Microvesicles

Subjects: Biochemistry & Molecular Biology

Contributor: Kerstin Menck , Suganja Sivaloganathan , Annalen Bleckmann , Claudia Binder

Extracellular vesicles (EV) are secreted by all cell types in a tumor and its microenvironment (TME) and play an essential role in intercellular communication and the establishment of a TME favorable for tumor invasion and metastasis. They encompass a variety of vesicle populations, among them the well-known endosomal-derived small exosomes (Exo), but also larger vesicles (diameter >100 nm) that are shed directly from the plasma membrane, the so-called microvesicles (MV). Increasing evidence suggests that MV, although biologically different, share the tumor-promoting features of Exo in the TME. Due to their larger size, they can be readily harvested from patients' blood and characterized by routine methods such as conventional flow cytometry exploiting the plethora of molecules expressed on their surface. In this review we summarize the current knowledge about the biology and the composition of MV as well as their role within the TME. We highlight not only the challenges and potential of MV as novel biomarkers for cancer, but also discuss their possible use for therapeutic intervention.

microvesicles biomarker cancer tumor microenvironment therapy

1. Introduction

On the basis of various characteristics, ranging from size, biogenesis, cell of origin, morphology, and content, EV are categorized into four main classes: endosomal-derived small exosomes (Exo) (50-150 nm), plasma membrane-derived middle-sized microvesicles (MV) (100-1000 nm), and large oncosomes (LO) (1000-10,000 nm), as well as apoptotic bodies (500-4000 nm) that are released from dying cells[1][2]. Although commonly used to categorize EV, this classification has been challenged by recent evidence demonstrating that, for instance, the Exo contain further subtypes with different biological and biochemical properties[3].

While much attention has been paid to the role of Exo in cancer, the function of the larger MV is still poorly defined. This seems surprising, since MV, in contrast to Exo, are easily accessible in patients' blood and are characterizable by routine methods that should make them ideal candidates for "liquid biopsies". Additionally, MV have long been known for their involvement in metastasis formation. This was initially attributed to their procoagulant activity, favoring the formation of microthrombi and facilitating the extravasation of the thus captured circulating tumor cells (reviewed in[4]). However, more recently, accumulating evidence points to a plethora of different ways in which MV are involved in the various steps of tumor progression.

2. Preparation of MV

In order to decipher the role of MV in cancer development and progression, effective methods are required that allow for the stringent isolation of pure MV populations from different cell types and biological fluids. A major caveat in MV research is that the currently available isolation methods potentially co-isolate LO or Exo, yielding a mixed population of EV. This may explain many of the apparently conflicting results in the field of EV research. To address this major challenge, new technologies are under development, but they are not yet suitable for laboratory use. In an endeavor to standardize the experimental procedures and limit experimental variability in the field, scientists of the International Society of Extracellular Vesicles (ISEV) published a position paper indicating the appropriate methods for isolation of EV from cells or biological fluids and highlighting the current knowledge and major caveats of these procedures[5]. A variety of methods are available on the basis of different principles for enriching the various EV subpopulations, including density gradient centrifugation, size-exclusion chromatography, precipitation via volume-excluding polymers, immunoaffinity capture methods, high-pressure liquid chromatography, field flow fractionation, and flow cytometry[5][6].

