *Exp. Diabetes Res.* 2012.

Jiang, X.L., Zhang, Y., Tan, B., Luo, C.L., and Wu, X.H. (2014). Renal tumor-derived exosomes inhibit hepaCAM expression of renal carcinoma cells in a p-AKT-dependent manner. *Neoplasma* 61, 416–423.

de Jong, O.G., Verhaar, M.C., Chen, Y., Vader, P., Gremmels, H., Posthuma, G., Schiffelers, R.M., Gucek, M., and van Balkom, B.W.M. (2012). Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *J. Extracell. Vesicles* 1.

Juan, T., and Fürthauer, M. (2015). [The ESCRT complex: from endosomal transport to the development of multicellular organisms]. *Biol. Aujourd'hui* 209, 111–124.

Junquero, D., and Rival, Y. (2005). Syndrome métabolique: quelle définition pour quel(s) traitement(s)? *médecine/sciences* 21, 1045–1053.

Karolina, D.S., Tavintharan, S., Armugam, A., Sepramaniam, S., Pek, S.L.T., Wong, M.T.K., Lim, S.C., Sum, C.F., and Jeyaseelan, K. (2012). Circulating miRNA Profiles in Patients with Metabolic Syndrome. *J. Clin. Endocrinol. Metab.* 97, E2271–E2276.

Khalyfa, A., Kheirandish-Gozal, L., Khalyfa, A.A., Philby, M.F., Alonso-Álvarez, M.L., Mohammadi, M., Bhattacharjee, R., Terán-Santos, J., Huang, L., Andrade, J., et al. (2016). Circulating Plasma Extracellular Microvesicle MicroRNA Cargo and Endothelial Dysfunction in Children with Obstructive Sleep Apnea. *Am. J. Respir. Crit. Care Med.* 194, 1116–1126.

Kluge, M.A., Fetterman, J.L., and Vita, J.A. (2013). Mitochondria and Endothelial Function. *Circ. Res.* 112, 1171–1188.

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., and Théry, C. (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl. Acad. Sci. U. S. A.* 113, E968-977.

Kraehling, J.R., Hao, Z., Lee, M.Y., Vinyard, D.J., Velazquez, H., Liu, X., Stan, R.V., Brudvig, G.W., and Sessa, W.C. (2016). Uncoupling Caveolae From Intracellular Signaling In Vivo. *Circ. Res.* 118, 48–55.

Kranendonk, M.E., de Kleijn, D.P., Kalkhoven, E., Kanhai, D.A., Uiterwaal, C.S., van der Graaf, Y., Pasterkamp, G., and Visseren, F.L. (2014). Extracellular vesicle markers in relation to obesity and metabolic complications in patients with manifest cardiovascular disease. *Cardiovasc. Diabetol.* 13, 37.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 87, 99–163.

Kunzelmann-Marche, C., Freyssinet, J.-M., and Martínez, M.C. (2002). Loss of Plasma Membrane Phospholipid Asymmetry Requires Raft Integrity ROLE OF TRANSIENT RECEPTOR POTENTIAL CHANNELS AND ERK PATHWAY. *J. Biol. Chem.* 277, 19876–19881.

Lai, C.P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C., Chen, J.W., Tannous, B.A., and Breakefield, X.O. (2014). Dynamic Biodistribution of Extracellular Vesicles In Vivo Using a Multimodal Imaging Reporter. *ACS Nano* 8, 483–494.

Laulagnier, K., Motta, C., Hamdi, S., Roy, S., Fauvelle, F., Pageaux, J.-F., Kobayashi, T., Salles, J.-P., Perret, B., Bonnerot, C., et al. (2004). Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochem. J.* 380, 161–171.

- Li, H., and Förstermann, U. (2013). Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease. *Curr. Opin. Pharmacol.* 13, 161–167.
- Li, P., Jiang, R., Li, L., Liu, C., Yang, F., and Qiu, Y. (2015). Correlation of serum adiponectin and adiponectin gene polymorphism with metabolic syndrome in Chinese adolescents. *Eur. J. Clin. Nutr.* 69, 62–67.
- Lindholm, L.H., Ibsen, H., Dahlöf, B., Devereux, R.B., Beevers, G., de Faire, U., Fyhrquist, F., Julius, S., Kjeldsen, S.E., Kristiansson, K., et al. (2002). Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet Lond. Engl.* 359, 1004–1010.
- Llorente, A., Skotland, T., Sylvänne, T., Kauhanen, D., Róg, T., Orłowski, A., Vattulainen, I., Ekroos, K., and Sandvig, K. (2013). Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids* 1831, 1302–1309.
- Lötvall, J., Hill, A.F., Hochberg, F., Buzás, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P., et al. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* 3.
- Luga, V., Zhang, L., Vilorio-Petit, A.M., Ogunjimi, A.A., Inanlou, M.R., Chiu, E., Buchanan, M., Hosein, A.N., Basik, M., and Wrana, J.L. (2012). Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration. *Cell* 151, 1542–1556.
- Manca, S., Giraud, D., and Zemleni, J. (2016). Bioavailability and Biodistribution of Fluorophore-labeled Exosomes from Cow's Milk after Intravenous and Oral Administration in C57Bl/6J Mice. *FASEB J.* 30, 690.8-690.8.
- Martinez, M.C., Tual-Chalot, S., Leonetti, D., and Andriantsitohaina, R. (2011). Microparticles: targets and tools in cardiovascular disease. *Trends Pharmacol. Sci.* 32, 659–665.
- Melo, S.A., Luecke, L.B., Kahlert, C., Fernandez, A.F., Gammon, S.T., Kaye, J., LeBleu, V.S., Mittendorf, E.A., Weitz, J., Rahbari, N., et al. (2015). Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 523, 177–182.
- Michel, T., and Vanhoutte, P.M. (2010). Cellular signaling and NO production. *Pflugers Arch.* 459, 807–816.
- Mirza, S., Qu, H.-Q., Li, Q., Martinez, P.J., Rentfro, A.R., McCormick, J.B., and Fisher-Hoch, S.P. (2011). Adiponectin/leptin ratio and Metabolic Syndrome in a Mexican American population. *Clin. Investig. Med. Med. Clin. Exp.* 34, E290.
- Mishra, P., and Chan, D.C. (2016). Metabolic regulation of mitochondrial dynamics. *J. Cell Biol.* 212, 379–387.
- Mitra, K., Wunder, C., Roysam, B., Lin, G., and Lippincott-Schwartz, J. (2009). A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc. Natl. Acad. Sci. U. S. A.* 106, 11960–11965.
- Mohan, V., Deepa, R., Pradeepa, R., Vimalaswaran, K.S., Mohan, A., Velmurugan, K., and Radha, V. (2005). Association of low adiponectin levels with the metabolic syndrome—the Chennai Urban Rural Epidemiology Study (CURES-4). *Metabolism* 54, 476–481.
- Molina, A.M., Lin, X., Korytowsky, B., Matczak, E., Lechuga, M.J., Wiltshire, R., and Motzer, R.J. (2014). Sunitinib objective response in metastatic renal cell carcinoma: analysis of 1059 patients treated on clinical trials. *Eur. J. Cancer Oxf. Engl.* 1990 50, 351–358.

- Montecalvo, A., Larregina, A.T., Shufesky, W.J., Stolz, D.B., Sullivan, M.L.G., Karlsson, J.M., Baty, C.J., Gibson, G.A., Erdos, G., Wang, Z., et al. (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 119, 756–766.
- Morel, O., Toti, F., Hugel, B., Bakouboula, B., Camoin-Jau, L., Dignat-George, F., and Freyssinet, J.-M. (2006). Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler. Thromb. Vasc. Biol.* 26, 2594–2604.
- Morelli, A.E., Larregina, A.T., Shufesky, W.J., Sullivan, M.L.G., Stolz, D.B., Papworth, G.D., Zahorchak, A.F., Logar, A.J., Wang, Z., Watkins, S.C., et al. (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* 104, 3257–3266.
- Motzer, R.J., Hutson, T.E., Tomczak, P., Michaelson, M.D., Bukowski, R.M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., Kim, S.T., et al. (2007). Sunitinib versus Interferon Alfa in Metastatic Renal-Cell Carcinoma. *N. Engl. J. Med.* 356, 115–124.
- Müller, G. (2012). Microvesicles/exosomes as potential novel biomarkers of metabolic diseases. *Diabetes Metab. Syndr. Obes. Targets Ther.* 5, 247–282.
- Nakase, I., and Futaki, S. (2015). Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. *Sci. Rep.* 5, 10112.
- Nakase, I., Kobayashi, N.B., Takatani-Nakase, T., and Yoshida, T. (2015). Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. *Sci. Rep.* 5, 10300.
- Nanbo, A., Kawanishi, E., Yoshida, R., and Yoshiyama, H. (2013). Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. *J. Virol.* 87, 10334–10347.
- van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., Marks, M.S., Rubinstein, E., and Raposo, G. (2011). The tetraspanin CD63 regulates ESCRT-independent and dependent endosomal sorting during melanogenesis. *Dev. Cell* 21, 708–721.
- Oksvold, M.P., Kullmann, A., Forfang, L., Kierulf, B., Li, M., Brech, A., Vlassov, A.V., Smeland, E.B., Neurauter, A., and Pedersen, K.W. (2014). Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. *Clin. Ther.* 36, 847–862.e1.
- Oliveira-Paula, G.H., Lacchini, R., and Tanus-Santos, J.E. (2016). Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS3 polymorphisms. *Gene* 575, 584–599.
- O'Neill, S., Bohl, M., Gregersen, S., Hermansen, K., and O'Driscoll, L. (2016). Blood-Based Biomarkers for Metabolic Syndrome. *Trends Endocrinol. Metab.* 27, 363–374.
- Ostrowski, M., Carmo, N.B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C.F., Schauer, K., Hume, A.N., Freitas, R.P., et al. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* 12, 19–30.
- Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524–526.
- Pan, B.-T., Teng, K., Wu, C., Adam, M., and Johnstone, R.M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* 101, 942–948.
- Pantuck, A.J., Klatter, T., Seligson, D., Atkins, M., and Beldegrun, A. (2008). Carbonic Anhydrase IX

As a Predictive Biomarker for Clear Cell Renal Cell Carcinoma. *J. Clin. Oncol.* 26, 3105–3107.

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., et al. (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J. Biol. Chem.* 284, 34211–34222.

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Nitadori-Hoshino, A., et al. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 18, 883–891.

Phoonsawat, W., Aoki-Yoshida, A., Tsuruta, T., and Sonoyama, K. (2014). Adiponectin is partially associated with exosomes in mouse serum. *Biochem. Biophys. Res. Commun.* 448, 261–266.

Qu, L., Ding, J., Chen, C., Wu, Z.-J., Liu, B., Gao, Y., Chen, W., Liu, F., Sun, W., Li, X.-F., et al. (2016). Exosome-Transmitted IncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. *Cancer Cell* 29, 653–668.

Raimondo, F., Morosi, L., Corbetta, S., Chinello, C., Brambilla, P., Della Mina, P., Villa, A., Albo, G., Battaglia, C., Bosari, S., et al. (2013). Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol. Biosyst.* 9, 1220–1233.

Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C., and Desaynard, C. (1997). Accumulation of Major Histocompatibility Complex Class II Molecules in Mast Cell Secretory Granules and Their Release upon Degranulation. *Mol. Biol. Cell* 8, 2631–2645.

Reaven, G.M. (1988). Role of Insulin Resistance in Human Disease. *Diabetes* 37, 1595–1607.

Record, M., Carayon, K., Poirot, M., and Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologicals. *Biochim. Biophys. Acta* 1841, 108–120.

Roucourt, B., Meeussen, S., Bao, J., Zimmermann, P., and David, G. (2015). Heparanase activates the syndecan-syntenin-ALIX exosome pathway. *Cell Res.* 25, 412–428.

Safiedeen, Z., Rodríguez-Gómez, I., Vergori, L., Soleti, R., Vaithilingam, D., Douma, I., Agouni, A., Leiber, D., Dubois, S., Simard, G., et al. (2017). Temporal Cross Talk Between Endoplasmic Reticulum and Mitochondria Regulates Oxidative Stress and Mediates Microparticle-Induced Endothelial Dysfunction. *Antioxid. Redox Signal.* 26, 15–27.

SANTULLI, G. (2016). MicroRNAs and Endothelial (Dys) Function. *J. Cell. Physiol.* 231, 1638–1644.

Sapet, C., Simoncini, S., Lloriod, B., Puthier, D., Sampol, J., Nguyen, C., Dignat-George, F., and Anfosso, F. (2006). Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. *Blood* 108, 1868–1876.

Savina, A., Fader, C.M., Damiani, M.T., and Colombo, M.I. (2005). Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic Cph. Den.* 6, 131–143.

Schmidt, O., and Teis, D. (2012). The ESCRT machinery. *Curr. Biol.* 22, R116–R120.

Sebbagh, M., Renvoizé, C., Hamelin, J., Riché, N., Bertoglio, J., and Bréard, J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat. Cell Biol.* 3, 346–352.

Segura, E., Nicco, C., Lombard, B., Véron, P., Raposo, G., Batteux, F., Amigorena, S., and Théry,

- C. (2005). ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* *106*, 216–223.
- Setti, M., Osti, D., Richichi, C., Ortensi, B., Del Bene, M., Fornasari, L., Beznoussenko, G., Mironov, A., Rappa, G., Cuomo, A., et al. (2015). Extracellular vesicle-mediated transfer of CLIC1 protein is a novel mechanism for the regulation of glioblastoma growth. *Oncotarget* *6*, 31413–31427.
- Sheldon, H., Heikamp, E., Turley, H., Dragovic, R., Thomas, P., Oon, C.E., Leek, R., Edelmann, M., Kessler, B., Sainson, R.C.A., et al. (2010). New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* *116*, 2385–2394.
- Shenouda, S.M., Widlansky, M.E., Chen, K., Xu, G., Holbrook, M., Tabit, C.E., Hamburg, N.M., Frame, A.A., Caiano, T.L., Kluge, M.A., et al. (2011). Altered Mitochondrial Dynamics Contributes to Endothelial Dysfunction in Diabetes Mellitus. *Circulation* *124*, 444–453.
- Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* *82*, 291–295.
- Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Carter, B.S., Krichevsky, A.M., and Breakefield, X.O. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* *10*, 1470–1476.
- Skotland, T., Ekroos, K., Kauhanen, D., Simolin, H., Seierstad, T., Berge, V., Sandvig, K., and Llorente, A. (2017). Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. *Eur. J. Cancer Oxf. Engl. 1990* *70*, 122–132.
- Smyth, T., Kullberg, M., Malik, N., Smith-Jones, P., Graner, M.W., and Anchordoquy, T.J. (2015). Biodistribution and Delivery Efficiency of Unmodified Tumor-Derived Exosomes. *J. Control. Release Off. J. Control. Release Soc.* *199*, 145–155.
- Soleti, R., Lauret, E., Andriantsitohaina, R., and Carmen Martínez, M. (2012). Internalization and induction of antioxidant messages by microvesicles contribute to the antiapoptotic effects on human endothelial cells. *Free Radic. Biol. Med.* *53*, 2159–2170.
- Soung, Y.H., Ford, S., Zhang, V., and Chung, J. (2017). Exosomes in Cancer Diagnostics. *Cancers* *9*.
- Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* *10*, 513–525.
- Stocker, R., and Keaney, J.F. (2005). New insights on oxidative stress in the artery wall. *J. Thromb. Haemost. JTH* *3*, 1825–1834.
- Sun, X., Belkin, N., and Feinberg, M.W. (2013). Endothelial MicroRNAs and Atherosclerosis. *Curr. Atheroscler. Rep.* *15*.
- Suzuki, J., Umeda, M., Sims, P.J., and Nagata, S. (2010). Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* *468*, 834–838.
- Svensson, K.J., Christianson, H.C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L.M., Mörgelin, M., and Belting, M. (2013). Exosome Uptake Depends on ERK1/2-Heat Shock Protein 27 Signaling and Lipid Raft-mediated Endocytosis Negatively Regulated by Caveolin-1. *J. Biol. Chem.* *288*, 17713–17724.
- Takahashi, Y., Nishikawa, M., Shinotsuka, H., Matsui, Y., Ohara, S., Imai, T., and Takakura, Y. (2013). Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in

- mice after intravenous injection. *J. Biotechnol.* *165*, 77–84.
- Tang, X., Luo, Y.-X., Chen, H.-Z., and Liu, D.-P. (2014). Mitochondria, endothelial cell function, and vascular diseases. *Front. Physiol.* *5*.
- Taylor, D.D., and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* *110*, 13–21.
- Taylor, D., Zacharias, W., and Gercel-Taylor, C. (2011). Exosome Isolation for Proteomic Analyses and RNA Profiling. In *Serum/Plasma Proteomics*, R.J. Simpson, and D.W. Greening, eds. (Humana Press), pp. 235–246.
- Théry, C., Amigorena, S., Raposo, G., and Clayton, A. (2001). Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. In *Current Protocols in Cell Biology*, (John Wiley & Sons, Inc.), p.
- Théry, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* *2*, 569–579.
- Tian, T., Wang, Y., Wang, H., Zhu, Z., and Xiao, Z. (2010). Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. *J. Cell. Biochem.* *111*, 488–496.
- Tian, T., Zhu, Y.-L., Zhou, Y.-Y., Liang, G.-F., Wang, Y.-Y., Hu, F.-H., and Xiao, Z.-D. (2014). Exosome Uptake through Clathrin-mediated Endocytosis and Macropinocytosis and Mediating miR-21 Delivery. *J. Biol. Chem.* *289*, 22258–22267.
- Toti, F., Satta, N., Fressinaud, E., Meyer, D., and Freyssinet, J.M. (1996). Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. *Blood* *87*, 1409–1415.
- Tounian, P., Aggoun, Y., Dubern, B., Varille, V., Guy-Grand, B., Sidi, D., Girardet, J.P., and Bonnet, D. (2001). Presence of increased stiffness of the common carotid artery and endothelial dysfunction in severely obese children: a prospective study. *Lancet Lond. Engl.* *358*, 1400–1404.
- Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger, B., and Simons, M. (2008). Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science* *319*, 1244–1247.
- Tsukamoto, M., Iinuma, H., Yagi, T., Matsuda, K., and Hashiguchi, Y. (2017). Circulating Exosomal MicroRNA-21 as a Biomarker in Each Tumor Stage of Colorectal Cancer. *Oncology* *92*, 360–370.
- Urbanelli, L., Magini, A., Buratta, S., Brozzi, A., Sagini, K., Polchi, A., Tancini, B., and Emiliani, C. (2013). Signaling Pathways in Exosomes Biogenesis, Secretion and Fate. *Genes* *4*, 152–170.
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., and Lötvall, J.O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* *9*, 654–659.
- Vernay, M., Salanave, B., Peretti, C. de, Druet, C., Malon, A., Deschamps, V., Hercberg, S., and Castetbon, K. (2013). Metabolic syndrome and socioeconomic status in France: The French Nutrition and Health Survey (ENNS, 2006–2007). *Int. J. Public Health* *58*, 855–864.
- Vidal, M., Sainte-Marie, J., Philippot, J.R., and Bienvenue, A. (1989). Asymmetric distribution of phospholipids in the membrane of vesicles released during in vitro maturation of guinea pig reticulocytes: evidence precluding a role for “aminophospholipid translocase.” *J. Cell. Physiol.* *140*, 455–462.

- Wang, Y., Cheng, Q., Liu, J., and Dong, M. (2016). Leukemia Stem Cell-Released Microvesicles Promote the Survival and Migration of Myeloid Leukemia Cells and These Effects Can Be Inhibited by MicroRNA34a Overexpression. *Stem Cells Int.* 2016, 9313425.
- Weiss, R., Dziura, J., Burgert, T.S., Tamborlane, W.V., Taksali, S.E., Yeckel, C.W., Allen, K., Lopes, M., Savoye, M., Morrison, J., et al. (2004). Obesity and the metabolic syndrome in children and adolescents. *N. Engl. J. Med.* 350, 2362–2374.
- Widlansky, M.E., Gokce, N., Keaney Jr, J.F., and Vita, J.A. (2003). The clinical implications of endothelial dysfunction. *J. Am. Coll. Cardiol.* 42, 1149–1160.
- Wiklander, O.P.B., Nordin, J.Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., et al. (2015). Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J. Extracell. Vesicles* 4.
- Willms, E., Johansson, H.J., Mäger, I., Lee, Y., Blomberg, K.E.M., Sadik, M., Alaarg, A., Smith, C.I.E., Lehtiö, J., Andaloussi, S.E., et al. (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci. Rep.* 6, srep22519.
- Wolf, P. (1967). The nature and significance of platelet products in human plasma. *Br. J. Haematol.* 13, 269–288.
- Yamashita, F., Azuma, K., Yoshida, T., Yamada, K., Kawahara, A., Hattori, S., Takeoka, H., Zaizen, Y., Kawayama, T., Kage, M., et al. (2013). Prognostic Value of EGFR Mutation and ERCC1 in Patients with Non-Small Cell Lung Cancer Undergoing Platinum-Based Chemotherapy. *PLOS ONE* 8, e71356.
- Yang, J.S., Lee, J.C., Byeon, S.K., Rha, K.H., and Moon, M.H. (2017). Size Dependent Lipidomic Analysis of Urinary Exosomes from Patients with Prostate Cancer by Flow Field-Flow Fractionation and Nanoflow Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Chem.* 89, 2488–2496.
- Yang, Y.-M., Huang, A., Kaley, G., and Sun, D. (2009). eNOS uncoupling and endothelial dysfunction in aged vessels. *Am. J. Physiol. - Heart Circ. Physiol.* 297, H1829–H1836.
- Zhang, D.X., and Gutterman, D.D. (2007). Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* 292, H2023-2031.
- Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469, 221–225.

Annexe

Antioxidants & Redox Signaling

Antioxidants & Redox Signaling: <http://mc.manuscriptcentral.com/liebert/ARS>

EXTRACELLULAR VESICLES: MECHANISMS IN HUMAN HEALTH AND DISEASE

Journal:	<i>Antioxidants and Redox Signaling</i>
Manuscript ID	Draft
Manuscript Type:	Comprehensive Review Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Malloci, Marine; INSERM UMR 1063 Perdomo, Liliana; INSERM UMR 1063 Veerasamy, Maëva; INSERM UMR 1063 Andriantsitohaina, Ramaroson; INSERM UMR 1063 Simard, Gilles; INSERM UMR 1063, ; Laboratoire de Biochimie, Biochimie et génétique Martinez, M. Carmen; INSERM UMR 1063, ;
Keyword:	Biomarkers, Cardiovascular, Central Nervous System, Diabetes, Nitric Oxide, Regenerative medicine, Vascular
Manuscript Keywords (Search Terms):	Microvesicles, Exosomes, Biomarkers, miRNA

SCHOLARONE™
Manuscripts

COMPREHENSIVE INVITED REVIEW

**EXTRACELLULAR VESICLES:
MECHANISMS IN HUMAN HEALTH AND DISEASE**

Marine Malloci^{1*}, Liliana Perdomo^{1*}, Maëva Veerasamy¹, Ramaroson Andriantsitohaina^{1,2},
Gilles Simard^{1,2}, M. Carmen Martínez^{1,2}

¹INSERM UMR 1063, Stress oxydant et pathologies métaboliques, UNIV Angers, Université
Bretagne Loire, F-49933, Angers, France; ²Centre Hospitalo-Universitaire d'Angers, F-
49933, Angers, France

*These authors contributed equally in this work

Running head: Microvesicles/exosomes: biomarkers and effectors

Corresponding author: M. Carmen Martínez, PhD, INSERM UMR 1063, Stress oxydant et
pathologies métaboliques, Institut de Biologie en Santé, 4 rue Larrey, F-49933 Angers,
France; Phone: +33 2 44 68 85 79; E-mail: carmen.martinez@univ-angers.fr

Word count: 17761

Number of references: 344

Numbers of greyscale illustrations: 1

Number of color illustrations: 14 (for online only)

Keywords: Microvesicles, exosomes, biomarkers, miRNA

Abstract

Significance: Secreted extracellular vesicles (EVs) have now considered as veritable entities for diagnosis, prognosis and therapeutics. These structures are able to interact with target cells and modify their phenotype and function.

Recent advances: miRNA- or drug-enriched bioengineered EVs display enhanced potential in therapy to deliver desirable information to target cells/tissues.

Critical issues: The challenges are related to redefine methods allowing specific EV isolation and identification in different biological fluids, including plasma, serum, cerebrospinal fluid and urine.

Future directions: Analysis of bidirectional interactions between hosts and resident microbial communities via EVs will allow, at least partially, understanding the progression of several pathologies opening the way for new therapeutic approaches.

I. INTRODUCTION

Cells are able to release into the intercellular space various types of vesicles from different biogenesis. Long considered as simple cell debris, there is now enough evidence that extracellular vesicles (EVs) participate in intercellular communication and thus modulate many cellular processes. Surrounded by a phospholipid bilayer, EVs are loaded with proteins and nucleic acids from donor cells, which are grouped in the database Vesiclepedia (<http://www.microvesicles.org>) that contains molecular data identified in all classes of EVs. Furthermore, it is described in numerous pathologies that EVs could be potential biomarkers, since they are easy to detect, more stable than other soluble biomarkers and accessible in a non-invasive manner in most biological fluids (1).

Although the interest in EV research is increasing, there is still no real nomenclature to classify the different types of vesicles. Many terms are used, varying from ectosomes, microparticles, microvesicles (MVs), nanovesicles, exosomes and membrane particles to exosome-like particles and exovesicles (2). Currently, EVs are commonly classified based on their intracellular origin. Thus, three principal populations of EVs are considered: apoptotic bodies, MVs and exosomes (3). Apoptotic bodies (size between 50-5000 nm) which are released by cells undergoing apoptosis, and characterized by permeable membrane, will not be covered in this review. This review focuses on the role of EVs as possible biomarkers and/or therapeutic targets in diabetes, obesity, atherosclerosis, neurodegenerative diseases and cancer. Also, we develop the use of EVs as therapeutic tools in these diseases.

II. MICROVESICLES (MVs)

MVs were originally identified by Peter Wolf in 1967 as “platelet dust” (4) and, for a long time, they were considered as cellular debris resulting from cell damage or dynamic plasma membrane incomings (5). They are characterized by preset of surface markers indicative of

their cellular origin (6). Indeed, MVs are rich in negatively charged phospholipids (*e.g.* phosphatidylserine (PS)) at their surface.

MVs are distinguished of exosomes by their size and the mechanisms involved in their generation. MVs are directly shed from the plasma membrane of cells and measure approximately 100-1000 nm (7) (Figure 1). The size range of MVs and exosomes (see below) may possibly overlap, for that reason high-sensitivity technics and novel instruments including nanoparticle tracking analysis, are useful to characterize and distinguish both population (8).

A. MV formation and shedding

The shedding of MVs is preceded by the budding of small cytoplasmic protrusions, which then detach by fission from different cell types. Platelets, leukocytes, erythrocytes, endothelial cells, and tumor cells are availed to induce *in vitro* MV shedding (9-13). *In vivo*, it has been also described the presence of MVs in different tissues such as atherosclerotic plaques (14) and liver (15). Regarding MVs on fluids, MVs derived from platelets represent approximately 70-90% of circulating MVs (16), while in urine, MVs are mainly from renal cells, mainly from podocytes, juxtaglomerular and mesangial cell origin. Mechanisms that lead to the formation of MVs are not completely elucidated even if MVs shedding quantity and phenotype are depending on method of production. Nevertheless, numerous studies have shown that shedding of MVs follows chemical and physical cell activation or apoptosis (reviewed by (17)).

Distinct stimuli such a tumor necrosis factor (TNF- α) (18) or actinomycin D (19) trigger an increase of cytosolic calcium concentration, which lead to MV formation (20-23). This influx of extracellular calcium is associated with calcium-dependent calpain activation, a proteolytic enzyme, which induces changes in the actin cytoskeleton stability (20, 22). In addition, the inhibition of cytosolic calcium increase might prevent MV formation (24).

The asymmetry of the lipid bilayer is regulated by three types of enzymes: translocases, flippases and scramblases. These enzymes are responsible for the transport of PS and phosphatidylethanolamine to the external layer and the rapid “flip-flop” of phospholipids between the membrane layers. Under basal conditions, PS is found preferentially, if not exclusively, on the inner leaflet of the plasma membrane of cells. Evidence suggests that PS exposure is a key mediator of the formation of MVs. However, some reports suggest that PS is not externalized in certain MV populations (25).

Nevertheless, shedding of MVs is induced by prolonged high intracellular calcium concentration, which involves different mechanisms such as the activation of tyrosine kinases. Martinez and colleagues (reviewed by (17)) have reviewed several molecular targets linked to the increase of intracellular calcium concentration and, MV formation and shedding. Some targets include extracellular signal-regulated kinase (ERK) pathway, small GTPase Rho A, myosin light chain and Rho-associated kinase (ROCK I), NADPH oxidase, and reactive oxygen species (ROS). However, calcium is not the only second messenger involved in MV shedding. Indeed, in dendritic cells (DCs), macrophages and microglia, activation of protein kinase C (PKC) by a calcium-independent mechanism, is also effective to induce the shedding of MVs (reviewed by (26)).

On the other hand, during apoptosis, MV formation and shedding is associated with the activation of ROCK I and caspase 3, which might lead to the disruption of the membrane skeleton structure and consequently, to the MV formation (27). During apoptosis-dependent MV formation, disturbed membrane follows cell contraction and DNA fragmentation (28).