To date, differential centrifugation is still the method of choice for isolating MV since it yields a reasonably good separation of MV from Exo with regard to protein and RNA content as well as function[7][8][9]. In principle, samples are spun down at 2000 g to initially pellet large EV such as LO, followed by centrifugation at 10,000-20,000 g to sediment the middle-sized MV, while ultracentrifugation at ≥100,000 g leads to a harvest of small Exo[6]. Although ultracentrifugation has been criticized for inducing vesicle aggregation and thus affecting downstream applications[10][11][12], these studies were conducted on the 100,000 g Exo pellet. It is unclear to what extent these problems also apply to the 10,000 g MV pellet as well. Another popular method to separate EV from contaminations with non-vesicular proteins or RNA aggregates is ultracentrifugation of EV samples on density gradients. However, the typically used sucrose gradients lack sufficient resolution to separate EV that have slightly different densities and are released by different mechanisms[13]. Since the density of MV and Exo is comparable, they cannot be separated on sucrose gradients easily[9]. Likewise, precipitation methods such as protein organic solvent precipitation (PROSPR) have been demonstrated to co-isolate MV, but the preparations were mainly enriched in smaller Exo, with MV being only a minor side population[14]. Immunoaffinity capture methods rely on antibodies for specific EV surface markers that are coupled to beads and used to isolate specific vesicle subpopulations. Due to the current lack of specific markers for MV, this method has been scarcely used for the preparation of MV. Currently, novel micro-/nano-based devices for MV isolation from clinical samples are being developed (reviewed in[15]). However, one major problem is that the thus isolated vesicle preparations have often been poorly characterized and it is unclear whether they yield MV with a purity comparable to sequential centrifugation protocols. The same applies to methods originally established for Exo isolation such as precipitation or size-exclusion chromatography. While none of the currently available isolation procedures for MV and Exo yield pure vesicle populations, but rather MV- or Exo-enriched fractions, more effective techniques that separate pure MV from Exo might help to shed light on their distinct cell biological features such as biogenesis, uptake, and cargo trafficking routes. Then again, from a clinical viewpoint, the isolation of pure MV populations might not be absolutely necessary for their use as biomarkers, since recent studies have demonstrated the potential of MV for cancer diagnostics or therapy monitoring, even when the preparations contain some smaller Exo.

No matter which method is chosen for EV isolation, the researchers of the ISEV community highly recommend validating the obtained EV by different techniques[5]. This includes the analysis of marker protein expression comprising (i) transmembrane, (GPI)-anchored, as well as cytosolic EV marker proteins (e.g., CD63, CD9, Alix, Syntenin, Rgap1, along with negative controls consisting of (ii) non-EV co-isolated proteins (e.g. ApoB, albumin), (iii) proteins typically present in non-EV subcellular structures such as the Golgi or endoplasmatic reticulum (e.g., GM130, Calreticulin, histones), and (iv) secreted proteins (e.g., collagen, epidermal growth factor (EGF), interleukins). Moreover, EV should be characterized by at least two distinct techniques including their visualization by, for instance, electron or atomic force microscopy, as well as the analysis of their biophysical properties via, for example, nanoparticle tracking analysis (NTA) or Raman spectroscopy. Nanoparticle tracking analysis (NTA) is the most suitable method for analyzing the isolated MV in terms of quantification and sizing. NTA tracks the Brownian movement of laser-illuminated particles and calculates the diameter based on the Stokes–Einstein equation[16]. While NTA remains the most commonly used method for quantitative MV analysis, other methods such as tunable resistive pulse sensing or dynamic light scattering are also available[17]. However, a major limitation of these methods is that they cannot efficiently analyze larger vesicles and that they do not yield any information on the molecular composition of the MV. They are, therefore, combined with other methods such as tunable resistive pulse sensing and Raman spectroscopy or NTA with fluorescence labelling to obtain more information about the isolated MV[17].

A major caveat in MV research is that the currently available isolation methods potentially co-isolate LO or Exo, yielding a mixed population of EV. This may explain many of the apparently conflicting results in the field of EV research. To address this major challenge, new technologies are under development, but are not yet suitable for laboratory use. In an endeavor to standardize the experimental procedures and limit experimental variability in the field, scientists of the International Society of Extracellular Vesicles (ISEV) published a position paper that indicated the appropriate methods for isolation of EV from cells or biological fluids and highlighted the current knowledge and major caveats of these procedures[5]. Furthermore, the researchers of the ISEV community highly recommend validating different techniques for various cell types and biological fluids. A crowdsourcing knowledge base was established to create further transparency with regards to experimental and methodological parameters of EV isolation (http://evtrack.org)[18]. This platform encourages researchers to upload published and unpublished experiments, thereby creating an informed dialogue among researchers about relevant experimental parameters. This represents a major step in facilitating standardization in EV, as well as MV, research.

3. Biogenesis of MV

MV directly bud off from the outer cell membrane. The shedding process comprises molecular rearrangements of the plasma membrane regarding lipid and protein composition as well as Ca2+ levels. Ca2+-dependent aminophospholipid translocases, flippases, floppases, scramblases, and calpain drive the translocation of phosphatidylserine from the inner to the outer membrane leaflet, which is considered a typical characteristic of MV (comprehensively reviewed in[19]). Apoptotic bodies, which are larger in size, also externalize phosphatidylserine on their surface[20][21]. Therefore, the isolation of MV should be conducted solely from healthy and viable cells to

avoid contamination with apoptotic bodies, which otherwise can be difficult to discriminate from MV. Ca2+ levels regulate membrane rigidity and curvature and maintain physical bending of the membrane, which leads to restructuring and contraction of the underlying actin cytoskeleton enabling MV formation and pinching (reviewed in[22]). MV formation and release are also affected by the lipids ceramide and cholesterol[23]. Neutral sphingomyelinase activity, which hydrolyses lipid sphingomyelin into phosphorylcholine and ceramide, was shown to be involved in Exo and MV release. Inhibition of the enzyme led to a reduction in Exo release, while simultaneously increasing MV budding[24], which suggests that the release of both EV subpopulations is interconnected, albeit on the basis of distinct biogenetic mechanisms.

In addition to lipids, enzyme machineries involved in cytoskeletal regulation play a key role in MV formation and budding. One example is the small GTPase protein ADP-ribosylation factor 6 (ARF6), which stimulates phospholipase D (PLD) that subsequently associates with extracellular signal-regulated kinase (ERK) at the plasma membrane. ERK activates a signaling cascade downstream of the myosin light chain kinase (MLCK) that results in contraction of actinomyosin and enables MV release[25]. Similar to MV, LO are thought to derive from the plasma membrane. However, in contrast to MV, the shedding of LO has been exclusively attributed to aggressive cancer cells that have acquired an amoeboid phenotype to facilitate motility and invasiveness ([2]). Other Rho family small GTPases such as RhoA and RHO-associated protein kinases are equally important regulators of actin dynamics relevant for MV formation[26]. In addition, the endosomal sorting complex required for transport (ESCRT), which is mainly known for its role in the biogenesis of Exo[27], is also involved in MV formation and the last phase of their release. Interaction of arrestin domain-containing protein 1 (ARRDC1) with the late endosomal protein tumor susceptibility gene 101 (TSG101) results in relocation of TSG101 from the endosomal to the plasma membrane, which then induces the release of MV[28].

4. Membrane Composition of MV

EV-mediated cell–cell communication requires targeting and uptake into the recipient cell to deliver the bioactive cargo, which then induces functional and phenotypical changes. These events depend on the composition of the EV membrane, as surface molecules on EV are responsible for binding and docking to recipient cells[29][30]. The molecular composition of the MV membrane closely resembles that of the parental cell[31]. It is enriched in phospholipid lysophosphatidylcholine, sphingolipid sphingomyelins, acylcarnitine, and fatty acyl esters of L-carnitine[32]. ARF6, a key regulator of MV biogenesis, was shown to mediate MV surface molecule selection by recruiting proteins such as ß1 integrin receptor, major histocompatibility complex (MHC) class I and II molecules, membrane type 1-matrix metalloproteinase (MT1-MMP), vesicular SNARE (v-SNARE), and vesicle-associated membrane protein 3 (VAMP3) to tumor MV[25]. Moreover, the bioactive cargo of MV depends on the conditions the parental cells are subjected to, such as inflammation or other stressors. An additional example is hypoxia, which induces recruitment of the RAS-related protein Rab22a to the site of MV budding in breast cancer cells, thus influencing MV formation and loading[33].