Stimuli such as TRAIL/Apo2L, a cytokine belonging to the TNF- α superfamily, promote endothelial MV release by initiating the recruitment of adaptor proteins and the activation of nuclear factor kappa B (NF- κ B) (28). This capacity to induce MV production has been attributed to activation of different signaling cascades, including caspase, RhoA/ROCK, and

the p38-MAPK pathways (29). Interestingly, TNF- α treatment has a dual effect on the endothelial release of MVs. TNF- α can induce both miRNA-rich and miRNA-poor MVs displaying anti-apoptotic or pro-apoptotic effects, respectively, depending on the activated signal pathway. Indeed, TNF- α induces miRNA-rich MV release through the inhibition of caspase pathway, whereas when RhoA/ROCK pathway is suppressed, TNF- α induces miRNA-poor MV generation (30). This dual effect results in the anti-atherogenic and the pro-atherogenic effects of endothelial MVs on target cells.

B. MV composition

Proteomic analyses of several MV populations have been performed in order to improve MV molecular profile knowledge (31, 32). The different reports have confirmed that MV composition depends, not only on the origin cell type, but also on the conditions of cell stimulation (33, 34). In terms of composition, MVs present elements of their membrane origin, such ligands and receptors, miRNA and proteins from mitochondria (35-37), nucleus and cytosolic compartments (31). There are not a lot of specific protein markers of ubiquitous expression in all MV membranes, since protein composition depends on origin cell. However, a downright proteomic study of marker comparison has been recently performed in order to characterize heterogeneous populations of EV subtypes released by human DCs. This study has shown that MVs are specifically enriched in proteins such as actin-4 and -1, the chaperone endoplasmin, mitofilin, the eukaryotic translation elongation factor 2 and major vault protein. Nevertheless, MVs can express proteins shared by other types of EVs such as actin, tubulin, endoplasmin, glyceraldehyde-3-phosphate dehydrogenase and annexin II (32).

It should be noted that MVs express proteins related with the function of mother cells. Thus, MVs derived from neutrophils and tumors are enriched with matrix metalloproteinases (MMPs) and other proteolytic enzymes, which have a function in the digestion of the extracellular matrix necessary for the progress of inflammation and for cancer growth. MVs

derived from platelets harbor various integrins, the plasma membrane glycoproteins GPIb and GPIIb-IIIa and P-selectin, playing a role in coagulation. The vesicles shed from macrophages carry the ligand-1 of the P-selectin glycoprotein, which helps with their binding to platelets (reviewed by (26)).

Furthermore, lipid composition of MVs can vary depending on the environment nearby to cells. Thus, it has been shown that, after a high-fat meal, individuals display circulating MVs with higher apoB expression and weaker apoCII and ApoE expression when compared to pre-prandial conditions (38).

C. Isolation and characterization

Different biophysical and biochemical properties can be used to isolate EVs, including size, mass density, and shape, charge, and antigen exposure in order to distinguish MVs from exosomes or other type of EVs. The most common methods to isolate EVs are based on size, density, and immunophenotype separation. They include differential centrifugation, density gradient centrifugation, size exclusion chromatography, ultrafiltration, immunocapture assays and precipitation. Some of these methods may be used alone or in combination (reviewed by (39)). However, methods to obtain highly pure and well characterized vesicle populations are not well standardized.

Overall, MVs are isolated from cell culture supernatants or body fluids by a series of centrifugation steps in order to remove dead cells, large debris, and other cellular contaminants resulting from cell lysis (40). Isolation of circulating MVs includes process like do away blood cells from anticoagulated whole blood by low-speed centrifugation, at room temperature, in order to avoid *in vitro* platelet activation. However, time and speed of centrifugation change between protocols.

D. MV uptake by target cells

Several mechanisms for MV uptake have been proposed (41), though, the mechanisms by which MVs transfer biological information to target cells are not completely elucidated. It has been described that receptor-ligand interaction is an important mechanism to internalized MVs (42). Other mechanisms involved in MV interaction to the target cells are the transfer of components, the fusion of MVs and target-cell membranes and MV internalization (reviewed by (17)).

Receptor-ligand interaction leads to activation or inhibition of a transduction signal in target cells. However, the others mechanisms, above mentioned, can change the phenotype of target cells (43). Interaction by the transfer of MV components includes the transfer of surface receptors, proteins, mRNA, miRNA and bioactive lipids (reviewed by (26)).

III. EXOSOMES

Exosomes are described, for the first time three decade ago, as nanoscaled vesicles of endosomal origin, released as a result of the fusion of the multivesicular bodies (MVBs) with the plasma membrane in reticulocytes from rats (44) and sheeps (45). Indeed, by studying by electronic microscopy the intracellular traffic of transferrin receptor, it has been observed that transferrin receptor could be internalized to be recycled and, then, found on the surface of the exosomes in the extracellular medium. Since, *in vitro* release of exosomes is observed in most of the cells, such as hematopoietic (B-, T-, dendritic and mast cells, platelets), epithelial and neural cells, adipocytes, fibroblasts, stem cells and many types of tumor cells (46). In addition, exosomes are found in several biological fluids, such as blood, saliva, urine, seminal fluid, amniotic liquid, ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk and cerebrospinal fluid (CSF) (46). Since then, the multiple functions of exosomes in immune and cardiovascular diseases (CVD), tumorigenesis, and the propagation of neurodegenerative

diseases have become evident. This has sparked interest in exosomes as biomarkers and therapeutic targets.

Exosomes can be defined by their size comprised between 30-150 nm. The size of exosomes seems to depend on their origin cell type, being ~50 nm in reticulocytes (45), ~100 nm in B-cell lymphoma cells (47), or 80 to 120 nm in adipocytes (48). To identify exosomes, the analysis of at least three specific markers is advised in order to better identify your exosome populations (49). In fact, several proteins commonly enriched in exosomes from different origin cell types, can be considered as exosomal markers. Among them, we can find the tetraspanins CD9, CD81, CD63, the endosomal proteins TSG101 and ALIX, or Mfge8 (49). However, the relevance of these markers is currently discussed because they can be also found in MVs (32).

A. Exosome formation

In contrast to MVs (see above), exosomes come from the endosomal compartment and are formed as intraluminal vesicles (ILVs). ILVs are formed by inward budding of the early endosomal membrane, sequestering proteins, lipids, and cytosol, in order to degrade, recycle or exocytose this content. Early endosomes then mature in late endosomes and the accumulation of multitude of ILVs leads to the formation of MVBs. The main fate of MVBs is to fuse with lysosomes, ensuring the degradation of their content. However, MVBs can also fuse with the plasma membrane to release their sequester ILVs as exosomes in the extracellular space (50) (Figure 1). Several mechanisms have been proposed to participate in the exosome formation, and they can be classified in two groups depending on the endosomal sorting complex required for transport (ESCRT) involved.

ESCRT-dependent exosome formation (Figure 2): Involvement of ESCRT machinery in exosome biogenesis is initially inferred from proteomic studies showing the presence, in exosomes from different cell types, of ESCRT-associated proteins, TSG101 and ALIX

(51). The role of the ESCRT complexes is to ensure the transport of ubiquitinated proteins from endosomes to lysosomes, leading to their degradation. Thus, ESCRT proteins are responsible to sort proteins into ILVs by inward budding of the membrane and, by the way, exosome release. It has been suggested that ESCRT-0, -I and -II complexes form stable hetero-oligomers that function together to recognize and sequester ubiquitinated proteins in the endosomal membrane. Then, ESCRT-I together with ESCRT-II could initiate the budding process of the endosomal membrane. In addition, ESCRT-II initiates the assembly of the ESCRT-III complex, which in turn, mediates membrane budding and scission to form ILVs (52).

ESCRT-independent exosome formation (Figure 2): Alternative ESCRT-independent pathways have also been proposed. MCF7 tumor cells secrete exosomes through a syndecan-syntenin-ALIX pathway (53). Indeed, the syndecan heparan sulphate proteoglycans and their cytoplasmic adaptor syntenin can control the formation of exosomes by direct interaction with ALIX and supporting the intraluminal budding of endosomal membranes. These syndecan-positive exosomes are also enriched in CD63 and ceramide. Downstream of ALIX, charged multivesicular body protein, and vacuolar protein sorting-associated protein 4 knockdowns block their biogenesis (53), suggesting that these ESCRT-associated proteins are also involved in this formation way, although it is independent of the entire classical ESCRT pathway. Moreover, the enzymatic digestion of heparan sulfate chains on syndecans by heparanase facilitates both the endosomal membrane budding and the biogenesis of exosomes, and stimulates their release (54). Thereafter, it has also been described the GTPase ADP ribosylation factor 6 (ARF6) and its effector phospholipase (PL)D2 as regulators of this exosome biogenesis pathway (55). Both, ARF6 and PLD2, can affect exosomes by controlling the budding of ILVs into MVBs.

In oligodendroglial cell lines, Trajkovic and colleagues (56) have found that exosome release is reduced after inhibition of neutral sphingomyelinase (nSMase), an enzyme responsible of ceramide production. This lipid can facilitate the membrane invagination of ILVs through its cone-shaped structure and induce exosome formation. Exosomes derived from this way are enriched in proteolipoprotein (PLP), CD63, CD81 and TSG101.

In addition, the tetraspanins CD9 and CD63 have also been described as directly implicated in exosome formation. Indeed, DCs generate major histocompatibility complex (MHC)-II-exosomes through CD9-dependent pathway, without ESCRT machinery implication (57). In melanosome, CD63 directly participates in ESCRT-independent sorting of luminal domain of the premelanosome protein, a protein enriched in melanosomes, to ILVs (58).

Thus, the formation of exosomes seems to involve many mechanisms and remains incompletely elucidated. Moreover, these different ways of production influence exosome composition and, thus, induce the formation of different populations of exosomes. This also underlines the molecular and mechanical heterogeneity of the types of exosomes secreted by different cells.

B. Exosome release

To release exosomes, MVBs must be transported, docked and fuse with plasma membrane to liberate ILVs in the extracellular space. For that, it has been suggested that the proteins of Rab family, small GTPase involved in the trafficking of intracellular vesicles, their docking and fusion with plasma membrane (59), can be implicated (Figure 3). Indeed, Savina and colleagues (60) have shown that, in erythroleukemia cells, Rab11 regulates exosome secretion by acting in docking and fusion of MVBs which require also the presence of calcium. Beside, in oligodendrocyte, the inhibition of Rab35 function impairs exosome secretion (61). In addition, Rab27a and Rab27b promote exosome secretion in HeLa cells. These two Rab27 isoforms are involved in MVB docking at the plasma membrane (62). Exosomes released via

this way are enriched in late endosomal proteins (*e.g.* CD63, ALIX, and TSG101) (63). Furthermore, Rab7 has also been described as implicated in the release of exosomes containing ALIX and syntenin in breast tumor cells (53). However, other authors have shown that depletion of Rab7 does not affect exosome release in HeLa cells (62), suggesting this way can be dependent on the cell type.

Once again, the mechanisms allowing the secretion of the exosomes seems to be multiple, depending on the origin cells and are not yet completely elucidated.

C. Exosome composition

Like MVs (see above), exosomes carry multiple proteins and nucleic acids, protected by a lipid bilayer. However, their composition is a consequence of their formation through the endosomal compartment as well as the various mechanisms associated with their secretion. Thus, composition of exosomes differs from MVs and source cells. For example, a proteomic and lipidomic study has compared composition of exosomes, MVs and source cells for three different cell lines: U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells and human bone marrow-derived mesenchymal stem cells (MSCs) (64). The authors have found that protein patterns of exosomes are more likely different from their cells of origin than are the protein patterns of MVs. Moreover, exosomes seem enriched in proteins of extracellular matrix, heparin-binding, receptors, immune response and cell adhesion functions, whereas MVs are enriched in endoplasmic reticulum, proteasome and mitochondrial proteins. Exosomes and MVs also differ in their types of lipid contents. Furthermore, EV composition differs from one cell line to another, for instance, Huh7 and MSC exosomes are specifically enriched in cardiolipins whereas U87 exosomes are enriched in sphingomyelins (64).

Additionally, the composition of the vesicles may depend on the origin cell type but also on their specific function. Indeed, Conde-Vancells and colleagues (65) have compared exosomes from a mouse hepatic cell line and from a primary culture of rat hepatocytes, and they have

reported that hepatic-derived exosomes comport several common exosomal proteins as well as proteins not previously described in exosomes, such as proteins specifically found in hepatocytes and associated with the function of these cells. In adipocyte-derived EVs, it has been shown that exosomes and MVs exhibit specific protein signature and suggest that the differences in their content could predict the specific biological functions of the EVs (48).

Proteins carried by exosomes: Commonly found proteins in exosomes are logically those associated with the mechanisms responsible for their biogenesis and secretion, including proteins associated with the endosomal compartment. Thus, exosomes are enriched in proteins associated with ESCRT complexes (ALIX, TSG101), involved in the MVB structure (Lamp1 and Lamp2, CD9, CD81 and CD63 tetraspanins), proteins of membrane traffic (Rab GTPases, annexins), fusion proteins (soluble-N-ethylmaleimide sensitive factor attachment protein receptor (v-SNARES)), heat-shock proteins (HSP 90/70) or proteins of MHC-I and II, but also proteins from plasma membrane (flotillin-1, Mfge-8, caveolin-1) (for review see (66)).

In general, proteins associated with the endoplasmic reticulum, Golgi and nucleus are not found in exosomes (49); however exosomes carry transcription factors *e.g.* Notch, Wnt, which are normally found in the nucleus (63).

Lipid components in exosomes: Comparison of exosome composition produced *in vitro* from guinea pig primary reticulocytes and their parent cells show that exosomes and reticulocytes have a similar cholesterol composition, but phosphatidylethanolamine content is lower in exosomes than in parent cells (67). Since then, numerous studies have reported the lipid composition of exosomes derived from different cell types such adipocytes (48), MSCs (64), or cancer cells (68), mast cells and DCs (69). These comparisons show that, although lipid content of exosomes is distinct from that of the cell of origin, exosomes display some common lipid features independently of their origin. Indeed, exosomes are mainly enriched in cholesterol, sphingomyelin, ceramide and exposed PS at membrane surface. Otherwise, the

endosomal specific phospholipid, essentially localized in MVBs, bis(monoacylglycero)phosphate (BMP) could be a specific lipid marker of exosomes (69).

Moreover, fatty acids of exosome membranes are mainly saturated or mono-saturated reflecting a lateral segregation of these lipids in exosomes during maturation of MVBs (70). In addition, exosomes can also contain bioactive lipids. In exosomes from RBL-2H3 basophil cells, it has been shown the presence of enzymes involved in lipid metabolism including PLA2, PLC, and PLD, responsible of the release of signaling mediators from membrane phospholipids (71). Exosomes can also contain a large panel of free fatty acids such as arachidonic acid and prostaglandin E2.

Interestingly, lipid composition of exosomes can also serve as biomarkers and be involved in different pathologies (for review see (72)). Recently, it has been shown that lipid content of urinary exosomes change in patients with prostate cancer (73).

Nucleic acids in exosomes: Exosomes have the capacity to convey and to transfer genetic information, and thus to modulate the gene expression of the target cells. In a small number of cases, DNA has been found, including genomic and mitochondrial DNA (mtDNA), but exosomes contain mostly small RNAs, with many derived from ribosomal 18s and 28s rRNAs and tRNAs (63).

For the first time, mRNA and miRNA are found in exosomes from human and murine mastocytes (74). Montecalvo and colleagues (75) have described the transfer of functional miRNA between DCs through exosomes. Also, the different miRNA cargo profile observed according to the dendritic cell maturation suggests that cells could select miRNA content. In this context, in rats, it has also been shown that exosomal miRNA profile can be modulated by stimuli, as chronic ingestion of sucrose (76).

It has also been described that exosomes can contain many types of RNA such as long and short non-coding RNA, piwi-interacting RNA, vault RNA or Y RNA (63). In this respect, the

packaging of RNA in exosomes within the lipid bilayer membrane allows protecting it from RNase digestion once release into the extracellular environment. However, due to the variations on isolation techniques and the different cell types and culture conditions used to analyze exosomal content, the comparison of the existing results on the composition of these vesicles have to make with a critical eye.

D. Exosome uptake

Once released, exosomes can interact with recipient cells and trigger intracellular signaling pathways, by conveying message through the delivery of their content. Firstly, binding of exosomes to the cells surface may possible through a simple interaction with several contact proteins, surface receptors or ligands of target cells (Figure 4). Among all those which have been described, it is possible to retain the role of LFA-1 on DCs to capture intercellular adhesion molecule-1 (ICAM-1)-bearing dendritic exosomes (77). Moreover, blocking LFA-1, ICAM-1, α v or β 3 integrins, CD9 or CD81 tetraspanins decrease dendritic exosomes captured by DCs (78). Secondly, exosomes can also transmit their cargo by the directly fusion of membrane-membrane. It has been described in DCs (75) and melanoma cells (79). Finally, exosomes content can be internalized by different ways of endocytosis: caveolae-dependent endocytosis in epithelial cells (80), clathrin-dependent endocytosis in neurons (81) and PC12 cells (82), clathrin-independent but cholesterol- and lipid raft-dependent endocytosis in endothelial and some tumor cells (83), macropinocytosis by microglia (84) and tumor cells (85) or phagocytosis by macrophages (86) and PC12 cells (82).

To improve exosomes uptake, Nakase and colleagues (87) have proposed to combine cationic lipids and a pH-sensitive fusogenic GALA peptide which increase exosome binding at the plasma membrane and improve uptake via endocytic pathway. Interestingly, heparin blocks both binding to the membrane or recipient cells and uptake of glioblastoma-derived exosomes by human endothelial cells (88). Although the exact mechanism is not advanced, these authors

suggest that exosomes contain ligands which bind directly with heparin and impede exosomes binding with the receptors on recipient cells.

E. Biodistribution and clearance of exosomes

Independent studies indicate that the half-life of purified exogenous exosomes from splenocyte supernatants (89), after intravenous injection in mice, is about 2 minutes. Several biodistribution studies of intravenously injected exosomes demonstrate those vesicles are accumulated mainly in liver, spleen, and lungs (89-92). However, it has been shown different panel of exosome distribution after injection in mice of the same amount of exosomes through three different systemic delivery routes: intravenous, intraperitoneal, and subcutaneous (92).

Thus, biodistribution of exosomes depends on their origin, the administration way and the availability of different target cell types to internalize specific exosomes. Moreover, the slower uptake of exosomes in mice with an impaired innate immune system and a complement deficiency suggest that clearance of exosomes can be regulated by the innate immune system, likely mediated by complement opsonization (93).

F. Isolation of exosomes

Exosomes are usually purified from culture supernatant and biological fluids by serial steps of centrifugation and ultracentrifugation performed at 4°C and classically recovered at 100,000g during 70 min as pellets. However, the intensity or the duration of ultracentrifugation may vary depending on the type of original fluid. For further purification, ultracentrifugation on linear sucrose gradient is recommended, with exosomes floating to a density ranging from 1.13 to 1.19 g/mL. Some steps of serial centrifugations can be skipped through filtration on 0.22 µm pore filters (94). Other methods have been developed to isolate exosomes, such as immunoprecipitation or the use of antibody-loaded magnetic cell beads directed against proteins exposed on exosomal membranes (95). However, this approach allows isolating only a subpopulation of marker-positive exosomes. Finally, once captured on beads, exosomes

retaining full functionality may not be successfully eluted from the bead surface (94). Otherwise, the size-exclusion chromatography technique based on the separation of particles according to size, allows a better elimination of the possible contaminants isolated with the exosomes, while conserving their integrity and biological activity with no loss of sample (96). Moreover, this approach can easily be scaled for isolating large volumes of exosomes. It is described a more uniform vesicle size and superior recover of vesicle components (proteins and RNA) by chromatography *versus* centrifugation (97).

There are also different commercial kits, like more expensive, allowing the extraction of exosomes. It has been shown that when circulating exosomes are isolated by ExoQuick precipitation, exosomal RNAs and proteins present greater purity and quantity than chromatography and ultracentrifugation (97). Thus, extraction kits appear to be a good alternative when limited amounts of biological samples are available.

G. Characterization methods

Simple purification by differential ultracentrifugation is not sufficient to qualify vesicles as exosomes and a combination of quantitative protein composition, morphological and physical criteria must be used to identify exosomes among other EVs. Morphology and size of exosomes can be determined by transmission electron microscopy, which remains the only method that allows to directly visualizing in detail these vesicles. Employing immuno-gold labeling allows having biochemical information regarding exosome surface. Moreover, by using cryo-electron microscopy, samples are analyzed in frozen conditions without being stained or fixed (98). Nanoparticle tracking analysis (NTA) allows determining not only size (30-1000 nm), but also the exosome concentration of EVs at a concentration range of 10^8 - 10^9 (98). NTA software is then able to track individual EVs moving under Brownian motion, relates the movement to a particle size and calculates total concentration. By combining NTA with fluorescence measurement, it is also possible to determine EV phenotype with specific

antibodies (99). Finally, specific markers of exosomes can be identified and quantified by classical methods like western blot, global proteomic analysis using mass spectrometry techniques, or flow cytometry.

IV. EVs in DIABETES

Diabetes is a disease characterized by a blood glucose increase, which is associated with several complications, mainly cardiovascular alterations. There are two main types of diabetes: Type 1 diabetes (T1DM) is an autoimmune disease characterized by the destruction of pancreatic β -cells, which leads to a total failure of the insulin secretion (100), whereas Type 2 diabetes (T2DM) is characterized by failure of insulin action and/or secretion and, frequently, is associated with obesity and aging (101).

Sabatier and colleagues (102) have shown that both T1DM and T2DM are associated with increased levels of circulating MVs, mainly from, endothelial cells and platelets. These MVs from diabetic patients are different in their pro-coagulant activity being the pro-coagulant activity of circulating MVs increased in T1DM patients but not in T2DM patients. Interestingly, the pro-coagulant activity of MVs from T1DM is correlated with HbA1c levels, suggesting that this activity of MVs is associated with glucose imbalance. Even more, a recent meta-analysis that consists of 48 studies has shown that patients with T2DM display higher levels of circulating EVs, in particular MVs, than non-diabetic patients (103). Interestingly, enhanced endothelial-derived MV levels are reduced by anti-diabetic treatments such as pioglitazone but not by metformin. It suggests that although both drugs have similar effects on glycemic control, only pioglitazone is able to correct the imbalance between glucose and endothelial damage, as well as, to reduce circulating MVs derived from endothelial cells (104).

Even though some studies have shown the secretion of EVs under high glucose conditions, there is still a lack of knowledge regarding the role of EVs from tissues involved in insulin

resistance. An excessive consumption of refined carbohydrates, saturated fat and trans-fatty acids contribute to both weight gain and dyslipidemia, leading to insulin resistance and development of T2DM (105). Under this situation, storage capacity of white adipose tissue can become saturated which causes abnormal redistribution of lipids in other tissues (106). The effects of EVs from adipocytes have been poorly studied. However, it has been suggested that obesity-dysregulated microRNAs in visceral adipocyte-derived exosomes are predicted to impair insulin receptor signaling in obese subjects (107). Furthermore, circulating adipocyte-derived exosomal microRNAs targeting the canonical insulin receptor pathway are altered following bariatric surgery-induced weight loss (108). Additionally, white adipose tissue produces a wide variety of adipokines (leptin, resistin, adiponectin, interleukin (IL-6), TNF- α) which play a significant role in the development of peripheral insulin resistance. Likewise, elevated circulating levels of saturated free fatty acids (*e.g.* palmitic acid) induce inflammatory responses and cause insulin resistance in peripheral tissues. In this context, adipose tissue constitutes an important source of circulating exosomal miRNAs, which can regulate gene expression in distant tissues and thereby serve as a previously undescribed form of adipokines (109). Indeed, mice with Dicer adipose-specific knockout suffer from a severe metabolic phenotype with lipodystrophy which is associated with a huge decrease in serum exosomal miRNAs. Moreover, patients with congenital generalized lipodystrophy, who have been found to have decreased Dicer levels in adipose tissue, display a decreased expression of miRNAs in exosomes (109). Nevertheless, it has been described that exosomes from human adipose tissue, generated *ex vivo*, decrease or increase insulin-induced Akt phosphorylation depending on their adipokine content (110).

Individuals with T2DM have increased prevalence of chronic liver disease since the liver is a key organ in maintaining glucose homeostasis. EVs released by hepatocytes in response to palmitic acid and TNF- α might contribute to local macrophage activation, inflammation and

fibrosis. Even more, lipid-induced toxicity stimulates hepatocytes to release angiogenic MVs suggesting that, under lipotoxicity, MVs from hepatocytes may be critical signals to liver damage in steatohepatitis (111). Likewise, adipose-derived EVs may act into the liver and modulate signaling pathways associated with metabolism, inflammation and fibrosis, which may contribute to the initial stages of non-alcoholic steatohepatitis (NASH) (112). NASH has been described as a significant predictor factor for future T2DM in middle-aged health population, especially in women (113). In this respect, both exosomes and MVs enriched in miR-122 and miR-192 have been identified into the bloodstream during experimental NASH, suggesting an association between T2DM and these miRNA (15).

On the other hand, pancreatic β cells secrete insulin, which regulates hepatic production of glucose. However, when there is resistance to insulin in this tissue, there is a deregulation of hepatic gluconeogenesis contributing to the development of hyperglycemia (114). It has been demonstrated that EVs derived from endothelial progenitor cells (EPCs) enhance neo-angiogenesis in transplanted pancreatic islets through mRNA and microRNA transfer (115). Figliolin and colleagues (116) suggest that islet-derived EVs transport several miRNAs and mRNAs which may play a role in beta cell-endothelium cross-talk. EVs derived from pancreatic islets carry several mRNAs (vascular endothelial growth factor (VEGF), endothelial nitric oxide (NO \cdot) synthase (eNOS)) and microRNAs (miR-27b, miR-126, miR-130 and miR-296) involved in β -cell function, insulin secretion and angiogenesis, suggesting that their compartmentalization within EVs is not a random process. Some studies have shown that miRNAs carry on β -cells-derived EVs might modulate glucose homeostasis and interfere with the typical complications of diabetes. For instance, miR-375 (117) and miR-15a (118) are found to be positive regulators of insulin production in pancreatic β -cells by targeting myotrophin and UCP-2, respectively. Furthermore, Kamalden and colleagues (119) have provided data, *in vivo* and *in vitro*, which supports the concept that miR-15a contributes

to the pathogenesis of diabetes. miR-15a from the rat pancreatic β -cell line INS-1 can be packaged into exosomes, under high glucose culture conditions. Circulating miR-15a can travel through the circulation and play a role in diabetic retinopathy progression by their transfer to retinal cells, inducing oxidative stress and cell injury.

Diabetes also results in a health impact largely on its vascular complications, which include process such a retinopathy, nephropathy, and heart disease. Diabetic retinopathy is associated with ocular increases in oxidative stress, protein glycation, growth factors, inflammatory cytokines, and cell apoptosis, all of which stimulate the shedding of membrane MVs from retinal or vascular cells. In fact, it has been described the presence of MVs from platelets, endothelial and retinal cells in human vitreous samples of patients with diabetic retinopathy. Also, these MVs stimulate *in vitro* endothelial proliferation and *in vivo* new-vessel formation in a Matrigel plug model. This suggests a role of MVs from vitreous samples of patients with diabetic retinopathy in retinal angiogenesis by mechanisms involving VEGF (120). Also, it has been reported that miR-130a and miR-145 are enriched, while miR-155 and miR-424 reduced, in urinary exosomes from T1DM patients with microalbuminuria. In the same way, levels of miR-145 in urinary exosomes are increased in an animal model of diabetic nephropathy induced by streptozotocin. Furthermore, exposure of cultured mesangial cells to high glucose increases miR-145 content in both mesangial cells and mesangial cells derived exosomes, providing a potential mechanism for diabetes-induced miR-145 overexpression (121).

On the other hand, some studies have provided evidence of the role of EVs in the initiation of diabetic cardiomyopathy. Actually, maternal exosomes from diabetic pregnant C57BL/6 mice could cross the maternal-fetal barrier and infiltrated into the embryonic organs/tissues, including the heart, during embryonic development. Furthermore, exosomes from the maternal diabetic mice display upregulation of 126 miRNAs and downregulation of 92

miRNAs. Among these miRNAs, miR-133, miR-30, miR-99, miR-23 are found to be involved in cardiac development. This suggests that maternal exosomes from diabetic mice contribute to the cardiac development deficiency possibly by the transfer of miRNAs. Nonetheless, the relationship between the aberrant miRNAs and the pathways related to cardiac development (such as Notch, Pitx2, Wnt, NKX2.5 and GATA4) is still unknown (122). In addition, abnormal regulation of lipid uptake or its intracellular metabolism in cardiomyocytes may be one of the mechanisms underlying diabetic cardiomyopathy. Myocardial steatosis, defined as the accumulation of neutral lipids in the myocardium, has been observed in T2DM patients (123). In fact, circulating miR-1 and miR-133a levels are significantly elevated in T2DM patients compared to healthy subjects. High-fat diet-fed mice with myocardial steatosis also have presented elevated level of circulating miR-1 and miR-133a compared to control animals. Moreover, exosomes released from lipid-loaded HL-1 cardiomyocytes are enriched in miR-1 and miR-133a. These data suggest an association between exosomal miR-1 and miR-133a levels and myocardial steatosis in T2DM patients (124)

V. EVs in OBESITY

Defined as an excessive fat accumulation that may impair health and reduce life expectancy, obesity has become a true global epidemic, and worldwide obesity has more than doubled since 1980. Obesity causes are complex: besides nutrition, lack of physical activity and genetics, many environmental factors, such as insufficient sleep or endocrine disruptors seem to be also involved in the development of this chronic disease (125). Undeniably, obesity is favored by the modern lifestyle of industrialized countries, which is associated with an obesogenic environment leading to an imbalance between both energy intake and expenditure. Thus, when the former surpasses the second, excess fat will settle in peripheral tissues. One of the major anomalies characterizing people with obesity is inflammation related to progressive

accumulation of immune cells, particularly macrophages, in adipose tissue (126). It is associated with an abnormal production of adipokines which will contribute to generating complications in the other organs (127). Thus, obesity increases the likelihood of various diseases, particularly CVD like stroke, T2DM, obstructive sleep apnea (reviewed by (128)) but also certain types of cancer (129) and depression (130).

Changes to diet and exercising, and medications to reduce appetite or decrease fat absorption are the main treatments of obesity (131). If all these treatments are not sufficient, bariatric surgery may be performed to reduce stomach volume or bowel length. Currently, new approaches are needed to prevent and treat obesity development to stop the global epidemic. For this, accumulated evidences have shown that EVs could be interesting target.

Several studies have reported that patients with overweight, and also those with obesity, display enhanced circulating levels of procoagulant MVs expressing tissue factor, derived from platelets, endothelial cells and leukocytes, when compared with normal weight healthy subjects (132, 133). Whereas Stepanian and colleagues (132) show that MVs counts do not significantly vary in subjects who lost 25% of their excess weight, several studies have provided evidence that weight loss reduced EV rates in obese subjects. Indeed, Murakami's group has described those levels of platelet-derived MVs are significantly reduced after weight loss (134). In addition, it has been shown that, at one month after bariatric surgery, the decrease in body mass index (BMI) is associated with the improvement of glycaemia and reduction in endothelial-, platelet- and monocyte-derived MVs reflecting a diminished inflammation (135). More recently, it has been shown that, one year after gastrectomy, the decrease of BMI is correlated with a decline of circulating MVs from endothelial, platelet, leukocyte origins, and those expressing tissue factor and CD36. Moreover, the reductions of leukocyte-derived and CD36⁺ MVs are significantly correlated with hs-CRP decrease (136).

All these studies suggest that overweight and obesity are directly involved in the modulation of levels and subsets of circulating MVs.

Among the circulating EVs, adipocyte-derived exosomes (137) have been also described. Thus, it has been reported the detection of increased levels of exosomes expressing adiponectin in blood from *ob/ob* mice when compared with wild-type mice suggesting that adipocytes release important amounts of EVs during obesity (137). In addition, *in vitro* secretion of adipocyte-derived EVs is enhanced following exposure to different biological stimuli related to the chronic inflammation state associated with obesity such TNF- α and palmitic acid (48). All these results shed light on the fact that EVs, and particularly adipocyte-derived EVs, can be considered as true biomarkers of obesity.

Moreover, EVs could participate in the establishment of metabolic alterations. In fact, circulating MVs from obese insulino-resistant rats induce ROS production and vascular cell adhesion molecule (VCAM)-1 expression in cardiac endothelial cells (138) (Figure 5). Moreover, injection of adipose tissue-derived exosomes from obese mice in lean mice leads to the development of an insulin resistance, linked to the polarization of macrophages to an inflammatory phenotype (139). Indeed, it has been shown that adipocyte-derived exosomes from obese mice induce M1 macrophage phenotype through secreted miR-155 (140) (Figure 5). Thus, EVs could participate to the imbalance in the M1-to-M2 macrophage ratio in adipose tissue, which causes chronic inflammation and local insulino-resistance in obesity.

Changes of miRNA patterns in EVs are also described in obese patients, suggesting that EVs can carry other miRNAs which may participate to the development of obesity complications. Indeed, in adipocyte-derived exosomes from young lean and obese patients, 55 miRNAs are differentially expressed. Transforming growth factor (TGF)- β and Wnt/ β -catenin signaling emerged as top canonical pathways targeted by these miRNAs. These pathways appear to be important in the development and progression of chronic inflammation and fibrotic disease

(107). Moreover, Hubal and colleagues (108) have also shown that pattern of miRNA from adipocyte-derived exosomes change after 1-year of gastric bypass surgery and are correlated to improved post-surgery insulin resistance. Finally, in circulating exosomes of patients with metabolic troubles, a significant dysregulation of seven miRNAs associated with risks involved in the manifestation of metabolic syndrome has been reported when compared with exosomes from healthy subjects. Among them, miR-197, miR-23a, and miR-509-5p are correlated with BMI and may be considered as potential contributors of dyslipidemia in metabolic syndrome (141) (Figure 5).

VI. EVs in ATHEROSCLEROSIS

Atherosclerosis is the underlying pathology of CVD. The main risk factors for atherosclerosis are T2DM (102), hypertension (142), hyperlipidemia, hypercholesterolemia, sleep apnea (143) and obesity/metabolic syndrome (134). Atherosclerosis is considered a chronic inflammatory disease that affects large- and medium-sized arterial vessels, leading to the formation of atherosclerotic plaques (144). Typical human atherosclerotic lesions contain different cell types, including smooth muscle cells (SMCs), endothelial cells, fibroblasts, foam cells and macrophages which are involved in the inflammation and the development of the plaque. In the last years, several studies have highlighted the role of EVs, particularly MVs, during the initiation and the progression of atherosclerotic lesion due to EV capacity to interact with the cellular components of vessels.

MVs are produced and accumulated in the human atherosclerotic plaques, particularly, from leukocytes; this process induces endothelial dysfunction and leukocyte recruitment (14). Mechanisms leading to MV formation in atherosclerotic plaque are unknown; however, lipids oxidation or modification, oxidative stress and pro-inflammatory cytokines may locally contribute to MV release from vascular cells or monocytes (7). Likewise, exosomes are present in human atherosclerotic plaques; however, no data is available of the mechanisms

that allow regulating exosome presence in the plaque lesion (reviewed by (39)). In this context, EVs affect different biological pathways involved in atherosclerosis such as inflammation, endothelial dysfunction, thrombosis, neovascularization and calcification.

A. EVs and atherosclerotic type I/ II and III lesions

In the earliest stage of the atherosclerosis, lesion is classified as type I/ II and type III and is characterized by a near-normal wall thickness and diffuse intimal thickening without calcification (145). EVs are related with some processes in early atherosclerotic lesion including endothelial dysfunction, inflammation, ROS production and monocyte-macrophage adhesion.

EVs: relationship with endothelial dysfunction: Under physiological conditions such as high shear stress or an increase in blood flow, there is an increase of NO \cdot release by endothelial cells (146) that negatively regulates endothelial ATP-binding cassette (ABC)A1 expression, limiting membrane remodeling and PS exposure, but also stabilizing actin at the membrane (Figure 6). Both mechanisms concur to limit MV release. However, under pathological conditions such as atherosclerosis, low shear stress increase endothelial ROCK and ERK1/2 activation. This is associated with decreased eNOS, resulting in the loss of the NO-mediated repression of ABCA1, leading to membrane remodeling and MV release (25) (Figure 6).

Oxidative theory of atherosclerosis includes a primary accumulation of LDL levels and their oxidation (ox-LDL) in the sub-endothelial matrix (147) leading to endothelial dysfunction, monocyte-macrophage adhesion (148), ROS production (149), and secretion of pro-inflammatory cytokines and growth factors (150). Endothelial permeability is increased when hemodynamic forces and endothelial shear stress are disturbed, allowing LDL diffusion through endothelial cell junctions (14, 151). Then, LDL undergoes several modifications, including oxidation, lipolysis, proteolysis, or aggregation. Some evidence suggests that MVs derived from endothelial cells, platelets and leukocytes might contribute to endothelial

dysfunction (reviewed by (7)) (Figure 7). It has been shown that MVs derived from metabolic syndrome patients, but not from healthy individuals, induce endothelial dysfunction, leading to a reduction of both NO \cdot and ROS production. It is associated with a decrease of eNOS activity due to an increase of eNOS phosphorylation at the inhibitory site (Thr495), in human cultured endothelial cells (152). In fact, a temporal cross talk between endoplasmic reticulum and mitochondrial takes place in human endothelial cells treated with the MVs from metabolic syndrome patients. In that way, MVs increase cytosolic and mitochondrial ROS contributing to decrease bioavailability of NO \cdot . Likewise, MVs activate the three canonical unfolded protein response pathways involving PERK and eIF2a activation, XBP1 splicing and nuclear ATF6 translocation human aortic endothelial cells (153). Additionally, MVs from metabolic syndrome patients are able to activate nSMase, which has been directly related to endothelial dysfunction. Inhibition of nSMase strongly blocks MV-induced endoplasmic reticulum stress and improves both eNOS activity and NO \cdot production. In addition, neutralization of Fas-ligand, carried by MVs, abolishes the effects induced by both MVs types (153). Moreover, in diabetic mimic conditions, endothelial-derived MVs leads to an impairment of endothelial-dependent relaxation evoked by acetylcholine, in aortic rings from ApoE $^{-/-}$ mice. Indeed, these MVs possess a pro-oxidant role triggering ROS production in endothelial cells and the subsequent activation of p38. A specific inhibition of p38 prevents the effects mediated by MVs (12). Finally, the interaction between ROS and NO \cdot sets up a feedback loop which results in further endothelial dysfunction and inflammation.

EVs: Inflammation and monocyte-macrophage adhesion (Figure 7): Animal and *in vitro* studies have suggested that oxidative stress and hypercholesterolemia might lead to both monocyte activation and enhanced accumulation of ox-LDL, which induce endothelial dysfunction (154). Ox-LDL induces the release of MVs from pro-inflammatory monocytes and up-regulation of CD36, chemokine receptors, as well as, pro-inflammatory factors

through CD36-dependent pathways. Likewise, it has been described an association between atherosclerosis progress in subjects with heterozygous familial hypercholesterolemia and their circulating levels of MVs derived from monocytes. It suggests a link between lipoprotein-associated oxidative stress, monocyte-macrophage activation and MV release that might reflect some of the underlying mechanisms involved in the atherosclerotic process (154).

In vitro experiments show that MVs can promote adhesion of monocytes to the endothelium, a crucial step for subsequent leukocyte diapedesis. MVs derived from endothelial cells, platelets or leukocytes can promote monocytes rolling and adhesion as well as the release of pro-inflammatory cytokines (reviewed by (7)). Injection of circulating MVs from metabolic syndrome patients into mice induces pro-inflammatory cytokine production in aorta, mainly IL-6 and monocyte chemoattractant protein-1 (35). Circulating MVs from obstructive sleep apnea syndrome patients increase the expression of pro-inflammatory proteins such as E-selectin, integrin alpha5 and ICAM-1 in human endothelial cells. This may initiate atherogenic processes in these patients (155). Likewise, injection of MVs derived from endothelial cells from human coronary arteries increases monocyte and macrophage infiltration in atherosclerotic lesions in ApoE^{-/-} mice. It is associated to an enhancement of the adhesion protein expression, ICAM-1 and VCAM-1, in blood vessels (12). MVs isolated from human atherosclerotic plaques enhance inflammatory responses by favoring the adhesion of monocytes to endothelial cells via the transfer of ICAM-1 molecules leading to atherosclerotic plaque progression (14). Indeed, ICAM-1 expression on EVs isolated from patients with coronary heart disease is remarkably higher than in healthy controls (156).

EVs: SMC migration and proliferation (Figure 7): The proliferation and migration of SMCs from the tunica media to the sub-endothelial region lead to vessel thickening and occlusion (150) playing a crucial role in vascular remodeling. Under physiologic conditions, intercellular signals between endothelial cells and SMCs are required for the upkeep of the

vascular function and remodeling. In fact, EVs can transfer miR-143/145 from endothelial cells to SMCs. This is regulated by endothelial Krüppel-like factor (KLF)2 expression which leads to an enhanced repression of miR-143/145 target genes and de-differentiation-associated gene expression (157). In addition, it has been suggested that miR-146a and KLF4 present a feedback loop to regulate each other expression and SMC proliferation. miR-146a promotes SMC proliferation and migration by targeting KLF4 and regulating the miR-146a promoter. Finally, transfection of antisense miR-146a oligonucleotide into balloon-injured rat carotid arteries markedly decreases neointimal hyperplasia (158).

It has been shown that MVs derived from high glucose-treated endothelial cells transfer miR-503 to pericytes and, subsequently, inhibit VEGF expression, resulting in impaired pericyte migration and proliferation (159). MVs from TNF- α -treated endothelial cell carry out miR-146a-5p which plays an important role in modulating SMC function, increasing the proliferation and migration of these cells. miR-146a-5p has been associated with the activation of the Mek1/2/ERK1/2 pathway and caspase-3/Bcl-2 regulation (160). Furthermore, foam cell-derived EVs from atherosclerotic patients could promote SMC migration and adhesion. These may be mediated by regulating the actin cytoskeleton and focal adhesion pathways after the integration of EVs into SMCs and the subsequent downstream activation of ERK and Akt (161). Altogether these results indicate that EVs from different origins (endothelial cells or foam cells) can alter SMC function by increasing their proliferation, suggesting that EVs can be active mediators in the development of the neointima during atherosclerosis.

B. EVs and atherosclerotic type IV to VIII lesions

In advanced atherosclerotic stage, type IV/V lesions are characterized by the accumulation of lipids and the formation of the lipid or necrotic core. Type VI lesion is a complex plaque with possible surface defect, hemorrhages or thrombus. Finally, type VII lesion is a calcified

plaque and type VIII is a fibrotic plaque (145, 162). As a consequence of all these processes, including chronic inflammatory response, plaque grows into confluent necrotic cores characterized by a great infiltration of macrophages and apoptosis of foam cells and SMCs.

EVs: Foam cells and “fatty streaks”, extracellular matrix synthesis and necrotic cores: Several findings indicate EVs are implicated in the advanced stages of atherosclerosis but their effect on LDL sub-endothelial modification has not been directly evaluated. LDL and EVs have been implicated in CVD though their relationship has not been well established. Recently, it has been reported that circulating EV levels increase significantly after a high-fat meal in healthy individuals. Even more important, it has been shown that these EVs (after identification by annexin V and anti-CD41 for MVs or anti-CD9 and anti-CD63 for exosomes), are enriched with high quantities of apoB lipoproteins, and low amounts of apoCII and ApoE. These results highlight that both types of circulating EVs, MVs and exosomes, might contain significant amount of lipoproteins related with LDL (38). MVs derived from human coronary endothelial cells exposed to high glucose concentrations induce atherosclerotic plaque formation in ApoE^{-/-} mice after 8-weeks of treatment. This lesion was characterized by a larger necrotic core as a marker for plaque instability (12). In human carotid plaque, MVs derived from activated or apoptotic leukocytes are mostly concentrated as the result of decrease macrophage phagocytic activity in lesion (163).

Neovascularization of the plaque: As atherosclerosis progresses, the intima thickens and oxygen diffusion is impaired which favors the neovascularization of the plaque (164). Molecular mechanisms responsible for angiogenesis are predominantly related to hypoxia, inflammation and activation of the Toll-like receptor (165). Indeed, that accumulation of EVs within the atherosclerotic plaque may represent an endogenous signal of neovascularization and vulnerability. Even more, MVs isolated from human atherosclerotic lesions, which

express CD40L, induce endothelial cell proliferation and promote *in vivo* angiogenesis, through mechanism involving VEGF and PI3K/Akt (166).

Formation of complex plaque with possible surface defect, hemorrhage or thrombus: The density of intra-plaque neo-vessels increase the risk of rupture and intra-plaque hemorrhage, which is the most frequent cause of thrombosis and cardiovascular complications (150). Circulating MVs might contribute to platelet deposition and thrombus formation under plaque ruptures, damage of vascular lumen or vascular stenosis. MVs are more abundant in atherosclerotic plaques than in plasma, playing an important role in the pro-coagulant activity of the lipid core. Among MVs, leukocytes-derived MVs are the major subset in patients with unstable carotid plaque compared with patients with stable plaque. MVs present have been associated with plaque vulnerability in patients with high-grade carotid stenosis (167). Interestingly, mildly-ox-HDL are able to antagonize agonist-induced platelet aggregation by inhibiting the release of pro-coagulant platelet-derived EVs. This may be the result of more efficient phospho- and sphingolipid platelet remodeling mediated by HDL binding to CD36 or scavenger receptor-B1 in the absence of ABCA1 on platelets (168).

The pro-coagulant effects of MVs and thrombus formation have been correlated with expressing PS, tissue factor, as well as other surface molecules. Moreover, von Willebrand factor expressed in endothelial-derived MVs binds to platelets and platelet-derived MVs to promote coagulation. Tissue factor on the surface of MVs can function as blood-borne tissue factor to initiate the clotting process (169). MVs exposing coagulant tissue factor have been reported in acute coronary syndromes (reviewed by (170)). Circulating MVs and specifically platelet-derived MVs, from patients with coronary artery disease, enhance platelet aggregation, coagulation, and thrombosis on atherosclerotic and damaged vessels (171). Actually, platelet-derived MVs induce an increase in platelet and fibrin deposition on human atherosclerotic plaques (171). In contrast, only low numbers of platelet-derived exosomes

expose a negatively charged lipid such as PS suggesting that exomes do not display a coagulant activity (172).

Plaque calcification: Calcification of atherosclerotic plaque lesions is the latest stage of atherosclerosis. Vascular calcification is a predictor of CVD and a major determinant of atherosclerotic plaque stability (8). EVs have been identified in calcified human aortic valves and medial arterial calcifications, as well as in atherosclerotic intimal plaques. Emerging evidence suggests that vascular wall cells, including SMCs and macrophages, release calcifying EVs. They are susceptible to aggregate in the extracellular matrix and to form the calcific plaque (173) suggesting that MVs may initiate the mineralization processes, and may induce the rupture of vulnerable plaques. EVs released by SMCs and macrophages in the vessel wall interact with fibrillar collagen, which might lead to the calcification. Mineralization of SMCs generates MVs, which enhance calcification *in vitro*. Furthermore, mineralization in SMCs requires active MVs and an interaction of the MVs with type I collagen (174). Additionally, during the early stages of calcification, MVs released from macrophages and SMCs may contribute to the calcification process. *In vitro* and *in vivo* studies have proposed the concept of an inflammation-dependent calcification paradigm. It suggests that macrophage infiltration and inflammation proceed to the calcification. Activated pro-inflammatory pathways induce osteogenic transformation of SMCs and the release of MVs from macrophages and SMCs (reviewed by (175)).

SMC-derived EVs enriched in alkaline phosphatase, members of the annexin family, and MMP-2 play a role in the process of matrix calcification, releasing required substrates and forming intermediates known to trigger calcium phosphate precipitation (22). In fact, annexins 2, -5, and -6 in MVs, might contribute to the formation of nucleation complex. They facilitate calcium influx and mineralization by binding to PS and forming ion channels in the MV membrane (175). On the other hand, collagen receptor discoidin domain receptor-1

(DDR-1) regulates collagen deposition and release of calcifying EVs by SMCs through the TGF- β pathway. DDR-1 is a connection between SMC-induced fibrosis and EV-mediated calcification in early-stage atherosclerotic plaque formation. By restricting TGF- β 1 release in SMCs, DDR-1 suppresses phosphorylation of pro-atherogenic p38 and increases phospho-Smad3, resulting in attenuated fibrosis and calcifying EV release (176). Finally, miRNAs 30, 125-b, 143, 145 and 155 influence the expression of a specific set of osteogenic markers such as Smad1, RUNX-2, ALP and osterix and altered concentrations of these miRNAs in EVs result in changes in calcium and MAPK signaling pathways implicated in SMC-mediated calcification (177, 178) (Figure 8).

VII. NEURODEGENERATIVE DISEASES and EVs

The central nervous system (CNS) is made of neurons and glial cells comprising astrocytes, oligodendrocytes, microglia and ependymal cells. All these cells are able to release vesicles into the extracellular space and recent evidences indicate that cells utilize EVs to exchange signals with neighboring cells or to dispose of unwanted proteins. Thus, EVs provide in the CNS an important mode in intercellular communication in particular in neuron-glia communication but are, also more and more, implicated in the progression of neurodegenerative diseases associated with misfolded protein.

A. CHARACTERISTICS OF EVs FROM CNS (Figure 9)

Exosomes from neuronal cells: Release of EVs from neurons with the characteristics of exosomes was first demonstrated in 2006 by Fauré and colleagues (179) using cultured cortical neurons from rat embryos. This exosomal release is regulated by glutamatergic synaptic activity and exosomes contain the cell adhesion molecule L1, the glycoposphatidylinositol (GPI)-anchored prion protein and AMPA-type glutamate receptor subunits GluR2/3 (180). Further studies have shown that neuronal exosomes may also contain proteins associated with neurodegenerative disorders. Following treatments with alkalizing

drugs, exosomes from primary neuronal culture cells and differentiated neuroblastoma contain amyloid precursor protein (APP), which metabolism is central to the pathogenesis of Alzheimer disease, as well its derivatives, the amyloid intracellular domain and the APP C-terminal fragments (APP-CTFs) (181). Neurons have also the capacity to release exosomes with possible neuroprotective effects. Cystatin C, a protein implicated in the processes of neuronal repair of the nervous system (182) is secreted by neurons in association with exosomes (183). Nedd4 family-interacting protein, an adaptor protein required for the secretion of Nedd4, an ubiquitin ligase important for protein degradation, is detectable in exosomes secreted from primary neurons (184).

Through neuronal exosomes secretion, miR-124a can be transferred into astrocytes where it significantly increases the expression of glutamate transporter GLT1, an essential transporter for synaptic modulation (185). Exosomes are also implicated in the miRNA release from depolarized neurons (186). By example, miR-29, miR-99a and miR-125a are increased and functionally active in the synaptosome supernatant after depolarization in a calcium-dependent manner (187). Since release of exosomes from neurons can be modulated by synaptic activity, exosomes can play a key role in synaptic function and in interneuronal communication.

Exosomes from glial cells: In the absence of neurons, primary cultures of oligodendrocytes release exosomes which, in addition to cholesterol and phosphatidylcholine, are enriched in the classic myelin lipids such as galactosylceramide and sulfatide (188). Oligodendroglial exosomes are also characterized by the presence of major myelin proteins; although the relative abundance does not exactly reflect the stoichiometry of the myelin membrane as myelin basic protein, PLP, myelin oligodendrocyte glycoprotein and 2', 3'-cyclic nucleotide 3'-phosphodiesterase. Moreover, a proteomic analysis of these exosomes identifies a plethora of chaperones and enzymes involved in the management of the oxidative stress (188).

Astrocytes, the most abundant glial cells, are housekeeper of the CNS providing variety of supportive functions to neurons (189). Astrocytes secrete exosomes that contain functional excitatory amino-acid transporters, EAAT-1 and EAAT2 that are vital for neurotransmission. Importantly, the enrichment of EAAT-1, and probably also EAAT2, into exosomes is under the positive control of PKC (190). Interestingly, mtDNA (191) and miRNAs (192) have also been found in these exosomes. Exosomes are also released from astrocytes in response to oxidative stress. Cultured astrocytes subjected to hyperthermia release increasing amount of HSP70 associated with exosomes (193). Synapsin-I, an oligomannose binding protein, playing an important role in modulating nervous system development, is present in exosomes isolated from astrocyte-enriched cultures derived from cortex of neonatal mice and treated with hydrogen peroxide (194).

Microglia cells are tissue-resident macrophages in the CNS that play an important role in managing synaptic development and connectivity besides their function of primary mediators of the CNS immune defense system. Exosomes isolated from culture supernatant of N9, a well characterized-murine microglial cell line, are rich in chaperones, tetraspanins or membrane receptors previously reported in B cells and dendritic cell-derived exosomes, the lactate transporter MCT1 and the enkephalin-degrading- aminopeptidase CD13 (195).

MVs from brain cells: Besides exosomes, both neurons (196) and astrocytes (197) release shed MVs that contain fibroblast growth factor-2 and VEGF. Recent evidence also demonstrates that glial cells release MVs upon appropriate stimulation. For example, activation of astrocytes via the activation of the ATP receptor P2X₇ results in an increase release of MVs containing the proinflammatory cytokine IL-1beta (198). The signaling pathway downstream of P2X₇ receptor involves p38-dependent activation of acid SMase and is accompanied by the translocation of the enzyme to the outer membrane leaflet. This results in sphingomyelin breakdown and ceramide generation that facilitate membrane blebbing.

Upon stimulation by ATP, the N9 murine microglial cell release MVs containing IL-1beta (199). In both cases, the MV formation seems to be the predominant mechanism of IL-1beta secretion in glial cells.

B. FUNCTIONAL ROLES of EVs in CNS

A reciprocal communication between the different classes of brain cells is determinant in the development and physiology of human nervous system. In recent years, EVs have emerged as a novel and critical form of communication in the nervous system (reviewed in (200-202)). However, most studies evaluating the EV function are performed in cell cultures and need *in vivo* confirmation. An unresolved question indeed relates to the role of the recipient cells in a possible selection of the receiving cargo. Moreover, the majority of these studies focused on exosomes. In this part, we summarize the possible physiological roles of EVs in the CNS.

EVs from neuronal cells: The basal level of exosomes released from mature cortical neuron is very low. In contrast, treatment of neurons with antagonists of GABAA receptor, which indirectly activates glutamatergic synapses, rapidly and massively increases the secretion of exosomes. The addition of antagonists of glutamate receptors AMPA or ADMA inhibits this increase confirming that glutamatergic synapse activation triggers exosomal release (180). These exosomes contain AMPA-type glutamate receptor subunits GluR2/3 and the loss of AMPA receptors upon extensive synaptic activation could be a mean to regulate excitability by controlling the amount of post synaptic glutamate receptors. In addition, a recent work reports that exosome released upon synaptic activation are specifically transferred to neurons (203). By analyzing the binding of exosomes to target cells, these authors demonstrate that exosomes from neuroblastoma cells bind indiscriminately to neurons and glial cells, whereas exosomes secreted from stimulated cortical neurons bind neurons only.

During depolarization, neurons also release exosomes enriched with functionally specific miRNAs and the synaptic-plasticity-associated protein MAP1b (186). As depolarization is

associated with a neurite restricted decrease in miRNA, it is plausible that the exosomal miRNA may be an efficient way for cells to rapidly eliminate miRNA activity. Interestingly, Konopka and colleagues (204) recently report that miRNA depletion enhances learning and memory in mice. It is therefore tempting to speculate that miRNA exosomal release could be an important regulatory component to facilitate synaptic plasticity.

Additionally, exosomes released by neurons may also contribute to the function of the nervous system by modifying supporting cells such as astrocytes. For example, exosomal transfer of neuronal miR-124a is able to regulate the expression of astroglial glutamate transporter-1 (185). The mechanism of neuronal exosomal miRNA release is unclear and seems not directly linked to neuronal activity.

EVs from oligodendrocytes: Oligodendrocyte exosomes are able to functionally transfer their content to neurons. More in detail, the transfer of superoxide dismutase (SOD) and, catalase is associated with an increased resistance of neurons against oxidative stress and starvation (81) improving the cellular viability. Oligodendrocyte exosomes may also promote physiological responses in neurons. After application of exosomes, analysis of neuronal electrical activity reveals an increase in the firing rate of neurons (205). Moreover, treatment of cultured neurons with oligodendroglial exosomes activates pro-survival activating pathways such as Akt and ERK and to influence neuronal gene expression (205). In addition, oligodendroglial exosomes can act in an autocrine fashion to inhibit myelin membrane sheath formation (206). The signal responsible for the exosome release is unknown but it may induce change in intracellular calcium level (188). These exosomes appear to control the growth properties of the cell and the myelin formation (206). These results support the idea that neurons may control terminal differentiation of oligodendrocytes by regulating the release of autoinhibitory exosomes from oligodendrocytes.

Finally, it has been shown that oligodendrocyte exosomes are specifically and efficiently taken by microglia both *in vitro* and *in vivo* (84). By such a mechanism, microglia participate in the degradation of excess myelin component secreted by oligodendrocyte exosomes in an immunologically “silent” manner.

EVs from astrocytes: Astrocyte-derived MVs carry factors as growth factor 2 and VEGF, known to contribute to regulate angiogenesis and neurogenesis. Astrocytes also release exosomes containing synapsin-I, a lectin like protein that promotes neurite outgrowth and neuronal survival (194). In the presence of high KCl concentration (75-80 mM) similar to recorded during propagation of spreading depression waves, synapsin-I is released from exosomes. Thus, under condition of high neuronal activity, synapsin-I may play a neuroprotective role by modulating the interaction between neurons and glia.

EVs from microglia cells: Some recent studies suggest that MVs from microglia can physiologically influence synaptic activity. Application of MVs generated either from microglia cell culture or from the N9 microglia cell line on 14-day-old cultured hippocampal neurons enhances spontaneous and evokes excitatory transmission (207). MVs mainly act at the presynaptic site by increasing the probability of synaptic vesicle release. Both SMase and ceramidase activities are required for the presynaptic action of MVs indicating that sphingosine production from sphingomyelin plays a crucial role in the effect. Consistent with this finding, it is reported that sphingosine, but not ceramide, can enhance vesicle exocytosis in brain synaptosomes by activating vesicle-associated membrane protein-2 to form SNARE complex assembly (208). However, additional studies are warranted to better understand the signaling pathway linking MVs to sphingolipid metabolism in neurons.