Since both LO and MV are derived from the plasma membrane, it is not surprising that some transmembrane proteins are present on either of these EV. Analysis of protein marker expression on LO and MV revealed a

common signature, underlining the fact that the definition of MV-specific markers remains challenging. Of note, some of the markers initially thought to be specific for Exo, including tetraspanins (CD9, CD63, CD81, HSP60, HSP70, HSP90), membrane transporters and fusion proteins (annexin, flotillin), and multivesicular body (MVB) synthesis proteins (Alix, TSG101) were also found in varying amounts on MV and LO[7]. The fact that, despite their different routes of origin, EV share some common surface molecules and MV-specific markers are still lacking represents major challenges in EV research. It further emphasizes that to correctly characterize EV populations it is indispensable to combine a variety of parameters in addition to marker expression, such as size, sedimentation coefficient, and others.

References

1. Yáñez-Mó, M.; Siljander, P.R.-M.; Andreu, Z.; Bedina Zavec, A.; Borràs, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J.; et al. Biological properties of extracellular vesicles and their physiological functions. J. Extracell. Vesicles 2015, 4, 27066.
2. Minciacchi, V.R.; Freeman, M.R.; Di Vizio, D. Extracellular Vesicles in Cancer: Exosomes, Microvesicles and the Emerging Role of Large Oncosomes. Semin. Cell Dev. Biol. 2015, 40, 41–51
3. Sang-Soo Lee; Jong-Hoon Won; Gippeum J. Lim; Jeongran Han; Ji Youn Lee; Kyung-Ok Cho; Young-Kyung Bae; A novel population of extracellular vesicles smaller than exosomes promotes cell proliferation. *Cell Communication and Signaling* **2019**, *17*, 1-15, 10.1186/s12964-019-0401-z.
4. Soraya Mezouar; Diane Mège; Roxane Darbousset; Dominique Farge; P. Debourdeau; Françoise Dignat-George; Laurence Panicot-Dubois; Christophe Dubois; Involvement of Platelet-Derived Microparticles in Tumor Progression and Thrombosis. *Seminars in Oncology* **2014**, *41*, 346-358, 10.1053/j.seminoncol.2014.04.010.
5. Clotilde Théry; Kenneth Witwer; Elena Aikawa; Maria Jose Alcaraz; Johnathon D Anderson; Ramaroson Andriantsitohaina; Anna Antoniou; Tanina Arab; Fabienne Archer; Georgia K Atkin-Smith; et al.D Craig AyreJean-Marie BachDaniel BachurskiHossein BaharvandLeonora BalajShawn BaldacchinoNatalie N BauerAmy A BaxterMary BebawyCarla BeckhamApolonija Bedina ZavecAbderrahim BenmoussaA C BerardiPaolo BergeseEwa BielskaCherie BlenkironSylwia Bobis-WozowiczEric BoilardWilfrid BoireauAntonella BongiovanniFrancesc E. BorrasSteffi BöshC. M. BoulangerXandra BreakefieldAndrew M BreglioMeadhbh Á BrennanDavid R BrigstockAlain BrissonMarike Ld BroekmanJacqueline F BrombergPaulina Bryl-GóreckaShilpa BuchAmy H BuckDylan BurgerSara BusattoDominik BuschmannBenedetta BussolatiEdit I BuzásJ. Brian ByrdGiovanni CamussiDavid Rf CarterSarah CarusoLawrence W. ChamleyYu-Ting ChangChihchen ChenShuai ChenLesley ChengAndrew R ChinAled ClaytonStefano P ClericiAlex CocksEmanuele CocucciRobert J CoffeyAnabela Cordeiro-Da-SilvaYvonne CouchFrank Aw