Microglia-derived MVs are also identified as an additional vehicle for transport of lipophilic compounds in the brain like endocannabinoids (209). Endocannabinoids are synthesized in the post synaptic compartment of neurons and retrogradely act to inhibit GABA (inhibitory

neurotransmitter) or glutamate (excitatory neurotransmitter) release. Vesicular endocannabinoids are biologically active and exposure of hippocampal neurons to microglia-derived MVs induces a significant decrease of miniature inhibitory post synaptic currents mimicking the effect of an agonist of the presynaptic type-1 cannabinoid receptor (CB1). Thus, MVs are able to stimulate CB1 and to inhibit presynaptic transmission in GABAergic neurons. However, the suppression of GABAergic tone does not contribute to the increase excitatory transmission elicited by MVs and described above (209). Collectively, these data suggest that microglia MVs may be involved in the modulation of the excitation/inhibition balance.

Microglia also releases exosomes that contain the lactate transporter MCT1. Since exosomes contain all the enzymes necessary for glycolysis and lactate production, lactate release through MCT1 could function as an auxiliary source of energy for neurons. Microglial exosomes also display CD13, an aminopeptidase responsible for enkephalin cleavage. Neurons express CD13 on the presynaptic membrane, but the presence of active CD13 in such a mobile particle could be physiologically advantageous to control the catabolism of enkephalins released at distance from synapses (195).

C. EVs as BIOMARKERS of NEURODEGENERATIVE DISEASES

For tissues not accessible to direct examination such as the CNS, analysis of EVs released in the blood or in the CSF by neural and non-neural cells has been proposed as a promising diagnostic/prognostic markers for neurological disorders (for review (210)). In cerebrovascular disorders, it is suggested that endothelial- or platelet-derived MVs could represent one useful marker to stratify and identify patients in which initial thrombolysis treatment of the acute phase of ischemic stroke may not be indicated (211). In the diagnosis and treatment of inflammatory diseases of CNS, analysis of exosomes in the CSF may also help diagnostic. For instance, it has recently reported that exosomal proteome analysis of CSF

detects distinct biosignatures of neuromyelitis optica and multiple sclerosis (212), two disorders that are often indistinguishable by their clinical features (213). Reliable markers are also urgent needed to improve the diagnosis of AD and Parkinson's disease (PD) before the appearance of clinical symptoms (214). In CSF, Tau phosphorylated at Thr-181, an established biomarker for AD, has been shown to be significantly enriched relative to total Tau in exosomal fraction in the early stages of AD (215). Circulating exosomes contain proteins such as cathepsin D, LAMP-1, the levels of which are significantly higher in preclinical AD, ten years before diagnosis (216). To improve the specificity of exosome analysis in these diseases, isolation methodologies are developed to obtain plasma EVs enriched for neuronal origin (217). By analyzing such enriched exosomes, it would be possible to identify AD patients at a preclinical state, to predict the conversion from mild cognitive impairment to dementia (218) or to differentiate AD from frontotemporal dementia (219). Using the same approach, plasma exosomes containing elevated levels of α -synuclein are detected in PD patients when compared to healthy subjects (220). Expression of miRNA in exosomes might be useful for the diagnosis of PD (221) and AD (222). However, further works is still required to make exosome utilization a reality in the clinics. In particular, the yield isolation of exosomes crossing the blood brain barrier from brain into circulation needs to be improved.

D. IMPLICATION of EVs in NEUROLOGICAL DISORDERS

The involvement of EVs in neurological disorders including neurodegenerative diseases brain cancer, neuro inflammation has drawn particular attention in recent years. Indeed, it was proposed that exosomes might participate to the progression and the dissemination of the diseases. In particular, proteins associated with neurodegenerative diseases such as AD, PD and amyotrophic lateral sclerosis (ALS) have been shown to be released by cells in association with EVs.

Prion disease: Prions are infectious agents that are responsible for transmissible neurodegenerative diseases such as Creutzfeldt-Jakob disease of humans or bovine spongiform encephalopathy of cattle. The infectious particle is the abnormal protein isoform (PrP^{Sc}) of the host encoded cellular prion protein (PrP). Prion diseases are caused by misfolded forms of PrP that can induce the misfolded state in preexisting normally folded protein of the same type. Misfolded proteins trigger a chain reaction of refolding and self-aggregation, spreading the pathophysiological status to other cells and resulting in a formation of stable protein aggregates with a fibrillary amyloid-like structure (223). The precise mechanisms by which prions are transmitted from cell to cell remain to be fully elucidated. Fevrier and colleagues (224) suggest that exosomes may contribute to intercellular membrane exchange and the spread of prions. Through the analysis of two distinct cellular models expressing ovine PrP (rabbit epithelial cells and mice neuroglial cells), they found that normal PrP and PrP^{Sc} are released by cells in association with exosomes and that intracerebral inoculation of these exosomes causes clinical disease in mice. Vella and colleagues (225) have provided the first evidence on the transmission of exosome-associated PrP^{Sc} to heterologous cell types in addition to homologous cell types. Recently, it has been demonstrated that the MVB is the major internal site of prion conversion (226) and that stimulation of the release of exosomes increases the infectiousness of PrP^{Sc} (227). Moreover, PrP^{Sc} is detected in EVs obtained from plasma samples from preclinical and clinically sick mice (228). Together, these studies highlight the potential role of exosomes in prion transmission.

Alzheimer' disease (Figure 10): AD is characterized by the deposition in the brain of the 40-42 amino acid amyloid- β peptide ($A\beta$) in extracellular plaques and of the microtubule-binding protein Tau in intracellular neurofibrillary tangles. $A\beta$ is produced via the sequential enzymatic cleavage of the amyloid precursor protein (APP) by two proteases, β and γ

secretases. HeLa cells expressing the Swedish mutant of APP and neuroblastoma cells N2a provide evidence that A β peptides are produced in the early endosome and are directed to MVBs and that a small portion of A β (<1%) is secreted into the extracellular medium bound to exosomes upon fusion of MVB with the plasma membrane (229). In addition, subsequent studies demonstrated that APP, APP metabolites and key enzymes that cleave full-length APP are secreted in exosomes using APP overexpressing cell lines (181, 230). Because exosomal markers such as ALIX and flotillins have been found to accumulate in amyloid plaques (231), it has been suggested that exosomes might participate in the pathogenesis of AD. Neurotoxicity of exosomes in AD is consistent with recent evidences indicating that prevention of neuronal exosome secretion is associated with lower amyloid plaque load (232) and improvement of cognition in the 5XFAD mouse model of AD (233). In these studies, the reduction of exosomes secretion is obtained by pharmacological inhibition of nSMase2, a critical enzyme for exosome biogenesis or by generating genetically nSMase2-deficient mice. However, the exact role of exosomes in AD remains controversial (234). Several studies have indeed reported that exosomes may also have a protective function. Yuyama and colleagues (235) initially demonstrated that exosomes released from neuroblastoma N2a can bind extracellular soluble A β , promoting A β fibril formation on their surface and incorporation in microglia for lysosomal degradation. In a more recent study, they show that exosomes isolated from primary neuron cultures have also the capability to capture A β and, clearly provide evidence that, intracerebral infusion of these exosomes can lead to decrease A β accumulation in the brains of APP transgenic mice (236). Thus, the binding of A β to neuronal exosome might be a way to increase A β clearance by microglia leading them to hypothesize that the decrease in exosome secretion observed in aging could contribute to the plaque formation (236). Furthermore, An and colleagues (237) have demonstrated that, in rats, the intracerebroventricularly infusion of exosomes from N2a cells or healthy CSF can prevent the

synaptic plasticity disruption caused by injection of A β derived from AD brain extracts. Mechanistically, the sequestration of A β and mostly the generation of fibrillar A β at the exosomal surface may explain the protective function attributed to exosomes by reducing the level of the most neurotoxic species of A β , the oligomeric forms of A β (A β O) (237, 238). Interestingly, it has been reported that PrPc associated with exosomes from neuronal cell lines (N2a and SH-SY5Y) accelerates fibrillization of A β (238) suggesting a protective role of exosomal PrPc in AD. Nevertheless, it has recently demonstrated that MVs released by cultured microglia strongly increase A β neurotoxicity *in vitro* by promoting the formation of soluble toxic forms of A β from extracellular insoluble aggregates and that microglial MVs also contain toxic forms generated from internalized A β (234). Taken together, these results indicate that the action of EVs on AD may vary between vesicle populations and support the concept that the EVs may have a “double-edged sword effect” on the process of AD depending on the cell origins, the relative abundance of EVs, and the condition of their formation (239).

Intracellular neurofibrillary tangles consisting of hyperphosphorylated Tau are the second major hallmarks of AD. Exosomes are proposed to be a vehicle for spreading Tau (Figure 11). Saman and colleagues (215) first reported that Tau can be exported via an exosome-mediated mechanism in the M1C neuroblastoma tauopathy model. Moreover, it has been reported that exosomes released by cultured primary neurons or by N2a cells overexpressing different Tau constructs contribute to the transmission of Tau from neuron to neuron via a trans-synaptic pathway (240). However, exosomes containing Tau or phosphorylated Tau have been found to be released by astrocytes (241) and microglia (242), suggesting also the possibility of an exo-synaptic propagation of Tau. By using an adeno-associated virus based model exhibiting rapid Tau propagation, it has been observed that depletion of microglia suppresses the propagation of Tau (242). Since microglia spread Tau via exosomes secretion, it has been

proposed that microglia phagocytose neuron-contained Tau and then, secrete Tau in exosomes which, in turn, efficiently transmit Tau to neurons.

Parkinson's disease: PD is a neurodegenerative disease clinically characterized by tremor, bradykinesia and postural instability in which the aggregation of protein α -synuclein has been found to be central in the progression of the disease. Analysis of the distribution pattern of α -synuclein aggregates have led to the deduction that PD may start in either the olfactory bulbs or the enteric nervous system before spreading to the midbrain and neocortex (243). Using an inducible α -synuclein SH-SY5Y cell line, Emmanouilidou's group (244) have shown that α -synuclein can be exported of the cells via an exosomal pathway. This finding, confirmed by others (for review (245)), suggests that exosomes might play a role in the transmission of PD. Although exosomal α -synuclein only represents a small pool of the total extracellular α -synuclein, several studies have highlighted the importance of this fraction to provide a platform for the propagation of PD (246) and to catalyze the aggregation of free α -synuclein (247). Moreover, it has been observed that, *in vitro*, decreasing lysosomal activity promoted the exosome formation (248). Interestingly, impairment of the autophagy-lysosome pathway is a hallmark of sporadic and genetics forms of PD suggesting that *in vivo* exosome release may be increased in PD. Thus, exosome is the most studied mode of α -synuclein spread (249).

Amyotrophic lateral sclerosis: ALS is a fatal neurodegenerative disease characterized by the progressive loss of motor neurons in the brain, brainstem and spinal cord. SOD1 and TDP-43 are two proteins mutated in the familial forms of ALS and, both of them have been found associated with exosomes suggesting that exosomes could propagate the proliferation of misfolded SOD1 (250) and TDP43 (251). By example, when human TDP-43-transfected mouse neuroblastoma Neuro2a cells are exposed to exosomes from ALS, but not for healthy brain, TDP-43 redistribute in the cytoplasm, suggesting that TDP-43 may propagate via exosomes. However, inhibition of exosomes secretion using GW4869, an inhibitor of

nSMase2, provokes formation of TDP-43 aggregates in Neuro2a cells and exacerbates pathological defects in TDP-43A315T transgenic mice (251). Thus, it seems that in the transgenic mouse model, exosome secretion may also protect the cells by eliminating unwanted protein products.

VIII. EVs AND CANCER

Tumor cells as well as tumor microenvironment cells are prone to produce EVs, that are involved in both tumorigenesis and cancer progression. More in details, exosomes derived from cancer cells are able to transfer pathophysiological information to normal “health” cells favoring their transformation in malignant cells. Also, cells from tumor microenvironment generate EVs which increase angiogenesis in order to induce new blood vessel generation necessary to supply oxygen and nutrients facilitating growth of cancer cells. Also, EV levels can be used as biomarkers of cancer progression and efficacy of anti-tumoral treatments. Due to the huge number of data concerning EVs on cancer, in this part of this review, we will analyze some works showing the potential role of EVs (i) as biomarkers of cancer progression and (ii) as shuttles of information involved in growth of tumors.

A. EVs as biomarkers for cancer diagnosis and prognosis

It has been reported elevated levels of EVs in different biofluids from patients with either solid or non-solid tumors (for review see (252, 253)) (Figure 12). In particular, elevated levels of CD41⁺ MVs from platelets, and CD14⁺ and CD62P⁺ MVs from monocytes and activated platelets have been detected in patients with gastric (254) and lung cancer (255), respectively. Notably, plasma levels of CD41⁺ MVs were markedly correlated with the advanced stage of gastric cancer and with the prediction of distant metastasis (254). Also, levels of glioblastoma-derived exosomes expressing epidermal growth factor receptor (EGFR)vIII mRNA are correlated with the severity of this cancer (256) and, interestingly, can predict the efficacy of *in vivo* treatment with the alkylating agent, temozolomide (257). Analysis of the

subsets of circulating MVs in patients with advanced non-small cell lung cancer (NSCLC) has shown that circulating levels of MVs from platelets (CD31⁺CD42b⁺) and endothelial cells (CD31⁺CD42b⁻) are significantly higher in NSCLC patients than in control subjects, and returned to control levels at 3 months after surgical intervention (258). However, only CD31⁺CD42b⁻Annexin V⁻ MVs from activated endothelial cells are significantly correlated to liver metastasis, disease progression after complete course of first-line treatment, and one-year mortality suggesting that this MP subset may be used a biomarker predictive of one-year mortality in end stage-NSCLC patients (259). By a proteomic study performed on urinary exosomes obtained from renal cell carcinoma patients, Raimondo and colleagues (260) have identified 9 overexpressed proteins including MMP-9, ceruloplasmin, podocalyxin, Dickkopf related protein 4 and carbonic anhydrase IX and 5 down-expressed proteins (aquaporin-1, MMP inducer, neprilysin, dipeptidase 1 and syntenin-1). However, further studies are needed in order to establish whether the urinary levels of these markers are modified depending on the patient's outcome.

Concerning the non-solid tumors, the characterization of EVs from peripheral blood of patients with hematological malignancies highlights the fact that markers of EVs are specific for each pathology. Indeed, high levels of CD19⁺ EVs were detected in B cell neoplasms, CD38⁺ EVs in multiple myeloma (MM), CD13⁺ EVs in myeloid tumors, and CD30⁺ EVs in Hodgkin's lymphoma (261). In agreement with this data, other authors have described comparable results in patients or in animal models (262-265). The study of miRNA carried by EVs in the prognosis of cancer is promising. By analyzing the profile of miRNA into exosomes from normal and MM-derived exosomes, Roccaro and colleagues (266) have shown that levels of miR-15a are significantly decreased in MM-derived exosomes, suggestive of a tumor-suppressive role of exosome-associated miR-15a. Also, two miRNAs, let-7b and miR-18a, in circulating exosomes can predict progression-free survival and overall

survival in patients with newly diagnosed MM (267). Also, in patients with NSCLC, it has been proposed that plasma exosomes rich in miR-21 and miR-4257 could be potential biomarkers to predict tumor recurrence (268). Remarkably, miR-21 is associated with tumor size and tumor-node-metastasis stage, while miR-4257 shows an association with histological subtypes, lymphatic invasion and tumor-node-metastasis stage. Moreover, in patients receiving curative resection, high exosomal miR-21 or miR-4257 levels are correlated with lower disease-free survival rates than those with low exosomal miR-4257 levels. This suggests that plasma exosomal miR-21 and mir-4257 expression has potential as a predictive biomarker for recurrence in NSCLC patients. miR-21 associated to exosomes is also a useful biomarker for the prediction of recurrence and poor prognosis in colorectal cancer patients with tumor-node-metastasis stage II, III, or IV (269). In another study, it has been shown that, in colon cancer patients developing liver metastases, expression of miR-328 into exosomes is greater in plasma from mesenteric veins than in peripheral veins indicating a possible role of miR-328 in the development of liver metastases (270).

Finally, the analysis of the 36 most abundant lipid species in urinary exosomes from prostate cancer patients reveals that nine lipids species are different to those of healthy patients. Whereas levels of PS and phosphatidylethanolamine are decreased in exosomes from prostate cancer patients, phosphatidylcholine, hexosylceramide and lactosylceramide are enhanced. These data show the potential of specific molecular lipid species in urinary exosomes as biomarkers for prostate cancer (73). Altogether, these data suggest that detection of specific proteins, miRNAs or even lipids in EVs can be used as non-invasive predictor methods of cancer outcome or response to therapy (253).

B. EVs as shuttle of information involved in growth of tumors

Implication of EVs on malignant transformation of cells: Choi and colleagues (271) have compiled information concerning the mechanisms by which EVs can modulate oncogenic

transformation. Indeed, EVs from solid or non-solid tumors carry transforming molecules including oncoproteins (such as RAS, EGFR, Akt, TAX), oncogenic RNA and miRNA or even oncogenic DNA sequences. For instance, adult T-cell leukemia/lymphoma cells release exosomes containing TAX, an oncoprotein able to activate the expression of viral and cellular genes involved in several pathways including the permanent activation of the NF- κ B pathway (272). When delivered to MSCs, these exosomes induce phenotypic changes favoring cell proliferation and creating a more favorable environment for leukemia (273). Also, exosomes from prostate cancer cells overexpressing both miR-125b and miR-130b, involved in the down-regulation of the tumor repressors, reprogram adipose stem cells from cancer patients by conferring genetic instability and oncogenic transformation, and reproducing aggressive tumors in mice (274) (Figure 13). Other authors have shown that after injection of exosomes carrying BCR/ABL DNA and generated from human erythroleukemic cells K562, mice display symptoms characteristic of chronic myeloid leukemia (275). Moreover, they have detected both BCR/ABL mRNA and protein into mouse neutrophils. Interestingly, these effects are directly associated with the transfer of BCR/ABL DNA since inhibition of de novo synthesis of BCR/ABL protein prevents the abnormalities caused by K562 EVs in mice. In patients with B-cell chronic lymphocytic leukemia, it has been shown that plasma MVs, mainly from platelets and B cells, not only activate the Akt/mTOR/p70S6K/HIF-1 α axis in bone marrow stromal cells but also transfer, into these cells, the protein kinase receptor Axl in its phosphorylated form (276).

The ability of EVs to transform target cells is largely dependent of the susceptibility to EV uptake. Lee and colleagues (277) have shown that several biological barriers protect normal cells from horizontal transformation by extracellular trafficking of the H-ras oncogene. Indeed, epithelial cells and astrocytes are resistant to EV uptake, whereas primary and immortalized fibroblasts are susceptible to the EV uptake, retention of H-ras DNA and

phenotypic transformation. Notably, uptake of H-ras-containing EVs stimulates but fails to transform primary endothelial cells. Altogether these data suggest EVs derived from cancer cells contribute to cellular transformation, however, the horizontal transfer of oncogenic information through EVs is limited depending on target cells.

Implication of EVs on proliferation of tumor cells: In MM, exosomes isolated from bone marrow-derived MSCs induce *in vivo* tumor growth in mice and promote dissemination of tumor cells and metastasis to distant bone marrow niches (266). Analysis of miRNA content indicates that miR-15a is down-modulated in exosomes isolated from bone marrow-derived MSCs, being confirmed the role of miR-15 in MM progression by loss- and gain- of function studies. Indeed, when miR-15a is depleted in primary normal bone marrow-derived MSCs, the generated exosomes display proliferative activity, and inversely, inhibition of miR-15a in exosomes from MM decreases their proliferative activity (266). Similarly, *in vitro* studies have shown that exosomes from leukemia stem cells promote proliferation and migration and inhibit apoptosis of acute myeloid leukemia cells; these effects are associated with miR-34a down-expression since when exosomes are enriched in miR-34a these effects are prevented (278).

Furthermore, exosomes and MVs from glioblastoma express chloride intracellular channel 1 (CLIC1), which has been involved in tumor growth (279). CLIC1-containing EVs stimulate *in vitro* glioblastoma cell proliferation and migration and *in vivo* tumor growth and, these effects are significantly decreased by treatment of glioblastoma cells with EVs derived from CLIC1-silenced cells. These findings suggest that CLIC1 carried by EVs may be a novel regulator of glioblastoma growth but the exact mechanism by which CLIC1 acts is not elucidated (279).

Through the transfer of receptors that platelet-derived MVs express at their surface, they are able to increase adhesion, proliferation, and survival of malignant cells by the activation of several kinase signaling cascades including MAPK p42/44, PI3K/Akt, and STAT proteins.

The biological effects of MVs derived from platelets are only partly reduced by heat inactivation or trypsin digest, indicating that proteins and lipids of MVs are responsible for their biological activity (280).

EVs from tumor cells are actively involved on tumor angiogenesis: Angiogenesis is a key step of solid tumor progression since unceasing growth of this type of tumor requires (i) large quantity of oxygen and nutrients, and (ii) removal of metabolite wastes. For this new formation of vessels, tumor and tumor environment cells release pro-angiogenic factors, mainly VEGF (for review see (281)).

EVs, mainly exosomes, from epithelial ovarian cancer cells are rich in CD147, an extracellular MMP inducer (Figure 13). This type of EVs promote an angiogenic phenotype of cultured human endothelial cells characterized by the increase of MMP-1 and MMP-2 expressions and the formation of capillary-like structures (282). In addition, the treatment of ovarian cancer cells with small interfering RNA against CD147 suppressed the angiogenic potential of EVs suggesting the direct implication of this protein in the pro-angiogenic effects of EVs from ovarian cancer cells (282). Interestingly, *in vitro* studies have shown that EVs from CSF, but not from blood, from glioblastoma patients promote endothelial migration and proliferation leading to angiogenesis by a beta-catenin/Akt pathway (283). Isolation and characterization by transcriptional microarray analysis of exosomes released by human SW480 colorectal cancer cell line show that exosomes are enriched in 241 mRNAs related to cell cycle processes when compared to parent cells. Moreover, when human umbilical vein endothelial cells (HUVECs) are treated with human SW480 colorectal cancer-derived exosomes, endothelial cells are in metaphase and cytokinesis with mitotic spindle formation reflecting enhanced proliferation which is an essential step in neovascularization (284).

Since hypoxia is proposed as a driving of cancer progression, Hsu's group (285) has characterized the effects of EVs (exosomes) released from lung cancer cells exposed to

hypoxia (1% O₂) on endothelial cells. These exosomes affect HUVEC function by increasing migration and tube formation, endothelial permeability and transendothelial migration of cancer cells. These effects are mediated by miR-23a carried by exosomes from hypoxic lung cancer cells suggesting that exosomal miR-23a may be a promising strategy for lung cancer treatment (285).

EVs from non-tumor cell origins are also able to modulate tumor angiogenesis. In particular, human platelet-derived MVs have been described to increase angiogenesis, but also proliferation and survival of leukemic cells as well as metastasis, in lung cancer (286). These MVs are able to induce the release of pro-angiogenic factors such as VEGF, MMP-9 and IL-8 in HUVECs (286).

Taken together, these data show that EVs from tumor cells, but also those from tumor environment cells, display pro-angiogenic properties that can enhance the ability of endothelial cells to form new vessels in order to allow the tumor growth.

Role of EVs on metastasis: Tumor cells have the ability to migrate, to access the blood or lymphatic routes, circulate and reach distant zones from the primary tumor to form metastasis. In a very elegant study, and by combining high-resolution intravital imaging with a Cre recombinase-based method, Zomer and colleagues (287) have shown the spread of metastatic behavior through EVs in living mice, and directly visualized the release of EVs by highly metastatic human MDA-MB-231 mammary tumor cells. These EVs induce changes on tumor cells with low potential malignant enhancing their migratory behavior and metastatic capacity (287).

Exosomes from mouse pancreatic ductal adenocarcinomas induce liver pre-metastatic niche formation in mice and, consequently, increase liver metastatic burden. Interestingly, these exosomes are uptake by Kupffer cells and, then, release TGF β leading to the activation of hepatic stellate cells. These events account for the recruitment of bone marrow-derived

macrophages to the liver, providing a favorable niche for liver metastasis. Furthermore, these effects seem to be dependent to exosome-associated migration inhibitory factor (288). Metastatic murine and human breast cancer cell lines as well as sera of mice bearing metastatic tumors display miR-200-enriched EVs (289). Notably, these EVs, via the transfer of miR-200 to non-metastatic cells, alter gene expression and promote mesenchymal-to-epithelial transition. Besides, EVs from human xenograft models expressing miR-200, promote metastasis. Other authors have shown that exosomes from MDA-MB-231 breast cancer metastatic cell line downregulate tight junction protein zonula occludens (ZO)-1 and increase endothelial permeability leading to alteration of the barrier function of endothelial monolayers by a mechanism dependent to the presence of miR-105 into exosomes (290). In addition, the pathophysiological relevance of these findings was demonstrated by the injection of miR105-enriched exosomes into mice. Indeed, increased miR-105 levels are detected in lung and brain of mice, which is accompanied by reduced ZO-1 expression in endothelial cells, enhanced vascular permeability and metastasis (290). Also, circulating miR-105 levels correlates with metastasis in early-stage breast cancer patients. These authors suggest developing personalized therapeutics based on a combination of miR-105 with other circulating miRNAs and/or proteins allowing the selection of breast cancer patients with high risk for metastasis. In this respect, it has been described that exosomes from breast cancer patients contain the RISC loading complex proteins, Dicer, TRBP, and AGO2, which process pre-miRNAs into mature miRNAs. Consequently, cancer exosomes alter the transcriptome of target cells in a Dicer-dependent manner, which stimulate non-tumorigenic epithelial cells to form tumors (291).

As described above for their pro-angiogenic potential, platelet-derived MVs can also enhance the invasive capacity of breast cancer cells (292). Although Janowska-Wieczorek and colleagues have only performed *in vitro* experiments, they have demonstrated that human

platelet-derived MVs enhance activation of AKT and ERK1/2 pathways and stimulate the production of MMPs in invasive breast cancer cell lines as well as their chemoinvasion across the reconstituted basement membrane Matrigel. These results underline the problems associated with platelet transfusion to cancer patients.

Other actions of EVs from tumor cells: Cancer is commonly associated with a hypercoagulable state leading to an elevated risk of thrombosis and venous thromboembolism (293). Since EVs, mainly MVs, carry pro-coagulant factors such as tissue factor and/or PS (48, 294, 295), it is possible that they can be involved in the thrombus generation. Indeed, although exosomes from melanoma cells are less prone to generate thrombin and fibrin than MVs, it is necessary to emphasize the potential of both types of EVs to contribute to the pro-thrombotic state of cancer patients (296).

MVs from prostatic carcinoma cell lines (PC3 and LnCaP) modify normal fibroblast function as evidenced by the promotion of MMP-9 expression and ERK1/2 phosphorylation, the increase of tissue factor expression and PS exposure and acquire partial resistance to apoptosis. The data highlight the potential pro-coagulant role of EVs in cancer. Furthermore, prostatic carcinoma cell line-derived MVs induce migration of fibroblasts that, in their turn, release MVs promoting migration and invasion of cancer cells by a mechanism mediated, at least in part, by CX3CR1 (297).

Finally, EVs have been also related to the development of resistance of cancer cells that are associated to poor outcome of patients. The mechanisms by which resistance occurs have not completely elucidated, but some evidences show that inhibition of EV generation by methotrexate or an inhibitor of peptidylarginine deiminases sensitizes prostate cancer cells to chemotherapy (298, 299). Recently, it has been shown that the transfer of functional miR-21 by EVs from macrophages associated to tumors to gastric cancer cells induces cisplatin resistance. Once exosome-associated miR-21 enters into gastric cancer cells, it is able to

modulate the PTEN/PI3K/Akt signaling pathway and enhances the Bcl-2 anti-apoptotic protein in gastric cancer cells, without affecting ABC transporter genes. The authors conclude that targeting exosomal miR-21 from tumor-associated macrophages may be a promising adjuvant therapeutic strategy for gastric cancer patients, especially those developing resistance to cisplatin (300). By contrast, other studies have described the presence of ABC transporters in exosomes released from breast and prostate cancer cells treated with docetaxel (301, 302) suggesting that the involvement of these transporters carried by EVs on multidrug resistance developed by cancer cells may be dependent of the type of tumor.

IX. EVs as THERAPEUTIC TOOLS

As discussed previously, there is an emerging literature concerning the role of EVs on pathological processes such as diabetes, obesity, cardiovascular or neurodegenerative diseases, and cancer. Since the past decades, new therapeutic strategies in the field of nanomedicine have been developed using the nanostructures as drug delivery systems in order to treat specifically these diseases. Synthetic nanoparticles, liposomes or adenovirus have been produced; however, the use of those nanoparticles is limited. On the one hand, their membrane composition does not facilitate the crossover between cells (303) and, on the other hand, repeated injections of those nanoparticles induce inflammatory-undesired responses. Therefore, the use of EVs as drug delivery systems in order to increase the specificity and efficiency of the treatment seems to be an interesting breakthrough.

A. EVs as tools for drug delivery (Figure 14)

After they administration, the intrinsic characteristics of EVs allow the delivery of endogenous or exogenous molecules into target cells and tissues. Thus, (i) membrane of EVs protects their encapsulated content against degradation, (ii) their small size decreases their clearance and allows that they passively enter in tissues, and (iii) the modification of the membrane surface enhances their ability to target several tissues (304). Altogether,

specifically targeted drug delivery systems via EVs may increase efficacy of therapies for CVD, cancer or neurodegenerative diseases.

Targeting strategy for drug delivery: Several targeting strategies can be used in order to increase the ability of EVs to interact with specific cells. In fact, Alvarez-Erviti and colleagues (305), have bioengineered the lysosomal-associated membrane protein 2b (Lamp2b) to express the peptide rabies virus glycoprotein (RVG) directly below the signal peptide sequence to target either brain or tumor tissues. Indeed, RVG binds to acetylcholine receptors in neurons, microglia, and oligodendrocytes after intravenous injection allowing a target delivery of EV content. This approach enables the correct insertion of the protein into the exosome membrane and prevents the cleavage of important regions in the targeting peptide sequence. Another strategy concerns the fusion of nanobody DNA sequences with GPI-anchor peptide sequences in Neuro2A cells resulting in the insertion of nanobodies into EV membranes. Due to this modification, cell targeting specificity is increased as demonstrated by the enhancement of the association of EVs with EGFR-expressing tumor cells, while interactions with EGFR-negative cells are unaltered (304). Alternative strategies to enhance targeting of EVs consist to stable conjugation of targeting ligands to the surface of isolated EVs by using chemical linkers such as N-hydroxy succinimidyl ester reactive groups, or by the post-insertion technique, once EVs are generated, by fusion with the distal ends of polyethylene glycol-phospholipids (304).

Therapeutic cargo and methods for loading EVs: The current interest in EVs derives from their ability to deliver innovative therapies to specific target cells. This strategy consists to load EVs with substantial amount of pharmacological drugs efficient to act on target cells. Several loading strategies depending on the type of cargo have been described.

Simple incubation of exosomes with the cargo is a method which has proved efficient result thanks to the particular characteristics of the bilayer membrane. For example, exosomes can

deliver anti-inflammatory drugs such as curcumin to activate myeloid cells *in vivo*. Thus, in a lipopolysaccharide (LPS)-induced septic shock mouse model, curcumin carried by exosomes from murine macrophage cell line decreases IL-6 and TNF- α levels in sera (306). Another study compares nanovesicles in a context of creating chemotherapeutic for malignant tumors and find that doxorubicin-loaded exosomes reduce more tumor growth than bioinspired exosome-mimetic nanovesicles (307). Commercial transfection reagent is also used in order to load EVs but this approach is inadequate for an efficient loading in a therapeutic purpose. Hence, electroporation is a safe method which can be used in a clinical setting but could alter EV integrity. For instance, RVG-loaded exosomes are charged with GAPDH siRNA by electroporation (305). Another alternative corresponds to the direct transfection or pharmacological treatment of cells producing EVs. In this way, enrichment of human blood cells and THP-1 cells with synthetic miR-150 enhances the packaging into MVs which are able to reduce c-Myb expression and enhance human microvascular endothelial cell migration. These results indicate that transfection of miR-150 into cells enable the secretion of MVs which can modulate recipient cell function and regulate gene expression (308). Also, adeno-associated viruses (AAVs) are more efficient for delivering cargo into recipient cells when encapsulated in EVs than free AAVs (309). Moreover, it has been demonstrated that the treatment of tumor cells with chemotherapeutics drugs such as doxorubicin induces the release of doxorubicin-rich exosomes , thus these exosomes can be collected and exploited to kill tumor cells (310). However, these techniques can promote adverse effects on the exosomes integrity and their cargoes , by inducing aggregation, for example (311). Finally, pharmacologic treatment of parental cells is also efficient to load generated exosomes with drugs. For instance, when HepG2 cells are incubated with different anticancer agents, such as paclitaxel, etoposide, irinotecan or cisplatin, released exosomes display strong anti-proliferative activity on the human pancreatic cell line CFPAC-1 (312). Also, pharmacologic

treatment of human T cells with the apoptotic inducer actinomycin D induces the generation of MVs with anti-proliferative and anti-angiogenic properties that have been proposed to be used in lung carcinoma (313). Interestingly, when human T cells are treated with a cocktail of mitogens and actinomycin D, the generated MVs are rich in Sonic hedgehog and display *in vitro* and *in vivo* pro-angiogenic actions (10, 265). Indeed, these MVs harboring Sonic hedgehog may represent new therapeutic tools in diseases related with failed angiogenesis such as peripheral ischemia or diabetes (314) (Figure 15).

Choice of donor cell type: The use of EVs produced from tumor cells is controverted by the fact that they induce inflammatory reaction during administration (315). To avoid this undesirable effect, some studies produce EVs with immunogenic properties from DCs at their immature state. Immature DCs produce large quantities of exosomes, that are immunologically inert, lacking T-cell activators such as MHC-I, MHC-II and CD86 (316). In this respect, exosomes from immature DCs promote graft survival (317) and reduce inflammation in animal models of inflammatory-bowel disease (318). Moreover, because EVs are natural transporters, they are less susceptible to trigger immune responses and exert toxicity. Also, it has been demonstrated that EVs from progenitor or MSCs have regenerative properties, particularly they reach injured sites (319, 320), promote functional recovery and modulate immune responses. Moreover, in a model mouse myocardial ischemia/reperfusion injury, exosomes from MSCs increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling (321). Also, in a liver injury mouse model, it has been demonstrated that MSC-derived exosomes can trigger hepatoprotective effects against toxic-induced injury, through an activation of proliferative and regenerative responses (322). Several studies promote the advantageous use of MSC-derived EVs, instead of stem cells, because they do not replicate and are not trapped into lungs after intravenous administration (323).

Recently, it has been describe that bovine milk can serve as a source of exosomes that act as carrier for chemotherapeutic agents showing no adverse immune and inflammatory response. Notable, exosomes from milk are biocompatible and represent excellent candidates, in terms of cost-effectiveness, to enhance oral bioavailability, improve efficacy and safety of drugs (324). After the discovery of exosomes in animal cells, exosomes have also been isolated from several edible plants (325). Oral administration of exosome-like nanoparticles isolated from grape juice by ultracentrifugation on a sucrose gradient leads to protection from dextran sulphate sodium-induced colitis in mice. Moreover, these exosomes can be used as immune modulators in the intestine and for oral delivery of small molecule drugs to attenuate inflammatory responses in human disease (326). Also, a very recent work has shown that miR-156a-rich exosomes isolated from watermelon are internalized into a proliferative subpopulation of Caco-2 cells, suggesting that dietary miRNAs can be transferred into human circulation by the internalization of exosomes (327).

Administration of EVs: EVs have to be stable and capable of delivering their content through the classic administration routes (intravenous, oral...) but also by unusual ways in order to address their cargo more specifically. In the case of malignancies, EVs are mostly administrated by intravenous injection. This method is especially adapted in porous blood vessels and to a defective lymphatic drainage. For example, tumor-bearing mice intravenously treated with exosomes loaded with doxorubicin, show an inhibition of the tumor growth. Moreover, the capacity of these vesicles to express iRGD peptide allows an efficient targeting to the tumor tissue and distribution of doxorubicin without any toxicity (328). In the case of accessible tumors, intratumoral injections are used and provide a secure specific delivery of the therapeutic molecules. Because of their protein and lipid composition, exosomes loaded with doxorubicin intratumorally injected into mice are more efficient internalized by tumor

cells than liposomes and allow an effective liberation of doxorubicin inducing a complete suppression of tumor growth (93).

The biodistribution of DiR-labeled milk exosomes administrated to mice differs depending on the type of administration (324). Indeed, DiR-labeled milk exosomes predominate in the liver after intravenous injection whereas the distribution of these exosomes is uniform in the other organs. A novel approach for treating brain inflammatory-related diseases consists to intranasally administrate exosomes loaded with curcumin in a context of LPS-induced injured brain model in mice. This strategy enables a rapid delivery of exosome-encapsulated drug to the brain, mainly into microglial cells and induce their apoptosis (329). Intraventricular administration of folate receptor-positive EVs is also performed in mouse brain parenchyma in order to restore a functional folate receptor which allows cerebral folate transport (330). Another type of strategy is rising up in order to improve the uptake of EVs by cells through the merge of exosomes with liposomes conferring a complex lipid composition to the bilayer membrane. This approach allows an optimization of the exosome surface properties in order to decrease its immunogenicity and increase its colloidal stability, thus improving its half-life in blood. Using the freeze-thaw method which is a simple physiochemical process, exosomes and liposomes membranes fuse causing significant structural and functional changes allowing distinct type of interaction between the hybrid exosomes and the target cell (331). This membrane-engineering method represents a new strategy to load exogenous hydrophobic lipids either hydrophilic cargoes within the exosomes as drug delivery systems.

B. EVs as therapeutic tools in pathophysiological contexts

As described above, natural or modified EVs can be used as therapeutic tools by modifying the phenotype and function of target cells in order to correct a dysfunction or treat cells with drugs. Here, we propose several examples of the use of EVs as therapeutic tools.

Cardiovascular diseases: EVs are a potential interest for cardiac regenerative therapies. It has been evidenced that the MSC secretome, mainly exosomes, trigger the beneficial regenerative effects of MSCs by reducing infarct size in animal models of myocardial ischemia/reperfusion (332, 333). Moreover, exosomes derived from MSCs inhibit vascular remodeling and hypertension after an intravenous injection in a mouse model of pulmonary hypertension by the inhibition of STAT3 signaling in pulmonary artery endothelial cells (334). In a model of mouse diabetic hind-limb ischemia, exosomes from induced pluripotent stem cells derived from MSCs are able to promote angiogenesis and protect limb from ischemic injury (335).

On the other hand, EVs specifically enriched in Sonic hedgehog obtained by pharmacological treatment of human T lymphocytes display protective properties on cardiovascular cells (336). Their pro-angiogenic capacities have illustrated after an intravenous injection in a mouse model hind limb ischemia (265). In mice, these EVs are able to improve endothelial function by increasing NO[•] release and by reducing oxidative stress through the induction of antioxidant messages in HUVECs (33).

CNS pathologies: Recent studies concerning the modulation of neurological disorders have been started using EVs. For example, exosomes from MSCs promote endogenous angiogenesis, neurogenesis and reduce inflammation in a rat model of traumatic brain injury contributing of a partial functional recovery (337). It has been demonstrated that EVs from microglia, which are implicated in the defense during inflammation, spread inflammatory reactions throughout the brain by diffusing inflammatory signals and upregulating the expression of enhancing genes inflammation (338, 339). A strategy using EVs against AD is proposed in order to enhance the level of neprilysin which is the most important A β degrading enzyme in the brain. Indeed, active neprilysin carried by exosomes from human adipose tissue-derived MSCs decreases A β secretion and its intracellular levels into neuroblastoma cells (340). Moreover, *in vivo* systemic administration of EVs from DCs,

previously enriched in an exogenous siRNA, induce a knockdown of BACE1, a therapeutic target in AD, in the mouse brain (305).

Cancer: Some clinical trials are lead to analyze the efficacy of exosomes as cell-free vaccine in patients with a late-stage of cancer and demonstrated the feasibility and the good tolerance of these vesicles (315, 341, 342). In addition, antiangiogenic therapy has been demonstrated potential effect in altering the pro-angiogenic properties of EVs which can be used as a novel approach to decrease tumor-associated vasculature and tumor resistance (343).

X. FUTURE DIRECTIONS

The main disadvantage to use EVs as biomarkers and/or tools against diseases is the lack of gold standards for isolation techniques (344). To address this problem, the International Society for Extracellular Vesicles (ISEV) gives directives to isolate and characterize EVs, as accurately as possible. However, they recognize there is no protocol standard, and invite the researchers to be cautious about the interpretation of results (49). Also, in order to use EVs in a clinical context as therapeutic tools, characterization of their content has to be exhaustive and, at the same time, easy to perform and not expensive. Only if the scientific community succeeds to homogenize the parameters of isolation of EVs and their characterization, EVs may be considered as excellent biomarkers for diagnosis and prognosis with a particular interest in the development of tools for theragnosis in personalized medicine.

Acknowledgements

This work was supported by Institut National de la Santé et de la Recherche Médicale, Université d'Angers, and Centre Hospitalo-Universitaire d'Angers. MM and MV are recipients of doctoral fellowships from INSERM and Conseil Régional du Pays de la Loire. LP is recipient of a postdoctoral fellowship from Angers Loire Métropole.

List of Abbreviations

Adeno-associated virus, AAV; ADP ribosylation factor 6, ARF6; Alzheimer's disease, AD; Amyloid- β peptide, A β ; Amyloid precursor protein, APP; Amyotrophic lateral sclerosis, ALS; ATP-binding cassette, ABC; Body mass index, BMI; Cannabinoid receptor 1, CB1; Cardiovascular diseases, CVD; Central nervous system, CNS; Cerebrospinal fluid, CSF; Chloride intracellular channel 1, CLIC1; Dendritic cells, DCs; Discoidin domain receptor-1, DDR-1; Endosomal sorting complex required for transport, ESCRT; Endothelial nitric oxide synthase, eNOS; Epidermal growth factor receptor, EGFR; Excitatory amino-acid transporter, EAAT; Extracellular signal-regulated kinase, ERK; Extracellular vesicles, EVs; Glycophosphatidylinositol, GPI; Heat shock protein, HSP; Human umbilical vein endothelial cells, HUVECs; InterCellular Adhesion Molecule-1, ICAM-1; Interleukin, IL; Intraluminal vesicles, ILVs; Krüppel-like factor, KLF; Lipopolysaccharide, LPS; Major histocompatibility complex, MHC; Matrix metalloproteinase, MMPs; Mesenchymal stem cells, MSCs; Microvesicles, MVs; Mitochondrial DNA, mtDNA; Multiple myeloma, MM; Multivesicular bodies, MVBs; Nanoparticle tracking analysis, NTA; Neutral sphingomyelinase, nSMase; Nitric oxide, NO; Non-small cell lung cancer, NSCLC; Nuclear factor kappa B, NF- κ B; Oligomeric forms of amyloid- β peptide, A β O; Parkinson's disease, PD; Phosphatidylserine, PS; Phosphoinositide-3-kinase, PI3K; Phospholipase, PL; Protein kinase C, PKC; Prion protein, PrP; Proteolipoprotein, PLP; Rabies virus glycoprotein, RVG; Reactive oxygen species, ROS; Rho-associated kinase, ROCK I; Scrapie Prion protein, PrPSc; Smooth muscle cells, SMCs; Soluble N-ethylmaleimide sensitive factor attachment protein receptor, SNARE; Superoxide dismutase, SOD; Transforming growth factor, TGF; Tumor necrosis factor, TNF; Type 1 diabetes mellitus, T1DM; Type 2 diabetes mellitus, T2DM; Vascular cell adhesion molecule, VCAM; Vascular endothelial growth factor, VEGF; Zonula occludens, ZO

References

1. Müller G. Microvesicles/exosomes as potential novel biomarkers of metabolic diseases. *Diabetes Metab Syndr Obes Targets Ther* 5:247-282, 2012.
2. van Dommelen SM, Vader P, Lakhal S, et al. Microvesicles and exosomes: Opportunities for cell-derived membrane vesicles in drug delivery. *J Controlled Release* 161:635-644, 2012.
3. Théry C. Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep* 3, 2011.
4. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol* 13:269-288, 1967.
5. Hargett LA, Bauer NN. On the origin of microparticles: From “platelet dust” to mediators of intercellular communication. *Pulm Circ* 3:329-340, 2013.
6. Hauser P, Wang S, Didenko VV. Apoptotic bodies: Selective detection in extracellular vesicles. *Methods Mol Biol* 1554:193-200, 2017.
7. Flamant S, Tamarat R. Extracellular vesicles and vascular injury: New Insights for radiation exposure. *Radiat Res* 186:203-218, 2016.
8. Yin M, Loyer X, Boulanger CM. Extracellular vesicles as new pharmacological targets to treat atherosclerosis. *Eur J Pharmacol* 763:90-103, 2015.
9. Badimon L, Suades R, Fuentes E, Palomo I, Padró T. Role of platelet-derived microvesicles as crosstalk mediators in atherothrombosis and future pharmacology targets: a link between inflammation, atherosclerosis, and thrombosis. *Front Pharmacol* 7, 2016.
10. Soleti R, Benameur T, Porro C, Panaro MA, Andriantsitohaina R, Martínez MC. Microparticles harboring Sonic Hedgehog promote angiogenesis through the upregulation of adhesion proteins and proangiogenic factors. *Carcinogenesis* 30:580-588, 2009.
11. Livaja Koshlar R, Somajo S, Norström E, Dahlbäck B. Erythrocyte-derived microparticles supporting activated protein c-mediated regulation of blood coagulation. *PLoS One* 9, 2014.
12. Jansen F, Yang X, Franklin BS, et al. High glucose condition increases NADPH oxidase activity in endothelial microparticles that promote vascular inflammation. *Cardiovasc Res* 98:94-106, 2013.
13. Liu S, Sun J, Lan Q. Glioblastoma microvesicles promote endothelial cell proliferation through Akt/beta-catenin pathway. *Int J Clin Exp Pathol* 7:4857-4866, 2014.
14. Rautou P-E, Vion A-C, Amabile N, et al. Microparticles, vascular function, and atherothrombosis. *Circ Res* 109:593-606, 2011.

15. Povero D, Eguchi A, Li H, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One* 9:e113651, 2014.
16. Flaumenhaft R, Dilks JR, Richardson J, et al. Megakaryocyte-derived microparticles: direct visualization and distinction from platelet-derived microparticles. *Blood* 113:1112-1121, 2009.
17. Martinez MC, Tual-Chalot S, Leonetti D, Andriantsitohaina R. Microparticles: targets and tools in cardiovascular disease. *Trends Pharmacol Sci* 32:659-665, 2011.
18. Brown MD, Fearheller DL, Thakkar S, Veerabhadrapa P, Park J-Y. Racial differences in tumor necrosis factor- α -induced endothelial microparticles and interleukin-6 production. *Vasc Health Risk Manag* 7:541-550, 2011.
19. Mostefai HA, Agouni A, Carusio N, et al. Phosphatidylinositol 3-kinase and xanthine oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells. *J Immunol* 180:5028-5035, 2008.
20. Pasquet J-M, Dachary-Prigent J, Nurden AT. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. *Eur J Biochem* 239:647-654, 1996.
21. Miyoshi H, Umeshita K, Sakon M, et al. Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. *Gastroenterology* 110:1897-1904, 1996.
22. Kapustin AN, Davies JD, Reynolds JL, et al. Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ Res* 109:e1-12, 2011.
23. Neri T, Cordazzo C, Carmazzi Y, et al. Effects of peroxisome proliferator-activated receptor- γ agonists on the generation of microparticles by monocytes/macrophages. *Cardiovasc Res* 94:537-544, 2012.
24. Campbell LE, Nelson J, Gibbons E, Judd AM, Bell JD. Membrane properties involved in calcium-stimulated microparticle release from the plasma membranes of S49 lymphoma cells. *Scientific World Journal* 2014:537192, 2014.
25. Vion A-C, Ramkhalawon B, Loyer X, et al. Shear stress regulates endothelial microparticle release. *Circ Res* 112:1323-1333, 2013.
26. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 19:43-51, 2009.
27. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol* 3:339-345, 2001.
28. Simoncini S, Njock M-S, Robert S, et al. TRAIL/Apo2L mediates the release of procoagulant endothelial microparticles induced by thrombin in vitro: a potential mechanism linking inflammation and coagulation. *Circ Res* 104:943-951, 2009.

29. Liu Y, Huang W, Zhang R, Wu J, Li L, Tang Y. Proteomic analysis of TNF- α -activated endothelial cells and endothelial microparticles. *Mol Med Rep* 7:318-326, 2013.
30. Alexy T, Rooney K, Weber M, Gray WD, Searles CD. TNF- α alters the release and transfer of microparticle-encapsulated miRNAs from endothelial cells. *Physiol Genomics* 46:833-840, 2014.
31. Dean WL, Lee MJ, Cummins TD, Schultz DJ, Powell DW. Proteomic and functional characterisation of platelet microparticle size classes. *Thromb Haemost* 102:711-718, 2009. doi:10.1160/TH09-04-243.
32. Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 113:E968-977, 2016.
33. Soleti R, Lauret E, Andriantsitohaina R, Carmen Martínez M. Internalization and induction of antioxidant messages by microvesicles contribute to the antiapoptotic effects on human endothelial cells. *Free Radic Biol Med* 53:2159-2170, 2012.
34. Bochmann I, Ebstein F, Lehmann A, et al. T lymphocytes export proteasomes by way of microparticles: a possible mechanism for generation of extracellular proteasomes. *J Cell Mol Med* 18:59-68, 2014.
35. Agouni A, Ducluzeau P-H, Benameur T, et al. Microparticles from patients with metabolic syndrome induce vascular hypo-reactivity via Fas/Fas-ligand pathway in mice. *PLoS One* 6:e27809, 2011.
36. Chen P, Douglas SD, Meshki J, Tuluc F. Neurokinin 1 receptor mediates membrane blebbing and shear stress-induced microparticle formation in HEK293 cells. *PLoS One* 7, 2012.
37. Diehl P, Fricke A, Sander L, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. *Cardiovasc Res* 93:633-644, 2012.
38. Sódar BW, Kittel Á, Pálóczi K, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. *Sci Rep* 6:24316, 2016.
39. Boulanger CM, Loyer X, Rautou P-E, Amabile N. Extracellular vesicles in coronary artery disease. *Nat Rev Cardiol* 14:259-272, 2017.
40. Hulsmans M, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. *Cardiovasc Res* 100:7-18, 2013.
41. Mulcahy LA, Pink RC, Carter DRF. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles* 3, 2014.
42. Svensson KJ, Belting M. Role of extracellular membrane vesicles in intercellular communication of the tumour microenvironment. *Biochem Soc Trans* 41:273-276, 2013.
43. Tual-Chalot S, Leonetti D, Andriantsitohaina R, Martínez MC. Microvesicles: intercellular vectors of biological messages. *Mol Interv* 11:88-94, 2011.

44. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 97:329-339, 1983.
45. Pan B-T, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol* 101:942-948, 1985.
46. Urbanelli L, Magini A, Buratta S, et al. Signaling Pathways in Exosomes Biogenesis, Secretion and Fate. *Genes* 4:152-170, 2013.
47. Oksvold MP, Kullmann A, Forfang L, et al. Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. *Clin Ther* 36:847-86, 2014.
48. Durcin M, Fleury A, Taillebois E, et al. Characterisation of adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and small extracellular vesicles. *J Extracell Vesicles* 6, 2017
49. Lötvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 3, 2014.
50. Juan T, Fürthauer M. [The ESCRT complex: from endosomal transport to the development of multicellular organisms]. *Biol Aujourdhui* 209:111-124, 2015.
51. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2:569-579, 2002.
52. Schmidt O, Teis D. The ESCRT machinery. *Curr Biol* 22:R116-R120, 2012.
53. Baietti MF, Zhang Z, Mortier E, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol* 14:677-685, 2012.
54. Roucourt B, Meeussen S, Bao J, Zimmermann P, David G. Heparanase activates the syndecan-syntenin-ALIX exosome pathway. *Cell Res* 25:412-428, 2015.
55. Ghossoub R, Lembo F, Rubio A, et al. Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nat Commun* 5:3477, 2014.
56. Trajkovic K, Hsu C, Chiantia S, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319:1244-1247, 2008.
57. Buschow SI, Nolte-t Hoen ENM, Van Niel G, et al. MHC II in dendritic cells is targeted to lysosomes or t cell-induced exosomes via distinct multivesicular body pathways. *Traffic* 10:1528-1542, 2009.
58. van Niel G, Charrin S, Simoes S, et al. The tetraspanin CD63 regulates ESCRT-independent and dependent endosomal sorting during melanogenesis. *Dev Cell* 21:708-721, 2011.

59. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* 10:513-525, 2009.
60. Savina A, Fader CM, Damiani MT, Colombo MI. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic Cph Den* 6:131-143, 2005.
61. Hsu C, Morohashi Y, Yoshimura S-I, et al. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J Cell Biol* 189:223-232, 2010.
62. Ostrowski M, Carmo NB, Krumeich S, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 12:19-30, 2010. doi:10.1038/ncb2000.
63. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, rna cargo selection, content, release, and uptake. *Cell Mol Neurobiol* 36:301-312, 2016.
64. Haraszti RA, Didiot M-C, Sapp E, et al. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J Extracell Vesicles* 5, 2016.
65. Conde-Vancells J, Rodriguez-Suarez E, Embade N, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J Proteome Res* 7:5157, 2008.
66. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 30:255-289, 2014.
67. Vidal M, Sainte-Marie J, Philippot JR, Bienvenue A. Asymmetric distribution of phospholipids in the membrane of vesicles released during in vitro maturation of guinea pig reticulocytes: evidence precluding a role for "aminophospholipid translocase." *J Cell Physiol* 140:455-462, 1989.
68. Llorente A, Skotland T, Sylvänne T, et al. Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* 1831:1302-1309, 2013.
69. Laulagnier K, Motta C, Hamdi S, et al. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochem J* 380(Pt 1):161-171, 2004.
70. Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. *Biochimie* 89:205-212, 2007.
71. Subra C, Grand D, Laulagnier K, et al. Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins. *J Lipid Res* 51:2105-2120, 2010.
72. Record M, Carayon K, Poirot M, Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiological processes. *Biochim Biophys Acta* 1841:108-120, 2014.

73. Skotland T, Ekroos K, Kauhanen D, et al. Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. *Eur J Cancer* 70:122-132, 2017.
74. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9:654-659, 2007.
75. Montecalvo A, Larregina AT, Shufesky WJ, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 119:756-766, 2012.
76. Brianza-Padilla M, Carbó R, Arana JC, et al. Inflammation related micrnas are modulated in total plasma and in extracellular vesicles from rats with chronic ingestion of sucrose. *BioMed Res Int* 2016, 2016.
77. Segura E, Nicco C, Lombard B, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* 106:216-223, 2005.
78. Morelli AE, Larregina AT, Shufesky WJ, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* 104:3257-3266, 2004.
79. Parolini I, Federici C, Raggi C, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 284:34211-34222, 2009.
80. Nanbo A, Kawanishi E, Yoshida R, Yoshiyama H. Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. *J Virol* 87:10334-10347, 2013.
81. Frühbeis C, Fröhlich D, Kuo WP, et al. Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. *PLoS Biol* 11:e1001604, 2013.
82. Tian T, Zhu Y-L, Zhou Y-Y, et al. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem* 289:22258-22267, 2014.
83. Svensson KJ, Christianson HC, Wittrup A, et al. Exosome uptake depends on erk1/2-heat shock protein 27 signaling and lipid raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem* 288:17713-17724, 2013.
84. Fitzner D, Schnaars M, van Rossum D, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *J Cell Sci* 124:447-458, 2011.
85. Nakase I, Kobayashi NB, Takatani-Nakase T, Yoshida T. Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. *Sci Rep* 5:10300, 2015.
86. Feng D, Zhao W-L, Ye Y-Y, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 11:675-687, 2010.
87. Nakase I, Futaki S. Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. *Sci Rep* 5:10112, 2015.

88. Atai NA, Balaj L, Veen H van, et al. Heparin blocks transfer of extracellular vesicles between donor and recipient cells. *J Neurooncol* 115:343-351, 2013.
89. Takahashi Y, Nishikawa M, Shinotsuka H, et al. Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J Biotechnol* 165:77-84, 2013.
90. Lai CP, Mardini O, Ericsson M, et al. Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter. *ACS Nano* 8:483-494, 2014.
91. Manca S, Giraud D, Zempeni J. Bioavailability and biodistribution of fluorophore-labeled exosomes from cow's milk after intravenous and oral administration in C57Bl/6J mice. *FASEB J* 30:690.8, 2016.
92. Wiklander OPB, Nordin JZ, O'Loughlin A, et al. Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles* 4, 2015.
93. Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchordoquy TJ. Biodistribution and delivery efficiency of unmodified tumor-derived exosomes. *J Control Release* 199:145-155, 2015.
94. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol Chapter 3:Unit 3.22*, 2006.
95. Clayton A, Court J, Navabi H, et al. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J Immunol Methods* 247:163-174, 2001.
96. Blans K, Hansen MS, Sørensen LV, et al. Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 6, 2017.
97. Taylor D, Zacharias W, Gercel-Taylor C. Exosome Isolation for Proteomic Analyses and RNA Profiling. In: Simpson RJ, Greening DW, eds. *Serum/Plasma Proteomics*. Methods in Molecular Biology. Humana Press, 2011, p235-246.
98. Momen-Heravi F, Balaj L, Alian S, Tigges J, Toxavidis V, Ericsson M, Distel RJ, Ivanov AR, Skog J, Kuo WP. Alternative Methods for Characterization of Extracellular Vesicles. *Front Physiol* 3:354, 2012.
99. Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine Nanotechnol Biol Med* 7:780-788, 2011.
100. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev* 91:79-118, 2011.
101. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444:840-846, 2006.