CoumansBeth CoyleRossella CrescitelliMiria Ferreira CriadoCrislyn D’Souza-SchoreySaumya DasAmrita Datta ChaudhuriPaola De CandiaEliezer F De SantanaOlivier De WeverHernando A Del PortilloTanguy DemaretSarah DevilleA. DevittBert DhondtDolores Di VizioLothar C DieterichVincenza DoloAna Paula Domínguez RubioMassimo DominiciMauricio R DouradoTom A. P. DriedonksFilipe V. DuarteHeather M DuncanRamon M. EichenbergerKarin EkstromSamir El AndaloussiCeline Elie-CailleUta ErdbrüggerJuan M. Falcón-PérezFarah FatimaJason E. FishMiguel Flores-BellverAndrás FörsönitsAnnie Frelet-BarrandFabia FrickeGregor FuhrmannSusanne GabrielssonAna Gámez-ValeroChris GardinerKathrin GärtnerRaphaël GaudinYong Song GhoBernd GiebelCaroline GilbertMario GimonaIlaria GiustiDeborah Ci GoberdhanAndré GörgensSharon M. GorskiDavid W. GreeningJulia Christina GrossAlice GualerziGopal N GuptaDakota GustafsonAase HandbergReka A HaraszтiPaul HarrisonHargita HegyesiAn HendrixAndrew HillFred H HochbergKarl F HoffmannBeth HolderHarry HolthoferBaharak HosseinkhaniGuoku HuYiyao HuangVeronica HuberStuart HuntAhmed Gamal-Eldin IbrahimTsuneya IkezuJameel M. InalMustafa IşınAlena IvanovaHannah K JacksonSøren JacobsenSteven M JayMuthuvel JayachandranGuido JensterLanzhou JiangSuzanne M. JohnsonJennifer C. JonesAmbrose JongTijana Jovanovic-TalismanStephanie JungRaghu KalluriShin-Ichi KanoSukhbir KaurYumi KawamuraEvan T KellerDelaram KhamariElena KhomyakovaAnastasia KhvorovaPeter KierulfKwang Pyo KimThomas KislingerMikael KlingebornDavid J KlinkeMiroslaw KornekMaja M. KosanovićÁrpád Ferenc KovácsEva-Maria Krämer-AlbersSusanne KrasemannMirja KrauseIgor V KurochkinGina D KusumaSören KuypersSaara LaitinenScott M. LangevinLucia R. LanguinoJoanne LanniganCecilia LässerLouise C LaurentGregory LavieuElisa Lazaro-IbanezSoazig Le LayMyung-Shin LeeYi Xin Fiona LeeDebora S LemosMetka LenassiAleksandra LeszczynskaIsaac Ts LiKe LiaoSten F LibregtsErzsebet LigetiRebecca LimSai Kiang LimAija LinēKaren LinnemannstönsAlicia LlorenteCatherine A LombardMagdalena J LorenowiczÁkos M. LőrinczJan LötvallJason LovettMichelle C LowryXavier LoyerQuan LuBarbara LukomskaTaral R LunavatSybren Ln MaasHarmeet MalhiAntonio MarcillaJacopo MarianiJavier MariscalElena Martens-UzunovaLorena Martín-JaularM Carmen MartinezVilma R MartinsMathilde MathieuSuresh MathivananMarco MaugeriLynda K McGinnisMark J McVeyDavid G MeckesKatie L MeehanInge MertensValentina R MinciacchiAndreas MöllerMalene Møller JørgensenAizea Morales-KastresanaJess MorhayimFrançois MullierMaurizio MuracaL. MusanteVeronika MussackDillon C MuthKathryn H. MyburghTanbir NajranaMuhammad NawazIrina NazarenkoPeter NejsumChristian NeriTommaso NeriRienk Nieuwland2Leonardo NimrichterJohn P NolanEsther Nm Nolte-’T HoenNicole Noren HootenLorraine O’DriscollTina O’GradyAna O'loghlenTakahiro OchiyaMartin OlivierAlberto OrtizLuis A. OrtizXabier OsteikoetxeaOle ØstergaardMatias OstrowskiJaesung ParkD. Michiel PegtelHéctor PeinadoFrancesca PerutMichael W. PfafflDonald G. PhinneyBartijn Ch PietersRyan PinkDavid S PisetskyElke Pogge Von StrandmannIva PolakovicovaIvan Ka Ho PoonBonita H PowellIlaria PradaLynn PulliamPeter QuesenberryAnnalisa RadeghieriRobert L RaffaiStefania RaimondoJanusz RakMarcel I RamirezGraça RaposoMorsi S RayyanNeta Regev-