102. Sabatier F, Darmon P, Hugel B, et al. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes* 51:2840-2845, 2002.
103. Li S, Wei J, Zhang C, Li X, Meng W, Mo X, Zhang Q, Liu Q, Ren K, Du R, Tian H, Li J. Cell-derived microparticles in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. *Cell Physiol Biochem* 39:2439-2450, 2016.
104. Esposito K, Maiorino MI, Di Palo C, et al. Effects of pioglitazone versus metformin on circulating endothelial microparticles and progenitor cells in patients with newly diagnosed type 2 diabetes--a randomized controlled trial. *Diabetes Obes Metab* 13:439-445, 2011.
105. Kastorini C-M, Panagiotakos DB. Dietary patterns and prevention of type 2 diabetes: from research to clinical practice; a systematic review. *Curr Diabetes Rev* 5:221-227, 2009.
106. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell* 148:852-871, 2012.
107. Ferrante SC, Nadler EP, Pillai DK, et al. Adipocyte-derived exosomal miRNAs: a novel mechanism for obesity-related disease. *Pediatr Res* 77:447-454, 2015.
108. Hubal MJ, Nadler EP, Ferrante SC, et al. Circulating adipocyte-derived exosomal MicroRNAs associated with decreased insulin resistance after gastric bypass. *Obes Silver Spring Md* 25:102-110, 2017.
109. Thomou T, Mori MA, Dreyfuss JM, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature* 542:450-455 2017,.
110. Kranendonk MEG, de Kleijn DPV, Kalkhoven E, et al. Extracellular vesicle markers in relation to obesity and metabolic complications in patients with manifest cardiovascular disease. *Cardiovasc Diabetol* 13:37, 2014.
111. Povero D, Eguchi A, Niesman IR, et al. Lipid-induced toxicity stimulates hepatocytes to release angiogenic microparticles that require Vanin-1 for uptake by endothelial cells. *Sci Signal* 6:ra88, 2013.
112. Huang-Doran I, Zhang C-Y, Vidal-Puig A. Extracellular vesicles: novel mediators of cell communication in metabolic disease. *Trends Endocrinol Metab* 28:3-18 2017,.
113. Tokita Y, Maejima Y, Shimomura K, Takenoshita S, Ishiyama N, Akuzawa M, Shimomura Y, Nakajima K. Non-alcoholic fatty liver disease is a risk factor for type 2 diabetes in middle-aged japanese men and women. *Intern Med* 56:763-771, 2017.
114. Michael MD, Kulkarni RN, Postic C, et al. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 6:87-97, 2000.
115. Cantaluppi V, Biancone L, Figliolini F, et al. Microvesicles derived from endothelial progenitor cells enhance neoangiogenesis of human pancreatic islets. *Cell Transplant* 21:1305-1320, 2012.

116. Figliolini F, Cantaluppi V, De Lena M, et al. Isolation, characterization and potential role in beta cell-endothelium cross-talk of extracellular vesicles released from human pancreatic islets. *PloS One* 9:e102521, 2014.
117. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432:226-230, 2004.
118. Sun L-L, Jiang B-G, Li W-T, Zou J-J, Shi Y-Q, Liu Z-M. MicroRNA-15a positively regulates insulin synthesis by inhibiting uncoupling protein-2 expression. *Diabetes Res Clin Pract* 91:94-100, 2011.
119. Kamalden TA, Macgregor-Das AM, Kannan SM, Dunkerly-Eyring B, Khaliddin N, Xu Z, Fusco AP, Yazib SA, Chow RC, Duh EJ, Halushka MK, Steenbergen C, Das S. Exosomal microRNA-15a transfer from the pancreas augments diabetic complications by inducing oxidative stress. *Antioxid Redox Signal*. 2017, doi: 10.1089/ars.2016.6844.
120. Chahed S, Leroyer AS, Benzerroug M, et al. Increased vitreous shedding of microparticles in proliferative diabetic retinopathy stimulates endothelial proliferation. *Diabetes* 59:694-701, 2010.
121. Barutta F, Tricarico M, Corbelli A, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. *PloS One* 8:e73798, 2013.
122. Shi R, Zhao L, Cai W, et al. Maternal exosomes in diabetes contribute to the cardiac development deficiency. *Biochem Biophys Res Commun* 483:602-608, 2017.
123. Ng ACT, Delgado V, Bertini M, et al. Myocardial steatosis and biventricular strain and strain rate imaging in patients with type 2 diabetes mellitus. *Circulation* 122:2538-2544, 2010.
124. de Gonzalo-Calvo D, van der Meer RW, Rijzewijk LJ, et al. Serum microRNA-1 and microRNA-133a levels reflect myocardial steatosis in uncomplicated type 2 diabetes. *Sci Rep* 7:47, 2017.
125. Keith SW, Redden DT, Katzmarzyk PT, Boggiano MM, Hanlon EC, Benca RM, Ruden D, Pietrobelli A, Barger JL, Fontaine KR, Wang C, Aronne LJ, Wright SM, Baskin M, Dhurandhar NV, Lijoi MC, Grilo CM, DeLuca M, Westfall AO, Allison DB. Putative contributors to the secular increase in obesity: exploring the roads less traveled. *Int J Obes* 30:1585-1594, 2006.
126. Boutens L, Stienstra R. Adipose tissue macrophages: going off track during obesity. *Diabetologia* 59:879-894, 2016.
127. Jung UJ, Choi M-S. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci* 15:6184-6223, 2014.
128. Poirier P, Giles TD, Bray GA, et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. *Circulation* 113:898-918, 2006.
129. Kyrgiou M, Kalliala I, Markozannes G, et al. Adiposity and cancer at major anatomical sites: umbrella review of the literature. *BMJ* 356:j477, 2017.

130. Luppino FS, Wit LM de, Bouvy PF, et al. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. *Arch Gen Psychiatry* 67:220-229, 2010.
131. Yanovski SZ, Yanovski JA. Long-term drug treatment for obesity: a systematic and clinical review. *JAMA J Am Med Assoc* 311:74-86, 2014.
132. Stepanian A, Bourguignat L, Hennou S, et al. Microparticle increase in severe obesity: Not related to metabolic syndrome and unchanged after massive weight loss. *Obesity* 21:2236-2243, 2013.
133. Campello E, Zabeo E, Radu CM, et al. Hypercoagulability in overweight and obese subjects who are asymptomatic for thrombotic events. *Thromb Haemost* 113:85-96, 2015.
134. Murakami T, Horigome H, Tanaka K, et al. Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity. *Thromb Res* 119:45-53, 2007.
135. Cheng V, Kashyap SR, Schauer PR, Kirwan JP, McCrae KR. Restoration of glycemic control in patients with type 2 diabetes mellitus after bariatric surgery is associated with reduction in microparticles. *Surg Obes Relat Dis* 9:207-212, 2013.
136. Campello E, Zabeo E, Radu CM, et al. Dynamics of circulating microparticles in obesity after weight loss. *Intern Emerg Med* 11:695-702, 2016.
137. Phoonsawat W, Aoki-Yoshida A, Tsuruta T, Sonoyama K. Adiponectin is partially associated with exosomes in mouse serum. *Biochem Biophys Res Commun* 448:261-266, 2014.
138. Heinrich LF, Andersen DK, Cleasby ME, Lawson C. Long-term high fat feeding of rats results in increased numbers of circulating microvesicles with pro-inflammatory effects on endothelial cells. *Br J Nutr* 113:1704-1711, 2015.
139. Deng Z, Poliakov A, Hardy RW, et al. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes* 58:2498-2505, 2009.
140. Zhang Y, Mei H, Chang X, Chen F, Zhu Y, Han X. Adipocyte-derived microvesicles from obese mice induce M1 macrophage phenotype through secreted miR-155. *J Mol Cell Biol* 8:505-517, 2016.
141. Karolina DS, Tavintharan S, Armugam A, et al. Circulating miRNA profiles in patients with metabolic syndrome. *J Clin Endocrinol Metab* 97:E2271-E2276, 2012. doi:10.1210/jc.2012-1996.
142. Preston RA, Jy W, Jimenez JJ, et al. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension* 41:211-217, 2003.
143. Trzepizur W, Martinez MC, Priou P, Andriantsitohaina R, Gagnadoux F. Microparticles and vascular dysfunction in obstructive sleep apnoea. *Eur Respir J* 44:207-216, 2014.
144. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340:115-126, 1999.

145. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death. *Arterioscler Thromb Vasc Biol* 20:1262-1275, 2000.
146. Heiss C, Rodriguez-Mateos A, Kelm M. Central role of eNOS in the maintenance of endothelial homeostasis. *Antioxid Redox Signal* 22:1230-1242, 2015.
147. Violi F, Pignatelli P. Clinical application of nox activity and other oxidative biomarkers in cardiovascular disease: a critical review. *Antioxid Redox Signal* 3:514-532, 2015.
148. Ma G, Pan B, Chen Y, et al. Trimethylamine N-oxide in atherogenesis: impairing endothelial self-repair capacity and enhancing monocyte adhesion. *Biosci Rep* 37, 2017.
149. Förstermann U, Xia N, Li H. Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circ Res* 120:713-735, 2017.
150. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. *Circ Re* 114:1852-1866, 2014.
151. Tarbell JM. Shear stress and the endothelial transport barrier. *Cardiovasc Res* 87:320-330, 2010.
152. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, et al. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am J Pathol* 173:1210-1219, 2008.
153. Safiedeen Z, Rodríguez-Gómez I, Vergori L, et al. Temporal cross talk between endoplasmic reticulum and mitochondria regulates oxidative stress and mediates microparticle-induced endothelial dysfunction. *Antioxid Redox Signal* 26:15-27, 2017.
154. Nielsen MH, Irvine H, Vedel S, Raungaard B, Beck-Nielsen H, Handberg A. The impact of lipoprotein-associated oxidative stress on cell-specific microvesicle release in patients with familial hypercholesterolemia. *Oxid Med Cell Longev* 2016;2016.
155. Priou P, Gagnadoux F, Tesse A, et al. Endothelial dysfunction and circulating microparticles from patients with obstructive sleep apnea. *Am J Pathol* 177:974-983, 2010.
156. Hosseinkhani B, van den Akker N, D'Haen J, et al. Direct detection of nano-scale extracellular vesicles derived from inflammation-triggered endothelial cells using surface plasmon resonance. *Nanomedicine* 13:1663-1671, 2017.
157. Hergenreider E, Heydt S, Tréguer K, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat Cell Biol* 14:249-256, 2012.
158. Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, Barnes S, Grizzle W, Miller D, Zhang HG. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther* 18:1606-1614, 2010.
159. Caporali A, Meloni M, Nailor A, et al. p75(NTR)-dependent activation of NF-κB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. *Nat Commun* 6:8024, 2015.

160. Pan Q, Liu H, Zheng C, et al. Microvesicles derived from inflammation-challenged endothelial cells modulate vascular smooth muscle cell functions. *Front Physiol* 7:692, 2016.
161. Niu C, Wang X, Zhao M, Cai T, Liu P, Li J, Willard B, Zu L, Zhou E, Li Y, Pan B, Yang F, Zheng L. Macrophage foam cell-derived extracellular vesicles promote vascular smooth muscle cell migration and adhesion. *J Am Heart Assoc* 5, 2016.
162. Hetterich H, Webber N, Willner M, et al. AHA classification of coronary and carotid atherosclerotic plaques by grating-based phase-contrast computed tomography. *Eur Radiol* 26:3223-3233, 2016.
163. Jansen F, Yang X, Hoelscher M, et al. Endothelial microparticle-mediated transfer of miRNA-126 promotes vascular endothelial cell repair via Spred1 and is abrogated in glucose-damaged endothelial microparticles. *Circulation* 128:2026-2038, 2013.
164. Camaré C, Pucelle M, Nègre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. *Redox Biol* 12:18, 2017.
165. Moreno PR, Purushothaman K-R, Sirol M, Levy AP, Fuster V. Neovascularization in human atherosclerosis. *Circulation* 113:2245-2252, 2006.
166. Leroyer AS, Isobe H, Lesèche G, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol* 49:772-777, 2007.
167. Sarlon-Bartoli G, Bennis Y, Lacroix R, et al. Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis. *J Am Coll Cardiol* 62:1436-1441, 2013.
168. Tafelmeier M, Fischer A, Orsó E, et al. Mildly oxidized HDL decrease agonist-induced platelet aggregation and release of pro-coagulant platelet extracellular vesicles. *J Steroid Biochem Mol Biol* 169:176-188, 2017.
169. Xu M-D, Wu X-Z, Zhou Y, Xue Y, Zhang K-Q. Proteomic characteristics of circulating microparticles in patients with newly-diagnosed type 2 diabetes. *Am J Transl Res* 8:209-220, 2016.
170. Gaceb A, Martinez MC, Andriantsitohaina R. Extracellular vesicles: new players in cardiovascular diseases. *Int J Biochem Cell Biol* 50:24-28, 2014.
171. Suades R, Padró T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. *Thromb Haemost* 108:1208-1219, 2012.
172. Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 94:3791-3799, 1999.

173. Hutcheson JD, Goettsch C, Bertazzo S, et al. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. *Nat Mater* 15:335-343, 2016.
174. Chen NX, O'Neill KD, Chen X, Moe SM. Annexin-mediated matrix vesicle calcification in vascular smooth muscle cells. *J Bone Miner Res* 23:1798-1805, 2008.
175. New SEP, Aikawa E. Role of extracellular vesicles in de novo mineralization: an additional novel mechanism of cardiovascular calcification. *Arterioscler Thromb Vasc Biol* 33:1753-1758, 2013.
176. Krohn JB, Hutcheson JD, Martínez-Martínez E, et al. Discoidin domain receptor-1 regulates calcific extracellular vesicle release in vascular smooth muscle cell fibrocalcific response via transforming growth factor- β signaling. *Arterioscler Thromb Vasc Biol* 36:525-533, 2016.
177. Krohn JB, Hutcheson JD, Martínez-Martínez E, Aikawa E. Extracellular vesicles in cardiovascular calcification: expanding current paradigms. *J Physiol* 594:2895-2903, 2016.
178. Benjamin EJ, Blaha MJ, Chiuve SE, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* 135:e146-e603, 2017.
179. Fauré J, Lachenal G, Court M, et al. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 31:642-648, 2006.
180. Lachenal G, Pernet-Gallay K, Chivet M, et al. Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci* 46:409-418, 2011.
181. Vingtdoux V, Hamdane M, Loyens A, et al. Alkalizing drugs induce accumulation of amyloid precursor protein by-products in luminal vesicles of multivesicular bodies. *J Biol Chem* 282:18197-18205, 2007.
182. D'Adamio L. Role of cystatin C in neuroprotection and its therapeutic implications. *Am J Pathol* 177:2163-2165, 2010.
183. Ghidoni R, Paterlini A, Albertini V, et al. Cystatin C is released in association with exosomes: a new tool of neuronal communication which is unbalanced in Alzheimer's disease. *Neurobiol Aging* 32:1435-1442, 2011.
184. Putz U, Howitt J, Lackovic J, et al. Nedd4 family-interacting protein 1 (Ndfip1) is required for the exosomal secretion of Nedd4 family proteins. *J Biol Chem* 283:32621-32627, 2008.
185. Morel L, Regan M, Higashimori H, et al. Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. *J Biol Chem* 288:7105-7116, 2013.

186. Goldie BJ, Dun MD, Lin M, et al. Activity-associated miRNA are packaged in Map1b-enriched exosomes released from depolarized neurons. *Nucleic Acids Res* 42:9195-9208, 2014.
187. Xu J, Chen Q, Zen K, Zhang C, Zhang Q. Synaptosomes secrete and uptake functionally active microRNAs via exocytosis and endocytosis pathways. *J Neurochem* 124:15-25, 2013.
188. Krämer-Albers E-M, Bretz N, Tenzer S, et al. Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons? *Proteomics Clin Appl* 1:1446-1461, 2007.
189. Verkhratsky A, Matteoli M, Parpura V, Mothet J-P, Zorec R. Astrocytes as secretory cells of the central nervous system: idiosyncrasies of vesicular secretion. *EMBO J* 35:239-257, 2016.
190. Gosselin R-D, Meylan P, Decosterd I. Extracellular microvesicles from astrocytes contain functional glutamate transporters: regulation by protein kinase C and cell activation. *Front Cell Neurosci* 7:251, 2013.
191. Guescini M, Genedani S, Stocchi V, Agnati LF. Astrocytes and glioblastoma cells release exosomes carrying mtDNA. *J Neural Transm* 117:1-4, 2010.
192. Lafourcade C, Ramírez JP, Luarte A, Fernández A, Wyneken U. MiRNAs in astrocyte-derived exosomes as possible mediators of neuronal plasticity. *J Exp Neurosci* 10:1-9, 2016.
193. Taylor AR, Robinson MB, Gifondorwa DJ, Tytell M, Milligan CE. Regulation of heat shock protein 70 release in astrocytes: role of signaling kinases. *Dev Neurobiol* 67:1815-1829, 2007.
194. Wang S, Cesca F, Loers G, et al. Synapsin I is an oligomannose-carrying glycoprotein, acts as an oligomannose-binding lectin, and promotes neurite outgrowth and neuronal survival when released via glia-derived exosomes. *J Neurosci* 31:7275-7290, 2011.
195. Potolicchio I, Carven GJ, Xu X, et al. Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *J Immunol* 175:2237-2243, 2005.
196. Schiera G, Proia P, Alberti C, Mineo M, Savettieri G, Di Liegro I. Neurons produce FGF2 and VEGF and secrete them at least in part by shedding extracellular vesicles. *J Cell Mol Med* 11:1384-1394, 2007.
197. Proia P, Schiera G, Mineo M, et al. Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. *Int J Mol Med* 21:63-67, 2008.
198. Bianco F, Perrotta C, Novellino L, et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J* 28:1043-1054, 2009.
199. Bianco F, Pravettoni E, Colombo A, et al. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J Immunol* 174:7268-7277, 2005, .

200. Rajendran L, Bali J, Barr MM, et al. Emerging roles of extracellular vesicles in the nervous system. *J Neurosci* 34:15482-15489, 2014.
201. Basso M, Bonetto V. Extracellular vesicles and a novel form of communication in the brain. *Front Neurosci* 10:127, 2016.
202. Schiera G, Di Liegro CM, Di Liegro I. Extracellular membrane vesicles as vehicles for brain cell-to-cell interactions in physiological as well as pathological conditions. *BioMed Res Int* 2015:152926, 2015.
203. Chivet M, Javalet C, Laulagnier K, Blot B, Hemming FJ, Sadoul R. Exosomes secreted by cortical neurons upon glutamatergic synapse activation specifically interact with neurons. *J Extracell Vesicles* 3:247222014, .
204. Konopka W, Kiryk A, Novak M, et al. MicroRNA loss enhances learning and memory in mice. *J Neurosci* 30:14835-14842, 2010.
205. Fröhlich D, Kuo WP, Frühbeis C, Sun JJ, Zehendner CM, Luhmann HJ, Pinto S, Toedling J, Trotter J, Krämer-Albers EM. Multifaceted effects of oligodendroglial exosomes on neurons: impact on neuronal firing rate, signal transduction and gene regulation. *Philos Trans R Soc Lond B Biol Sci* 369, 2014.
206. Bakhti M, Winter C, Simons M. Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *J Biol Chem* 286:787-796, 2011.
207. Antonucci F, Turola E, Riganti L, et al. Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism. *EMBO J* 31:1231-1240, 2012.
208. Darios F, Wasser C, Shakirzyanova A, et al. Sphingosine facilitates SNARE complex assembly and activates synaptic vesicle exocytosis. *Neuron* 62:683-694, 2009.
209. Gabrielli M, Battista N, Riganti L, et al. Active endocannabinoids are secreted on extracellular membrane vesicles. *EMBO Rep* 16:213-2202015, .
210. Kanninen KM, Bister N, Koistinaho J, Malm T. Exosomes as new diagnostic tools in CNS diseases. *Biochim Biophys Acta* 1862:403-410, 2016.
211. Colombo E, Borgiani B, Verderio C, Furlan R. Microvesicles: novel biomarkers for neurological disorders. *Front Physiol* 3:63, 2012.
212. Lee J, McKinney KQ, Pavlopoulos AJ, et al. Exosomal proteome analysis of cerebrospinal fluid detects biosignatures of neuromyelitis optica and multiple sclerosis. *Clin Chim Acta* 462:118-126, 2016.
213. Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. *Lancet* 89:1336-1346, 2017.
214. Kawikova I, Askenase PW. Diagnostic and therapeutic potentials of exosomes in CNS diseases. *Brain Res* 1617:63-712015, .

215. Saman S, Kim W, Raya M, et al. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J Biol Chem* 287:3842-3849, 2012.
216. Goetzl EJ, Boxer A, Schwartz JB, et al. Altered lysosomal proteins in neural-derived plasma exosomes in preclinical Alzheimer disease. *Neurology* 85:40-47, 2015.
217. Mustapic M, Eitan E, Werner JK, et al. Plasma extracellular vesicles enriched for neuronal origin: a potential window into brain pathologic processes. *Front Neurosci* 11:278, 2017.
218. Winston CN, Goetzl EJ, Akers JC, et al. Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome protein profile. *Alzheimers Dement* 3:63-72, 2016.
219. Goetzl EJ, Kapogiannis D, Schwartz JB, et al. Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease. *FASEB J* 30:4141-4148, 2016.
220. Shi M, Liu C, Cook TJ, et al. Plasma exosomal α -synuclein is likely CNS-derived and increased in Parkinson's disease. *Acta Neuropathol (Berl)* 128:639-650, 2014.
221. Cao X-Y, Lu J-M, Zhao Z-Q, et al. MicroRNA biomarkers of Parkinson's disease in serum exosome-like microvesicles. *Neurosci Lett* 644:94-99, 2017.
222. V VG, Ss A. Emergence of exosomal miRNAs as a diagnostic biomarker for Alzheimer's disease. *J Neurol Sci* 360:141-152, 2016.
223. Stroo E, Koopman M, Nollen EAA, Mata-Cabana A. Cellular Regulation of Amyloid Formation in Aging and Disease. *Front Neurosci* 11:64, 2017.
224. Fevrier B, Vilette D, Archer F, et al. Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* 101:9683-9688, 2004.
225. Vella LJ, Sharples RA, Lawson VA, Masters CL, Cappai R, Hill AF. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J Pathol* 211:582-590, 2007.
226. Yim Y-I, Park B-C, Yadavalli R, Zhao X, Eisenberg E, Greene LE. The multivesicular body is the major internal site of prion conversion. *J Cell Sci* 128:1434-1443, 2015.
227. Guo BB, Bellingham SA, Hill AF. Stimulating the release of exosomes increases the intercellular transfer of prions. *J Biol Chem* 291:5128-5137, 2016.
228. Saá P, Yakovleva O, de Castro J, et al. First demonstration of transmissible spongiform encephalopathy-associated prion protein (PrPTSE) in extracellular vesicles from plasma of mice infected with mouse-adapted variant Creutzfeldt-Jakob disease by in vitro amplification. *J Biol Chem* 289:29247-29260, 2014.
229. Rajendran L, Honscho M, Zahn TR, et al. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 103:11172-11177, 2006.

230. Sharples RA, Vella LJ, Nisbet RM, et al. Inhibition of gamma-secretase causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes. *FASEB J* 22:1469-1478, 2008.
231. Rajendran L, Honsho M, Zahn TR, et al. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 103:11172-11177, 2006.
232. Dinkins MB, Dasgupta S, Wang G, Zhu G, Bieberich E. Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* 35:1792-1800, 2014.
233. Dinkins MB, Enasko J, Hernandez C, et al. Neutral Sphingomyelinase-2 Deficiency Ameliorates Alzheimer's Disease Pathology and Improves Cognition in the 5XFAD Mouse. *J Neurosci* 36:8653-8667, 2016.
234. Joshi P, Benussi L, Furlan R, Ghidoni R, Verderio C. Extracellular vesicles in Alzheimer's disease: friends or foes? Focus on a β -vesicle interaction. *Int J Mol Sci* 16:4800-4813, 2015.
235. Yuyama K, Sun H, Sakai S, et al. Decreased amyloid- β pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. *J Biol Chem* 289:24488-24498, 2014.
236. Yuyama K, Sun H, Usuki S, et al. A potential function for neuronal exosomes: sequestering intracerebral amyloid- β peptide. *FEBS Lett* 589:84-88, 2015.
237. An K, Klyubin I, Kim Y, et al. Exosomes neutralize synaptic-plasticity-disrupting activity of A β assemblies in vivo. *Mol Brain* 6:47, 2013.
238. Falker C, Hartmann A, Guett I, et al. Exosomal cellular prion protein drives fibrillization of amyloid beta and counteracts amyloid beta-mediated neurotoxicity. *J Neurochem* 137:88-100, 2016.
239. Xiao T, Zhang W, Jiao B, Pan C-Z, Liu X, Shen L. The role of exosomes in the pathogenesis of Alzheimer' disease. *Transl Neurodegener* 6:3, 2017.
240. Wang Y, Balaji V, Kaniyappan S, et al. The release and trans-synaptic transmission of Tau via exosomes. *Mol Neurodegener* 12:52017, .
241. Chiarini A, Armato U, Gardenal E, Gui L, Dal Prà I. Amyloid β -exposed human astrocytes overproduce phospho-tau and overrelease it within exosomes, effects suppressed by calcilytic nps 2143-further implications for alzheimer's therapy. *Front Neurosci* 11:217, 2017.
242. Asai H, Ikezu S, Tsunoda S, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat Neurosci* 18:1584-1593, 2015.
243. Braak H, Rüb U, Gai WP, Del Tredici K. Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm* 110:517-536, 2003.

244. Emmanouilidou E, Melachroinou K, Roumeliotis T, et al. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci* 30:6838-6851, 2010.
245. Vella LJ, Hill AF, Cheng L. Focus on extracellular vesicles: exosomes and their role in protein trafficking and biomarker potential in Alzheimer's and Parkinson's Disease. *Int J Mol Sci* 17:173, 2016.
246. Danzer KM, Kranich LR, Ruf WP, et al. Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol Neurodegener* 7:42, 2012.
247. Grey M, Dunning CJ, Gaspar R, et al. Acceleration of α -synuclein aggregation by exosomes. *J Biol Chem* 290:2969-2982, 2015.
248. Alvarez-Erviti L, Seow Y, Schapira AH, et al. Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol Dis* 42:360-367, 2011.
249. Valdinocci D, Radford RAW, Siow SM, Chung RS, Pountney DL. Potential modes of intercellular α -synuclein transmission. *Int J Mol Sci* 18, 2017.
250. Silverman JM, Fernando SM, Grad LI, et al. Disease mechanisms in ALS: misfolded sod1 transferred through exosome-dependent and exosome-independent pathways. *Cell Mol Neurobiol* 36:377-381, 2016.
251. Iguchi Y, Eid L, Parent M, et al. Exosome secretion is a key pathway for clearance of pathological TDP-43. *Brain J Neurol* 139:3187-32012016, .
252. Gong J, Jaiswal R, Dalla P, Luk F, Bebawy M. Microparticles in cancer: A review of recent developments and the potential for clinical application. *Semin Cell Dev Biol* 40:35-40, 2015.
253. Boyiadzis M, Whiteside TL. The emerging roles of tumor-derived exosomes in hematological malignancies. *Leukemia* 31:1259-1268, 2017.
254. Kim HK, Song KS, Park YS, et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur J Cancer* 39:184-191, 2003.
255. Kanazawa S, Nomura S, Kuwana M, Muramatsu M, Yamaguchi K, Fukuhara S. Monocyte-derived microparticles may be a sign of vascular complication in patients with lung cancer. *Lung Cancer* 39:145-149, 2003.
256. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 10:1470-1476, 2008.
257. Shao H, Chung J, Balaj L, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med* 18:1835-1840, 2012.

258. Tseng C-C, Wang C-C, Hsiao C-C, et al. Time courses and value of circulating microparticles in patients with operable stage non-small cell lung cancer undergoing surgical intervention. *Tumour Biol* 37:11873-11882, 2016.
259. Wang C-C, Tseng C-C, Hsiao C-C, et al. Circulating endothelial-derived activated microparticle: a useful biomarker for predicting one-year mortality in patients with advanced non-small cell lung cancer. *BioMed Res Int* 2014:173401, 2014 .
260. Raimondo F, Morosi L, Corbetta S, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol Biosyst* 9:1220-1233, 2013.
261. Caivano A, Laurenzana I, De Luca L, et al. High serum levels of extracellular vesicles expressing malignancy-related markers are released in patients with various types of hematological neoplastic disorders. *Tumour Biol* 36:9739-9752, 2015.
262. De Luca L, D'Arena G, Simeon V, et al. Characterization and prognostic relevance of circulating microvesicles in chronic lymphocytic leukemia. *Leuk Lymphoma* 58:1424-1432, 2017.
263. Belov L, Matic KJ, Hallal S, Best OG, Mulligan SP, Christopherson RI. Extensive surface protein profiles of extracellular vesicles from cancer cells may provide diagnostic signatures from blood samples. *J Extracell Vesicles* 5:25355, 2016.
264. Oksvold MP, Kullmann A, Forfang L, et al. Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. *Clin Ther* 36:847-862, 2014
265. Benameur T, Soleti R, Porro C, Andriantsitohaina R, Martínez MC. Microparticles carrying Sonic hedgehog favor neovascularization through the activation of nitric oxide pathway in mice. *PLoS One* 5:e12688, 2010.
266. Roccaro AM, Sacco A, Maiso P, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest* 123:1542-1555, 2013.
267. Manier S, Liu C-J, Avet-Loiseau H, et al. Prognostic role of circulating exosomal miRNAs in multiple myeloma. *Blood* 129:2429-2436, 2017.
268. Dejima H, Iinuma H, Kanaoka R, Matsutani N, Kawamura M. Exosomal microRNA in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer. *Oncol Lett* 13:1256-1263, 2017.
269. Tsukamoto M, Iinuma H, Yagi T, Matsuda K, Hashiguchi Y. Circulating exosomal microRNA-21 as a biomarker in each tumor stage of colorectal cancer. *Oncology* 92:360-370, 2017.
270. Monzo M, Santasusagna S, Moreno I, et al. Exosomal microRNAs isolated from plasma of mesenteric veins linked to liver metastases in resected patients with colon cancer. *Oncotarget* 8:30859-30869, 2017.
271. Choi D, Lee TH, Spinelli C, Chennakrishnaiah S, D'Asti E, Rak J. Extracellular vesicle communication pathways as regulatory targets of oncogenic transformation. *Semin Cell Dev Biol* 67:11-22, 2017.

272. Wang C, Long W, Peng C, et al. HTLV-1 Tax functions as a ubiquitin e3 ligase for direct ikk activation via synthesis of mixed-linkage polyubiquitin chains. *PLoS Pathog* 12:e1005584, 2016.
273. El-Saghir J, Nassar F, Tawil N, El-Sabban M. ATL-derived exosomes modulate mesenchymal stem cells: potential role in leukemia progression. *Retrovirology* 13:73, 2016.
274. Abd Elmageed ZY, Yang Y, Thomas R, et al. Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes. *Stem Cells* 32:983-997, 2014.
275. Cai J, Wu G, Tan X, et al. Transferred BCR/ABL DNA from K562 extracellular vesicles causes chronic myeloid leukemia in immunodeficient mice. *PloS One* 9:e105200, 2014.
276. Ghosh AK, Secreto CR, Knox TR, Ding W, Mukhopadhyay D, Kay NE. Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression. *Blood* 115:1755-1764, 2010.
277. Th L, S C, B M, et al. Barriers to horizontal cell transformation by extracellular vesicles containing oncogenic H-ras. *Oncotarget* 7:51991-52002, 2016.
278. Wang Y, Cheng Q, Liu J, Dong M. Leukemia stem cell-released microvesicles promote the survival and migration of myeloid leukemia cells and these effects can be inhibited by microrna34a overexpression. *Stem Cells Int* 2016:9313425, 2016.
279. Setti M, Osti D, Richichi C, et al. Extracellular vesicle-mediated transfer of CLIC1 protein is a novel mechanism for the regulation of glioblastoma growth. *Oncotarget* 6:31413-31427, 2015.
280. Baj-Krzyworzeka M, Majka M, Pratico D, et al. Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. *Exp Hematol* 30:450-459, 2002.
281. Kholia S, Ranghino A, Garnieri P, et al. Extracellular vesicles as new players in angiogenesis. *Vascul Pharmacol* 86:64-70, 2016.
282. Millimaggi D, Mari M, D'Ascenzo S, et al. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia* 9:349-35, 2007.
283. Liu S, Sun J, Lan Q. Glioblastoma microvesicles promote endothelial cell proliferation through Akt/beta-catenin pathway. *Int J Clin Exp Pathol* 7:4857-4866, 2014.
284. Hong BS, Cho J-H, Kim H, et al. Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics* 10:556, 2009.
285. Hsu Y-L, Hung J-Y, Chang W-A, et al. Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1. *Oncogene* 2017, doi: 10.1038/onc.2017.105

286. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int J Cancer* 113:752-760, 2005.
287. Zomer A, Maynard C, Verweij FJ, et al. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* 161:1046-1057, 2015.
288. Costa-Silva B, Aiello NM, Ocean AJ, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 17:816-826, 2015.
289. Le MTN, Hamar P, Guo C, et al. miR-200-containing extracellular vesicles promote breast cancer cell metastasis. *J Clin Invest* 124:5109-5128, 2014.
290. Zhou W, Fong MY, Min Y, et al. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* 25:501-515, 2014.
291. Melo SA, Sugimoto H, O'Connell JT, et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell* 26:707-721, 2014.
292. Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M, Ratajczak MZ. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. *Transfusion (Paris)* 46:1199-1209, 2006.
293. Franco AT, Corken A, Ware J. Platelets at the interface of thrombosis, inflammation, and cancer. *Blood* 126:582-588, 2015.
294. Lea J, Sharma R, Yang F, Zhu H, Ward ES, Schroit AJ. Detection of phosphatidylserine-positive exosomes as a diagnostic marker for ovarian malignancies: a proof of concept study. *Oncotarget* 8:14395-14407, 2017.
295. Kapustin AN, Schoppet M, Schurgers LJ, et al. Prothrombin loading of vascular smooth muscle cell-derived exosomes regulates coagulation and calcification. *Arterioscler Thromb Vasc Biol* 37:e22-e32, 2017.
296. Mr M-S, Sc S, Ac D, Jm F, T K, Ad M. Procoagulant and immunogenic properties of melanoma exosomes, microvesicles and apoptotic vesicles. *Oncotarget* 7:56279-56294, 2016.
297. Castellana D, Zobairi F, Martinez MC, et al. Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: a role for activated fibroblasts and CX3CL1-CX3CR1 axis. *Cancer Res* 69:785-793, 2009.
298. Jorfi S, Ansa-Addo EA, Kholia S, et al. Inhibition of microvesiculation sensitizes prostate cancer cells to chemotherapy and reduces docetaxel dose required to limit tumor growth in vivo. *Sci Rep* 5:13006, 2015.
299. Kholia S, Jorfi S, Thompson PR, et al. A novel role for peptidylarginine deiminases in microvesicle release reveals therapeutic potential of PAD inhibition in sensitizing prostate cancer cells to chemotherapy. *J Extracell Vesicles* 4:26192, 2015 .

300. Zheng P, Chen L, Yuan X, et al. Exosomal transfer of tumor-associated macrophage-derived miR-21 confers cisplatin resistance in gastric cancer cells. *J Exp Clin Cancer Res* 36:53, 2017.
301. Mm L, Xy Z, Wx C, et al. Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein. *Tumour Biol* 35:10773-10779, 2014.
302. Corcoran C, Rani S, O'Brien K, et al. Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PLoS One* 7:e50999, 2012.
303. El Andaloussi S, Lakhali S, Mäger I, Wood MJA. Exosomes for targeted siRNA delivery across biological barriers. *Adv Drug Deliv Rev* 65:391-397, 2013.
304. Kooijmans SAA, Schiffelers RM, Zarovni N, Vago R. Modulation of tissue tropism and biological activity of exosomes and other extracellular vesicles: New nanotools for cancer treatment. *Pharmacol Res* 111:487-500, 2016.
305. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhali S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 29:341-345, 2011.
306. Sun D, Zhuang X, Xiang X, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther* 18:1606-1614, 2010.
307. Jang SC, Kim OY, Yoon CM, et al. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. *ACS Nano* 7:7698-7710, 2013.
308. Zhang Y, Liu D, Chen X, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol Cell* 39:133-144, 2010.
309. Maguire CA, Balaj L, Sivaraman S, et al. Microvesicle-associated AAV vector as a novel gene delivery system. *Mol Ther* 20:960-971, 2012.
310. Tang K, Zhang Y, Zhang H, et al. Delivery of chemotherapeutic drugs in tumour cell-derived microparticles. *Nat Commun* 3:1282, 2012.
311. Kooijmans SAA, Stremersch S, Braeckmans K, et al. Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. *J Control Release* 172:229-238, 2013.
312. Lv L-H, Wan Y-L, Lin Y, et al. Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem* 287:15874-15885, 2012.
313. Yang C, Xiong W, Qiu Q, et al. Anti-proliferative and anti-tumour effects of lymphocyte-derived microparticles are neither species- nor tumour-type specific. *J Extracell Vesicles* 3:2014, .

314. Martinez MC, Andriantsitohaina R. Microparticles in angiogenesis: therapeutic potential. *Circ Res* 109:110-119, 2011.
315. Escudier B, Dorval T, Chaput N, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J Transl Med* 3:10, 2005.
316. Quah BJC, O'Neill HC. The immunogenicity of dendritic cell-derived exosomes. *Blood Cells Mol Dis* 35:94-110, 2005.
317. Pêche H, Heslan M, Usal C, Amigorena S, Cuturi MC. Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection. *Transplantation* 76:1503-1510, 2003, .
318. Yang X, Meng S, Jiang H, Chen T, Wu W. Exosomes derived from interleukin-10-treated dendritic cells can inhibit trinitrobenzene sulfonic acid-induced rat colitis. *Scand J Gastroenterol* 45:1168-1177, 2010.
319. Kraitchman DL, Tatsumi M, Gilson WD, et al. Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* 112:1451-1461, 2005.
320. Kim SM, Jeong CH, Woo JS, Ryu CH, Lee J-H, Jeun S-S. In vivo near-infrared imaging for the tracking of systemically delivered mesenchymal stem cells: tropism for brain tumors and biodistribution. *Int J Nanomedicine* 11:13-23, 2015.
321. Arslan F, Lai RC, Smeets MB, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 10:301-312, 2013.
322. Tan CY, Lai RC, Wong W, Dan YY, Lim S-K, Ho HK. Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 5:76, 2014.
323. Hwang DW, Choi H, Jang SC, et al. Noninvasive imaging of radiolabeled exosome-mimetic nanovesicle using (99m)Tc-HMPAO. *Sci Rep* 5:15636, 2015.
324. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. *Cancer Lett* 371:48-61, 2016.
325. Ju S, Mu J, Dokland T, et al. Grape exosome-like nanoparticles induce intestinal stem cells and protect mice from DSS-induced colitis. *Mol Ther* 21:1345-1357, 2013.
326. Wang B, Zhuang X, Deng Z-B, et al. Targeted drug delivery to intestinal macrophages by bioactive nanovesicles released from grapefruit. *Mol Ther* 22:522-534, 2014.
327. The Fifth International Meeting of ISEV, ISEV2016, Rotterdam, *J Extracell Vesicles*. 2016;5:31552, .

328. Tian Y, Li S, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 35:2383-2390, 2014.
329. Zhuang X, Xiang X, Grizzle W, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* 19:1769-1779, 2011.
330. Grapp M, Wrede A, Schweizer M, et al. Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma. *Nat Commun* 4:2123, 2013.
331. Sato YT, Umezaki K, Sawada S, et al. Engineering hybrid exosomes by membrane fusion with liposomes. *Sci Rep* 6:21933, 2016.
332. Lai CP-K, Breakefield XO. Role of exosomes/microvesicles in the nervous system and use in emerging therapies. *Front Physiol* 3:228, 2012.
333. Lai RC, Chen TS, Lim SK. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* 6:481-492, 2011.
334. Lee C, Mitsialis SA, Aslam M, et al. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 126:2601-2611, 2012.
335. Hu G, Li Q, Niu X, et al. Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. *Stem Cell Res Ther* 6:10, 2015.
336. Martínez MC, Larbret F, Zobairi F, et al. Transfer of differentiation signal by membrane microvesicles harboring hedgehog morphogens. *Blood* 108:3012-3020, 2006.
337. Zhang Y, Chopp M, Meng Y, et al. Effect of exosomes derived from multipotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg* 122:856-867, 2015.
338. Verderio C, Muzio L, Turola E, et al. Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. *Ann Neurol* 72:610-624, 2012.
339. Prada I, Furlan R, Matteoli M, Verderio C. Classical and unconventional pathways of vesicular release in microglia. *Glia* 61:1003-1017, 2013.
340. Katsuda T, Tsuchiya R, Kosaka N, et al. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* 3:11972013, .
341. Dai S, Wei D, Wu Z, et al. Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol Ther* 16:782-790, 2008.
342. Morse MA, Garst J, Osada T, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* 3:9, 2005.

343. Hannafon BN, Carpenter KJ, Berry WL, Janknecht R, Dooley WC, Ding W-Q. Exosome-mediated microRNA signaling from breast cancer cells is altered by the anti-angiogenesis agent docosahexaenoic acid (DHA). *Mol Cancer* 14:133, 2015.
344. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J Extracell Vesicles* 2, 2013.

Figure legends




Figure 1: Origin of extracellular vesicles. Microvesicles result from the blebbing of the plasma membrane after cell activation. Exosomes come from the endosomal compartment and are formed as intraluminal vesicles (ILVs) by inward budding of the early endosomal membrane, sequestering proteins, lipids, and cytosol, in order to degrade, recycle or exocytose this content. Early endosomes then mature in late endosomes and the accumulation of multitude of ILVs leads to the formation of multivesicular bodies (MVBs). The main fate of MVBs is to fuse with lysosomes, ensuring the degradation of their content. However, MVBs can also fuse with the plasma membrane to release their sequester ILVs as exosomes in the extracellular space. ( ,  , Intracellular components of extracellular vesicles; ( , Membrane components of extracellular vesicles).




Figure 2: Different ways to form intraluminal vesicles (ILVs) in multivesicular bodies (MVBs). ILVs are classically formed via the ESCRT machinery: ESCRT-0 recognizes protein, ESCRT-I and II initiate the budding process and ESCRT-III finalizes ILV formation. Three ESCRT-independent pathways are also been described: (i) the complex syndecan-syntenin-ALIX are responsible of ILVs budding in MCF7 tumor cells, (ii) ceramide can facilitate ILV invagination in oligodendroglial cells, and (iii) the tetraspanin CD9 and CD63 are involved in exosome formation in dendritic cells and in melanosomes, respectively. ( ,  , Intracellular components of EVs; ( , Membrane components of extracellular vesicles).




Figure 3: Secretion mechanisms of exosomes. Exosomes can be released via different proteins of Rab family: Rab11, Rab35, Rab27a and RAb27b and Rab7 are responsible for the transport, the docking and the fusion of the multivesicular bodies (MVBs) with the plasma membrane. At the plasma membrane, the V-SNARE protein Vamp7 may also be implicated. ( ,  , Intracellular components of exosomes; ( , Membrane components of exosomes).




Figure 4: Extracellular vesicles (EVs) may interact with target cells and convey information through different mechanisms: (i) ligand-receptor interaction, (ii) direct fusion of EVs membrane with plasma membrane of target cell, and (iii) internalization. ( ,  , Intracellular components of EVs;  , Membrane components of EVs).

Figure 5: Extracellular vesicles are biomarkers and vectors of obesity. Adipocyte-derived exosomes from obese animals can increase reactive oxygen species (ROS) production and VCAM1 expression in cardiac endothelial cells and mediate polarization towards an inflammatory M1 macrophage phenotype via miR-155. In addition, circulating microvesicles (MVs) from obese patients contain miR-197, miR23a and miR-509-5p, which may be involved in dyslipidemia.

Figure 6: Role of shear stress on microvesicle (MV) release. High shear stress plays an atheroprotective role by increasing nitric oxide (NO[•]) production under physiological conditions. NO[•] negatively regulates endothelial ATP-binding cassette (ABC)A1 expression and limits membrane remodeling. Low shear stress plays a proatherogenic role by blocking NO[•] production and NO[•]-mediated repression of ABCA1. Also, low shear stress activates endothelial Rho kinase and ERK1/2, leading to membrane remodeling and an increase of MV release.

Figure 7: Extracellular vesicles (EVs) in early atherosclerotic lesion. (i) Oxidative theory of atherosclerosis includes a primary accumulation of oxidized-LDL (oxLDL), which induces endothelial dysfunction and local inflammation. This is also associated to endothelial EV shedding, as well as, high glucose concentration. Oxidative stress and hypercholesterolemia might lead accumulation of oxLDL. MVs derived from endothelial cells (EC), platelets (P) and leukocytes (L) might contribute to endothelial dysfunction, leading to a reduction of nitric oxide (NO[•]) and an increase of reactive oxygen species (ROS) production. (ii) The interaction between ROS and NO[•] sets up a feedback loop which results in further endothelial

dysfunction and inflammation. Both endothelial dysfunction and inflammation are associated to an increase of EV release. (iii) Intercellular signals between endothelial cells and smooth muscle cells (SMCs) involve EV-mediated mechanisms. During atherosclerotic plaque formation, EVs might transfer different miRNAs to SMCs to induce change in phenotypic from contractile to secreting phenotype.

Figure 8: Extracellular vesicles (EVs) in advance atherosclerotic lesion. The accumulation of the lipids and chronic inflammatory response induce that plaque grows into confluent necrotic cores and macrophages infiltration (i), surrounded by a fibrous cap (ii). Apoptotic foam cells and SMCs are associated with an increase of EV release. As atherosclerosis progresses, oxygen diffusion is impaired favoring neovascularization. Accumulation of platelet-derive EVs (P-EVs) within plaque represents an endogenous signal of neovascularization and vulnerability by inducing endothelial cell (EC) proliferation and angiogenesis, through mechanisms involving VEGF, PI3K/Akt and transfer de miRNAs. On the other hand, EVs from ECs and leukocytes (L-EVs) decrease nitric oxide (NO) and increase reactive oxygen species (ROS) which is associated to impaired angiogenesis. (iii) EVs released by ECs, SMCs and macrophages in the vessel wall interact with fibrillar collagen, which might lead to calcification. miR-30, 126b, 143, 145 and 155 influence the expression of a specific set of osteogenic markers. Altered concentrations of these miRNAs in EVs result in changes in calcium and MAPK signaling pathways implicated in SMC-mediated calcification.

Figure 9: Possible roles of extracellular vesicles (EVs) in the central nervous system. Examples of molecules present in microvesicles (MVs) or exosomes released by brain cells are given in the boxes.

Figure 10: Representation of the effects of exosomes microvesicles (MVs) on extracellular amyloid- β peptide ($A\beta$) level and assembly. (1) $A\beta$ is derived from the

proteolytic processing of the amyloid- β precursor peptide (β APP) in early endosomes and is transported to multivesicular bodies; (2) Once released, monomeric A β aggregates to form the toxic soluble A β oligomers then fibrillar A β ; (3) Uptake of monomeric A β by exosomes from neurons or astrocytes and generation at their surface of fibrillar A β ; (4) Fibrillar and soluble A β are internalized and degraded by microglia; (5) MVs released by microglia promote the formation of soluble A β from insoluble aggregates (5a) and also contain toxic forms generated from internalized A β (5b); (6) Soluble A β form and A β storing MVs are toxic for neurons.

Figure 11: Contribution of exosomes to the trans-synaptic and exo-synaptic Tau transmission in Alzheimer's disease. Exosomes containing Tau or phosphorylated Tau can be released by astrocytes and microglia, suggesting the possibility of an exo-synaptic propagation of Tau. Also, microglia phagocytose neuron-contained Tau and then, secrete Tau in exosomes which, in turn, efficiently transmit Tau to neurons.

Figure 12. Extracellular vesicles are biomarkers for cancer. Levels of CD41⁺ microvesicles (MVs), CD14⁺ and CD62P⁺ MVs, CD31⁺CD42b⁺ and CD31⁺CD42b⁻ MVs and EGFR⁺ exosomes correlate with cancer severity in gastric, lung, non-small cell lung cancer and glioblastoma.

Figure 13. Extracellular vesicles participate on malignant transformation and tumor angiogenesis. (A) Exosomes from prostate cancer cells rich in both miR-125b and miR-130b, involved in the down-regulation of the tumor repressors, reprogram adipose stem cells from cancer patients by conferring genetic instability and oncogenic transformation. (B) Exosomes from epithelial ovarian cancer cells overexpressing CD147, an extracellular matrix metalloproteinase inducer, promote an angiogenic phenotype of cultured human endothelial cells favoring angiogenesis.








Figure 14. Extracellular vesicles (EVs) as therapeutic tools. EVs isolated from different type of sources such as bovine milk, vegetables, biofluids or cultured cells can be modified in order to enhance their therapeutic properties. They can be genetically or biochemically modified and be loaded with **therapeutic** cargo by using transfection, electroporation or incubation methods. Then, the generated EVs can be administrated by several routes to target specific tissues. (   , small molecules;  , non coding RNAs).

Figure 15. Protective effects of microvesicles (MVs) carrying Sonic hedgehog (Shh) on endothelial cells. MVs generated by pharmacological treatment from human T cells are rich in Shh and antioxidant enzymes such as catalase and superoxide dismutase (SOD) isoforms. These MVs increase nitric oxide (NO[•]) production and decrease oxidative stress by increasing expression of Mn-SOD in endothelial cells. ( ,  , Intracellular components of MVs; ( , Membrane components of MVs).