RudzkiFranz L RicklefsPaul D RobbinsDavid D. RobertsSilvia C RodriguesEva RohdeSophie RomeKasper Ma RouschopAurelia RughettiAshley E RussellPaula SaáSusmita SahooEdison Salas-HuenuleoCatherine SánchezJulie A. SaugstadMeike J SaulRaymond M. SchiffelersRaphael SchneiderTine Hiorth SchøyenAaron ScottEriomina ShahajShivani SharmaOlga ShatnyevaFaezeh ShekariGanesh Vilas ShelkeAshok K. Shett Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **2018**, *7*, 1535750, 10.1080/20013078.2018.1535750.

6. Bogdan Mateescu; Emma J. K. Kowal; Bas W. M. Van Balkom; Sabine Bartel; Suvendra N. Bhattacharyya; Edit I. Buzás; Amy H. Buck; Paola De Candia; Franklin Wang-Ngai Chow; Saumya Das; et al.Tom A. P. DriedonksLola Fernández-MessinaFranziska HaderkAndrew HillJennifer C. JonesKendall R. Van Keuren-JensenCharles P. LaiCecilia LässerItalia Di LiegroTaral R. LunavatMagdalena J. LorenowiczSybren L. N. MaasImre MagerMaría MittelbrunnStefan MommaKamalika MukherjeeMuhammad NawazD. Michiel PegtelMichael W. PfafflRaymond M. SchiffelersHidetoshi TaharaClotilde ThéryJuan Pablo TosarMarca H. M. WaubenKenneth WitwerEsther N. M. Nolte-‘T HoenMuhammed Nawaz Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper. *Journal of Extracellular Vesicles* **2017**, *6*, 1286095, 10.1080/20013078.2017.1286095.
7. Kowal, J.; Arras, G.; Colombo, M.; Jouve, M.; Morath, J.P.; Primdal-Bengtson, B.; Dingli, F.; Loew, D.; Tkach, M.; Théry, C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc. Natl. Acad. Sci. USA 2016, 113, E968–E977.
8. Crescitelli, R.; Lässer, C.; Szabó, T.G.; Kittel, A.; Eldh, M.; Dianzani, I.; Buzás, E.I.; Lötvall, J. Distinct RNA profiles in subpopulations of extracellular vesicles: Apoptotic bodies, microvesicles and exosomes. J. Extracell. Vesicles 2013, 2, 20677.
9. Menck, K.; Scharf, C.; Bleckmann, A.; Dyck, L.; Rost, U.; Wenzel, D.; Dhople, V.M.; Siam, L.; Pukrop, T.; Binder, C.; et al. Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN. J. Mol. Cell Biol. 2015, 7, 143–153.
10. Linares, R.; Tan, S.; Gounou, C.; Arraud, N.; Brisson, A.R. High-speed centrifugation induces aggregation of extracellular vesicles. J. Extracell. Vesicles 2015, 4.
11. Nordin, J.Z.; Lee, Y.; Vader, P.; Mäger, I.; Johansson, H.J.; Heusermann, W.; Wiklander, O.P.B.; Hällbrink, M.; Seow, Y.; Bultema, J.J.; et al. Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. Nanomed. Nanotechnol. Biol. Med. 2015, 11, 879–883.
12. Mol, E.A.; Goumans, M.-J.; Doevendans, P.A.; Sluijter, J.P.G.; Vader, P. Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to

ultracentrifugation. Nanomed. Nanotechnol. Biol. Med. 2017, 13, 2061–2065.

13. Angélique Bobrie; Marina Colombo; Sophie Krumeich; Graça Raposo; Clotilde Théry; Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *Journal of Extracellular Vesicles* **2012**, *1*, null, 10.3402/jev.v1i0.18397.
14. Xavier Gallart-Palau; Aida Serra; Andrew See Weng Wong; Sara Sandin; Mitchell K. P. Lai; Christopher P. Chen; Oi Lian Kon; Siu Kwan Sze; Extracellular vesicles are rapidly purified from human plasma by PRotein Organic Solvent PRecipitation (PROSPR). *Scientific Reports* **2015**, *5*, 14664, 10.1038/srep14664.
15. Jina Ko; Erica Carpenter; David Issadore; Detection and isolation of circulating exosomes and microvesicles for cancer monitoring and diagnostics using micro-/nano-based devices.. *The Analyst* **2016**, *141*, 450-460, 10.1039/c5an01610j.
16. Beate Vestad; Alicia Llorente; Axl Neurauter; Santosh Phuyal; Bente Kierulf; Peter Kierulf; Tore Skotland; Kirsten Sandvig; Kari Bente F. Haug; Reidun Øvstebø; et al. Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *Journal of Extracellular Vesicles* **2017**, *6*, 1344087, 10.1080/20013078.2017.1344087.
17. Chris Gardiner; Dolores Di Vizio; Susmita Sahoo; Clotilde Théry; Kenneth Witwer; Marca Wauben; Andrew Hill; Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *Journal of Extracellular Vesicles* **2016**, *5*, 32945, 10.3402/jev.v5.32945.
18. Jan Van Deun; EV-TRACK Consortium; Pieter Mestdagh; Patrizia Agostinis; Özden Akay; Sushma Anand; Jasper Anckaert; Zoraida Andreu Martinez; Tine Baetens; Els Beghein; et al.Laurence BertierGeert BerxJanneke BoereStephanie BoukourisMichel BremerDominik BuschmannJ. Brian ByrdClara CasertLesley ChengAnna CmochDelphine DaveloosEva De SmedtSeyma DemirsoyVictoria DepoorterBert DhondtTom A. P. DriedonksAleksandra DudekAbdou ElSharawyIlaria FlorisAndrew D. FoersKathrin GärtnerAbhishek D. GargEdward GeeurickxJan GettemansFarzaneh GhazaviBernd GiebelTom Groot KormelinkGrace HancockHetty H. HelsmoortelAndrew HillVincent HyenneHina KalraDavid KimJoanna KowalSandra KraemerPetra LeidingerCarina LeonelliYaxuan LiangLien LippensShu LiuAlessandra Lo CiceroShaun MartinSuresh MathivananPrabhu MathiyalaganTámas MatusekGloria MilaniMarta Monguió-TortajadaLiselot M. MusDillon C MuthAndrea NémethEsther N. M. Nolte-Â€™T HoenLorraine O’DriscollRoberta PalmulliMichael W. PfafflBjarke Primdal-BengtsonErminia RomanoQuentin RousseauSusmita SahooNatalia SampaioMonisha SamuelBenjamin SciclunaBieke SoenAnneleen SteelsJohannes V. SwinnenMaarit TakataloSafia ThaminyClotilde ThéryJoeri TulkensIsabel Van AudenhoveSusanne Van Der GreinAlan Van GoethemMartijn J. C. Van HerwijnenGuillaume Van NielNadine Van RoyAlexander R. Van VlietNiels VandammeSuzanne VanhauwaertGlenn VergauwenFrederik Johannes

VerweijAnnelynn WallaertMarca WaubenKenneth WitwerMarijke I. ZonneveldOlivier De WeverJo VandesompeleAn Hendrix EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nature Chemical Biology* **2017**, *14*, 228-232, 10.1038/nmeth.4185.