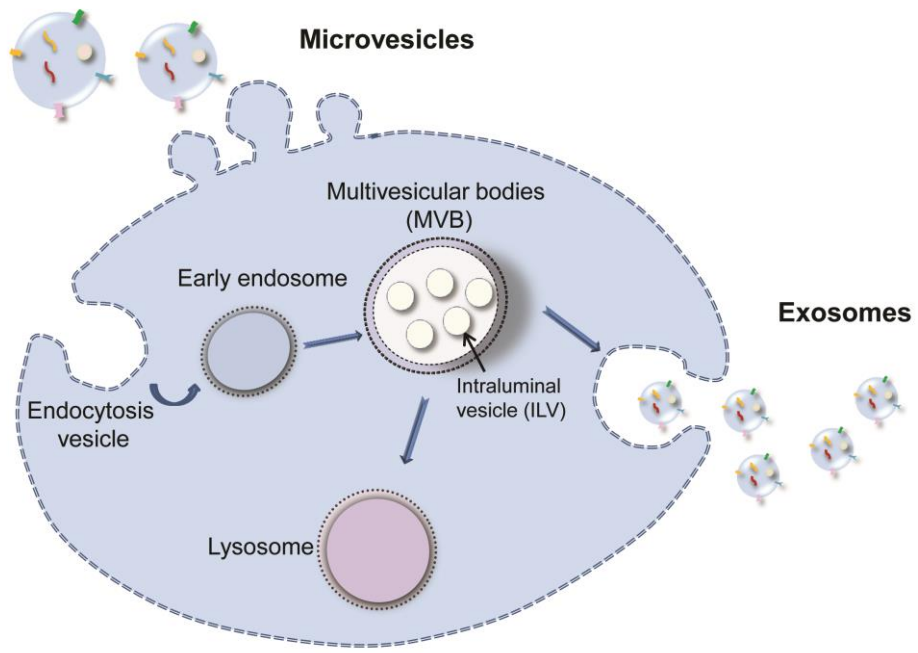


Figure 1

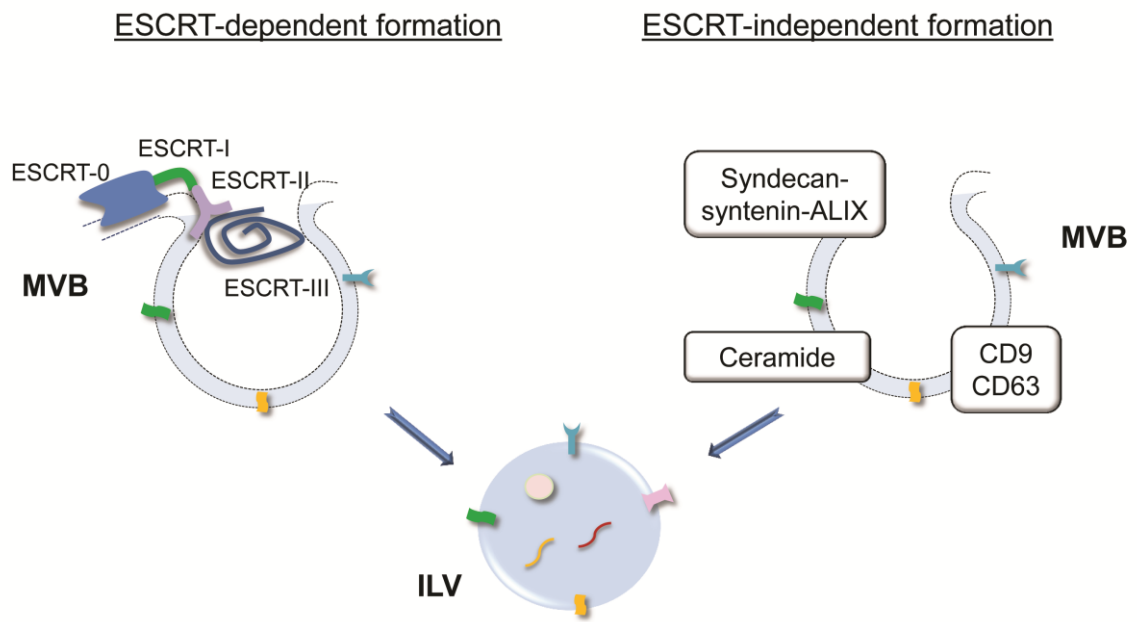


Figure 2

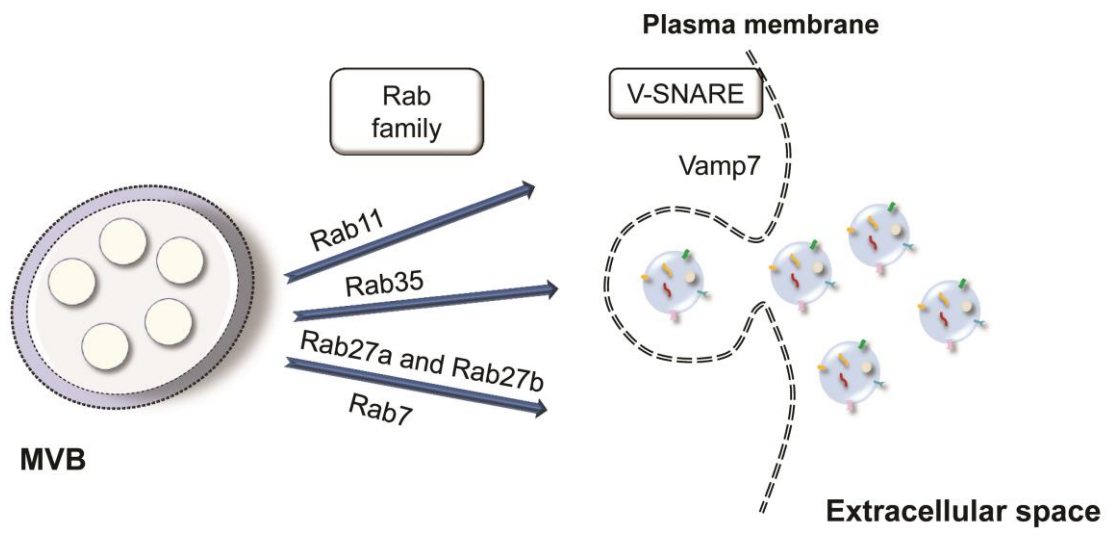


Figure 3

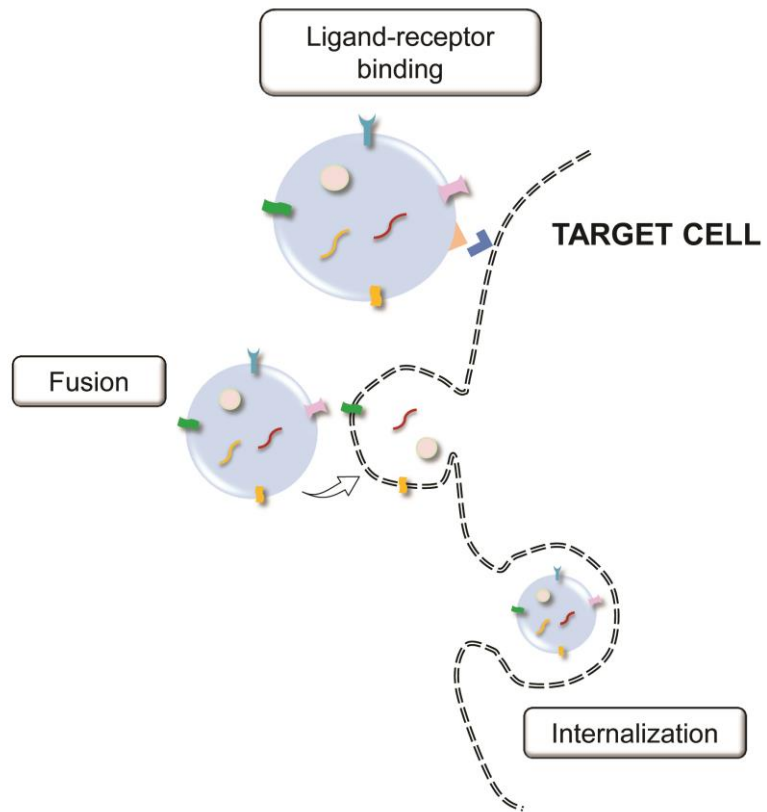


Figure 4

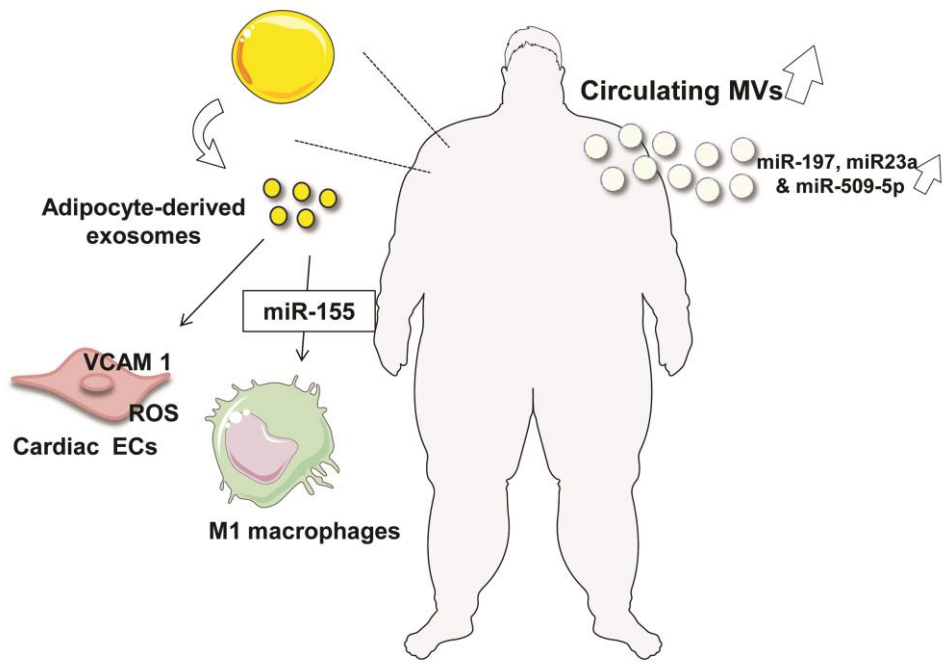


Figure 5

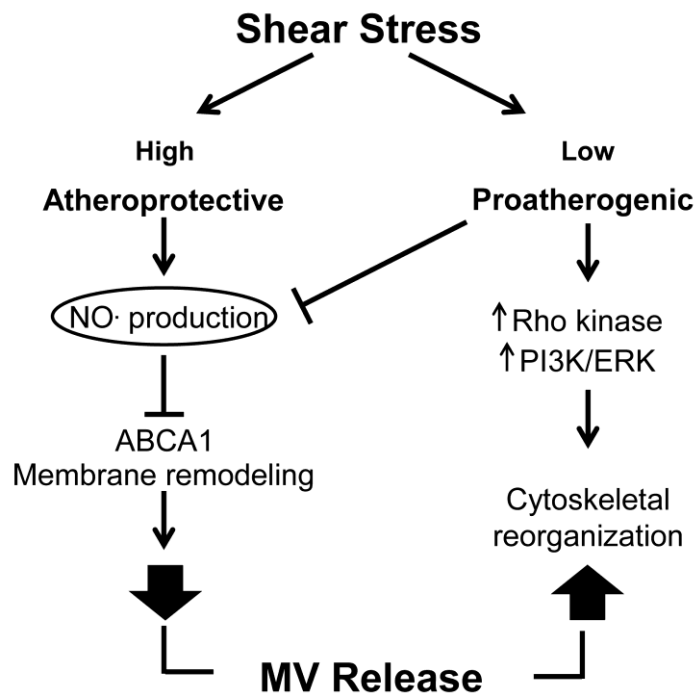


Figure 6

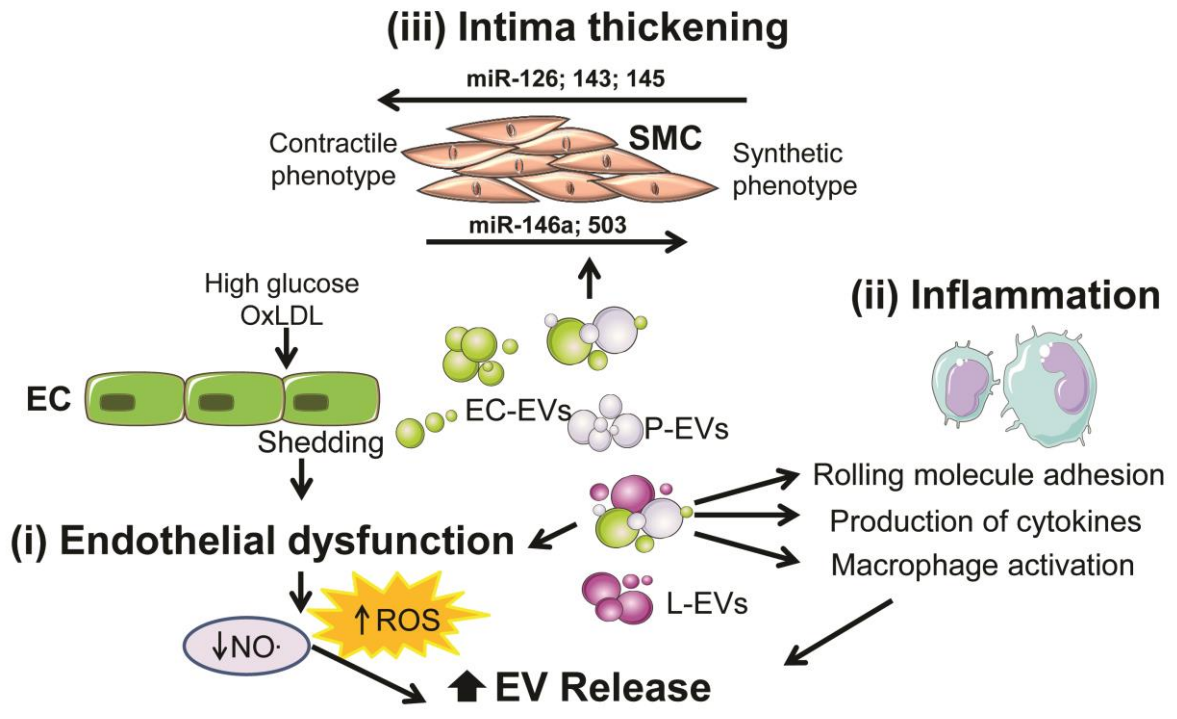


Figure 7

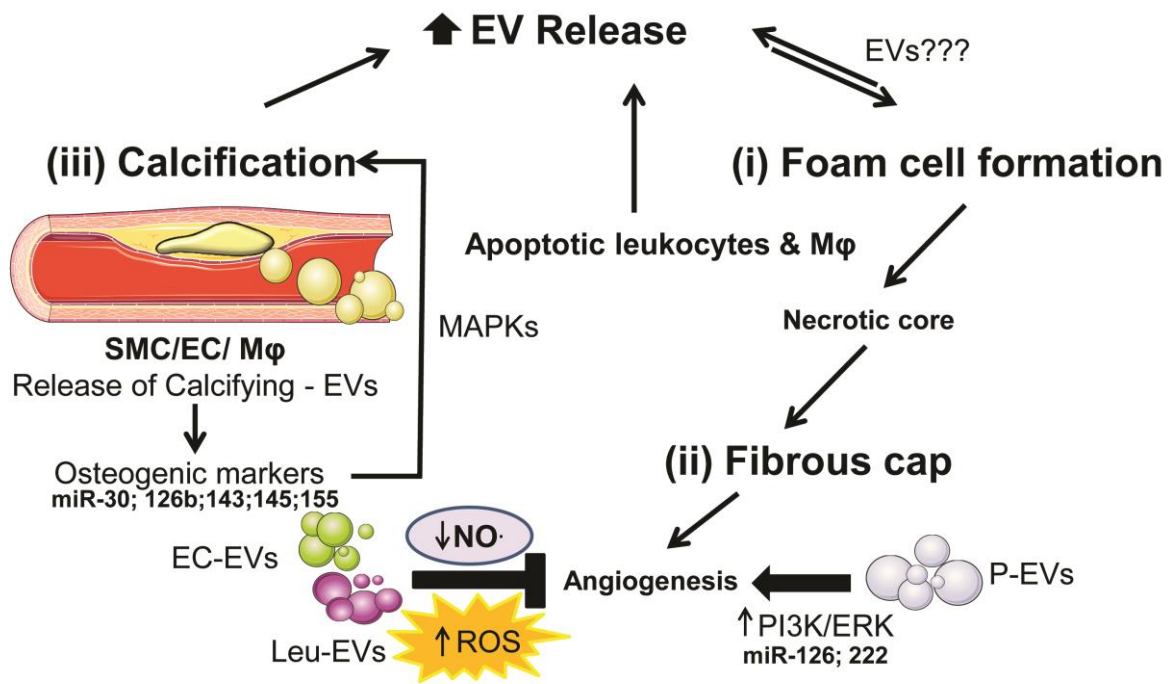


Figure 8

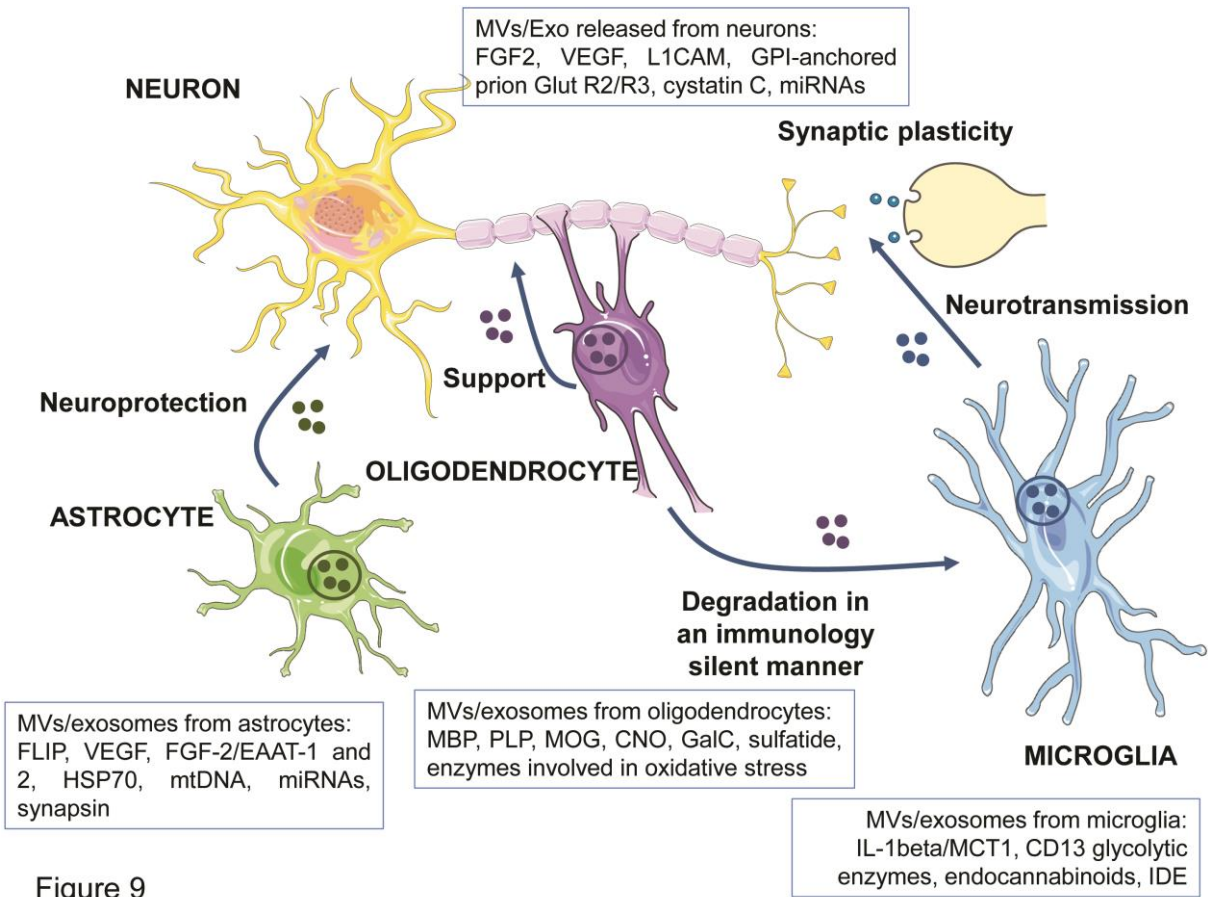


Figure 9

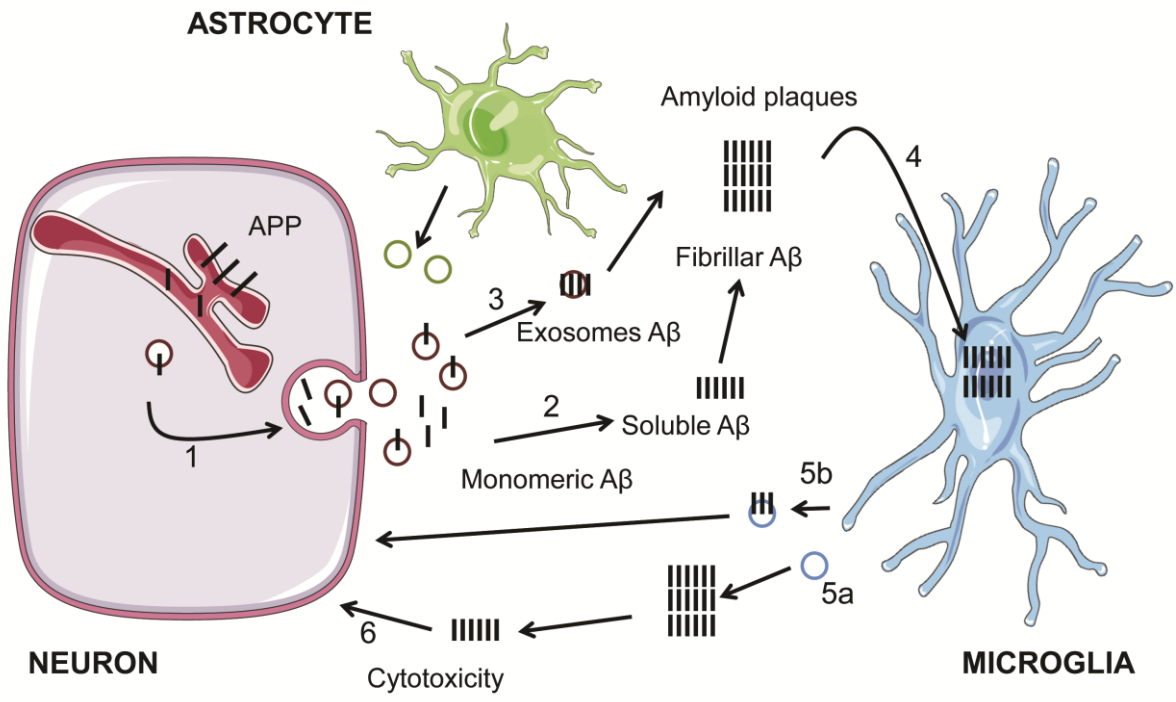


Figure 10

Microglial phagocytosis of
Tau-containing cytopathic
neurons or synapses

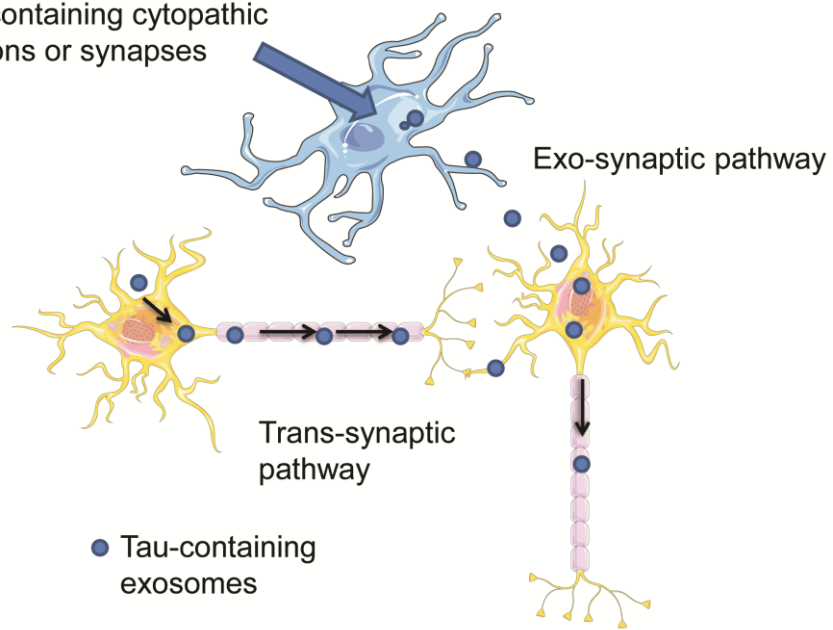


Figure 11

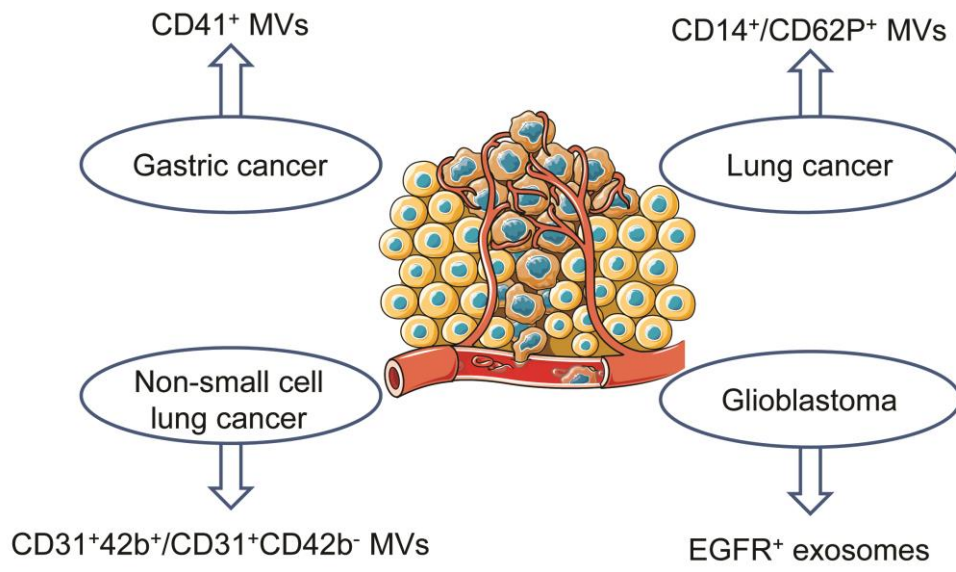


Figure 12

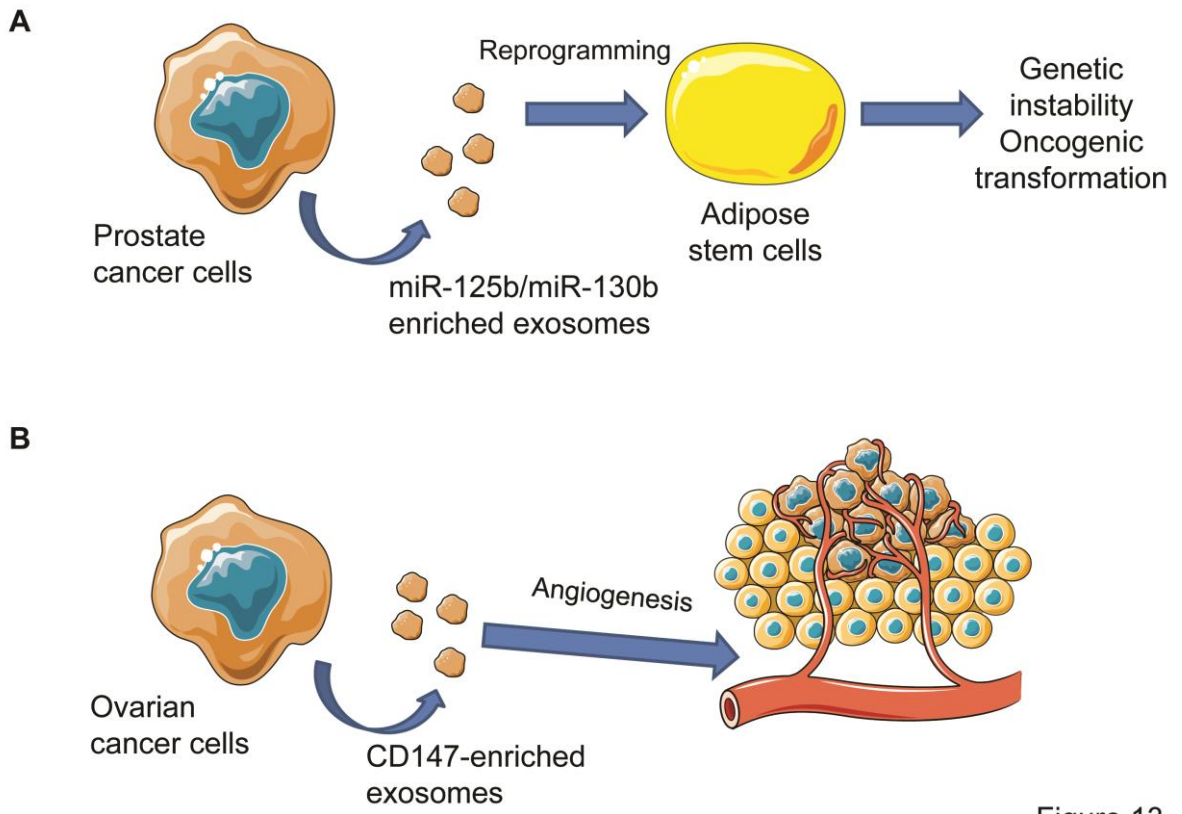


Figure 13

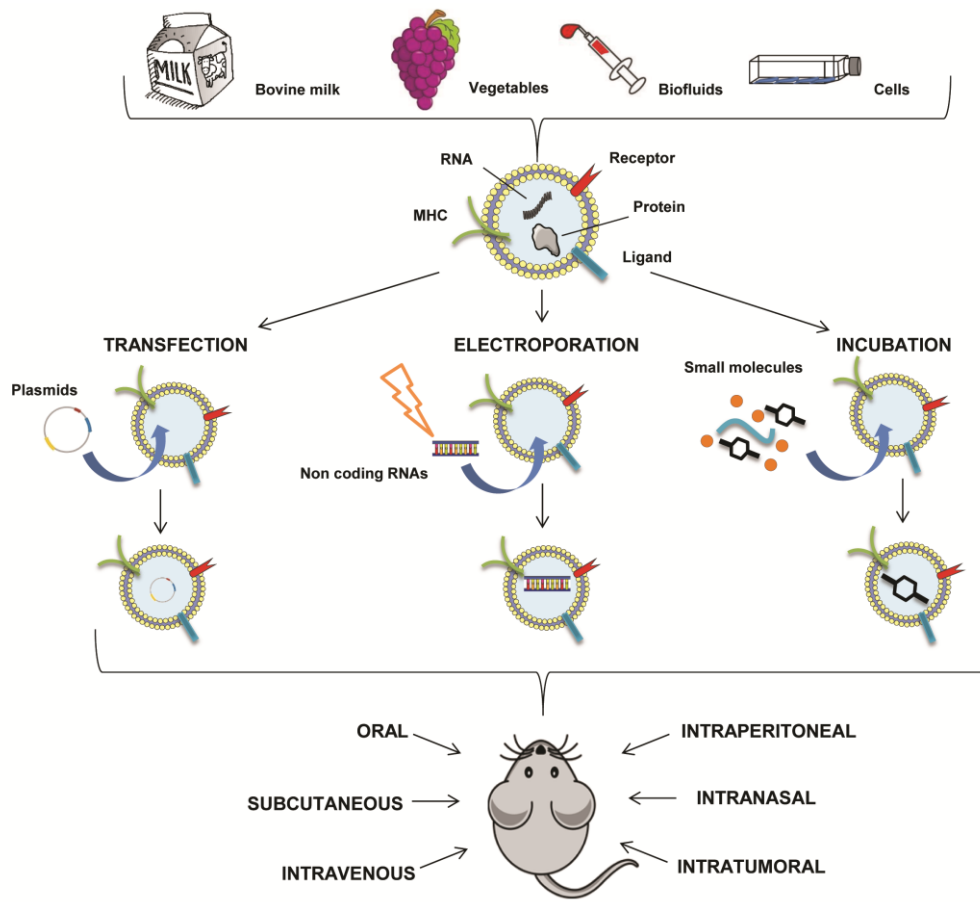


Figure 14

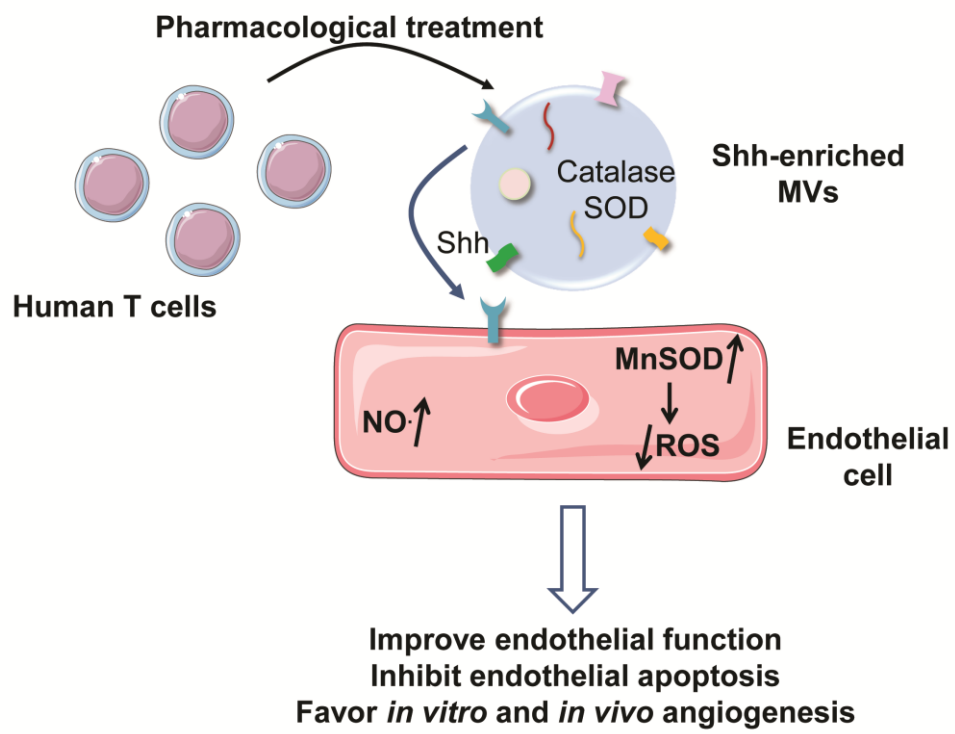


Figure 15

Thèse de Doctorat

Marine MALLOCI

Rôle des exosomes dans la dysfonction endothéliale associée au syndrome métabolique

Role of exosomes in endothelial dysfunction associated with metabolic syndrome

Résumé

Le syndrome métabolique (SM) est défini comme l'association de troubles favorisant les risques cardiovasculaires et le développement d'un diabète de type 2. Une personne atteinte de SM doit présenter au moins 3 critères sur les 5 suivants : une obésité abdominale; un taux élevé en triglycérides ; un faible taux en cholestérol HDL ; une hypertension et une hyperglycémie. Le SM est caractérisé par une dysfonction endothéliale, qui est définie par une diminution de la production de monoxyde d'azote (NO), et l'augmentation d'espèces réactives de l'oxygène, comme l'anion superoxyde (O_2^-), participant au stress oxydant.

Dans notre étude, nous avons émis l'hypothèse que les exosomes pouvaient participer à la dysfonction endothéliale chez ces patients.

Les exosomes sont des vésicules extracellulaires de petite taille (entre 30 à 100 nm), formées à partir du compartiment endo-lysosomal et libérées par la plupart des cellules. Suite à leur libération, elles peuvent interagir avec des cellules cibles et servir ainsi de vecteurs d'information biologique. Il a été montré que les exosomes pouvaient être impliqués dans de nombreuses pathologies.

Ainsi, nous avons montré que les patients atteints de SM présentent un taux d'exosomes circulants augmenté, dont la taille est diminuée. De plus, les exosomes provenant de patients SM diminuent la production de NO dans les cellules endothéliales, qui s'accompagne d'une augmentation de l' O_2^- mitochondriale. Chez la souris, les exosomes de patients SM induisent une diminution du relâchement vasculaire.

Les exosomes pourraient ainsi participer au développement de la dysfonction endothéliale chez les patients atteints de SM et pourraient être de biomarqueurs intéressants pour cette pathologie.

Mots clés

Syndrome métabolique, exosomes, dysfonction endothéliale, monoxyde d'azote, stress oxydant, mitochondrie.

Abstract

Metabolic syndrome (MetS) is defined as the combination of metabolic disorders leading to cardiovascular diseases and the development of type 2 diabetes. To be diagnosed with MetS, a person must present at least 3 of the following 5 metabolic risk factors: (i) abdominal obesity, (ii) high triglyceride levels, (iii) low HDL cholesterol levels, (iv) hypertension and (v) hyperglycaemia. MetS is characterized by endothelial dysfunction, which is defined by a decreased nitric oxide (NO) production, and an increased in reactive oxygen species such as superoxide (O_2^-), involved in oxidative stress. In our study, we hypothesized that exosomes could participate in the endothelial dysfunction observed in MetS patients. Exosomes are nanoscaled (30-100 nm) extracellular vesicles produced from the endo-lysosomal compartment and released by most of the cells of the organism. Following their release, they can interact with their target cells and be considered as biological information carriers. Exosomes have been shown to be involved in many pathologies. Thus, we have shown that MetS patients displayed an increased circulating rate of exosomes whose sizes were decreased. In addition, we have observed that exosomes from MetS patients induced (i) a decrease of NO production in endothelial cells, and (ii) an increase in mitochondrial O_2^- . Furthermore, the exosomes originating from MetS patients induced a decrease in vascular relaxation in mice. Thus, the exosomes could participate in the development of endothelial dysfunction in MetS patients and could be considered as biomarkers of interest for this pathology.

Key Words

Metabolic syndrome, exosomes, endothelial dysfunction, nitric oxide, oxidative stress, mitochondria.