19. Jack Taylor; Mary Bebawy; Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer. *PROTEOMICS* **2019**, *19*, 1800165, 10.1002/pmic.201800165.
20. Battistelli, M.; Falcieri, E. Apoptotic Bodies: Particular Extracellular Vesicles Involved in Intercellular Communication. Biology 2020, 9, 21.
21. Birge, R.B.; Boeltz, S.; Kumar, S.; Carlson, J.; Wanderley, J.; Calianese, D.; Barcinski, M.; Brekken, R.A.; Huang, X.; Hutchins, J.T.; et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell Death Differ. 2016, 23, 962–978.
22. Guillaume Van Niel; Gisela D'angelo; Graça Raposo; Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology* **2018**, *19*, 213-228, 10.1038/nrm.2017.125.
23. Alanna E. Sedgwick; Crislyn D'Souza-Schorey; The biology of extracellular microvesicles. *Traffic* **2018**, *19*, 319-327, 10.1111/tra.12558.
24. Kerstin Menck; Can Sönmezer; Thomas Stefan Worst; Matthias Schulz; Gry Helene Dihazi; Frank Streit; Gerrit Erdmann; Simon Kling; Michael Boutros; Claudia Binder; et al.Julia Christina Gross Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. *Journal of Extracellular Vesicles* **2017**, *6*, 1378056, 10.1080/20013078.2017.1378056.
25. Vandhana Muralidharan-Chari; James Clancy; Carolyn Plou; Maryse Romao; Philippe Chavrier; Graça Raposo; Crislyn D'Souza-Schorey; ARF6-Regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. *Current Biology* **2009**, *19*, 1875-1885, 10.1016/j.cub.2009.09.059.
26. Bo Li; Marc A. Antonyak; Jingwen Zhang; Richard A. Cerione; RhoA triggers a specific signaling pathway that generates transforming microvesicles in cancer cells. *Oncogene* **2012**, *31*, 4740-4749, 10.1038/onc.2011.636.
27. M. Colombo; Catarina Ferreira Moita; Guillaume Van Niel; Joanna Kowal; J. Vigneron; Philippe Benaroch; Nicolas Manel; Luis F. Moita; Clotilde Théry; G. Raposo; et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *Journal of Cell Science* **2013**, *126*, 5553-5565, 10.1242/jcs.128868.
28. Joseph F. Nabhan; Ruoxi Hu; Raymond S. Oh; Stanley N. Cohen; Quan Lü; Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proceedings of the National Academy of Sciences* **2012**, *109*, 4146-4151, 10.1073/pnas.1200448109.

29. Hoshino, A.; Costa-Silva, B.; Shen, T.-L.; Rodrigues, G.; Hashimoto, A.; Tesic Mark, M.; Molina, H.; Kohsaka, S.; Di Giannatale, A.; Ceder, S.; et al. Tumour exosome integrins determine organotropic metastasis. Nature 2015, 527, 329–335.
30. Nolte-‘t Hoen, E.N.M.; Buschow, S.I.; Anderton, S.M.; Stoorvogel, W.; Wauben, M.H.M. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. Blood 2009, 113, 1977–1981.
31. Emanuele Cocucci; Gabriella Racchetti; Jacopo Meldolesi; Shedding microvesicles: artefacts no more. *Trends in Cell Biology* **2009**, *19*, 43-51, 10.1016/j.tcb.2008.11.003.
32. Reka A Haraszti; Marie-Cecile Didiot; Ellen Sapp; John Leszyk; Scott A. Shaffer; Hannah E. Rockwell; Fei Gao; Niven R. Narain; Marian DiFiglia; Michael A. Kiebish; et al.Neil AroninAnastasia Khvorova High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *Journal of Extracellular Vesicles* **2016**, *5*, 32570, 10.340 2/jev.v5.32570.
33. Ting Wang; Daniele M. Gilkes; Naoharu Takano; Lisha Xiang; Weibo Luo; Corey J. Bishop; Pallavi Chaturvedi; Jordan J. Green; Gregg L. Semenza; Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proceedings of the National Academy of Sciences* **2014**, *111*, E3234-E3242, 10.1073/pnas.1410041111.