1  Title: **EXTRACELLULAR VESICLES IN HUMAN REPRODUCTION IN HEALTH**

2  **AND DISEASE.**

3

4  Short Title: **EVs IN HUMAN REPRODUCTION.**

5

6  Authors: **Simon C[1,2,3*], Greening DW[4*], Bolumar D[1*], Balaguer N[1], Salamonsen LA[5,6], Vilella F[1,3].**

7

8  [1]Igenomix Foundation, Instituto de Investigación Sanitaria Hospital Clínico (INCLIVA), Valencia,

9  Spain.

10  [2] Department of Pediatrics, Obstetrics and Gynecology, School of Medicine, Valencia University,

11  Valencia, Spain.

12  [3]Department of Obstetrics and Gynecology, Stanford University, USA

13  [4]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe

14  University, Bundoora, Melbourne, Australia.

15  [5]Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria, Australia.

16  [6]Department of Molecular and Translational Science, and Department of Obstetrics and Gynaecology,

17  Monash University, Clayton, Victoria, Australia.

18

19  *** Simon C., Greening DW & Bolumar D contributed equally to this work.

20  **Keywords:** Extracellular vesicles, human reproduction, exosomes, microvesicles, apoptotic bodies.

29

30  **Disclosure Summary**: The authors have no competing financial interests.

31  **Corresponding author** and requests for materials should be addressed to F.V (e-mail:

32  felipe.vilella@igenomix.com).

**Abstract**

Extensive evidence suggests that the release of membrane enclosed compartments, more commonly known as extracellular vesicles (EVs), is a potent newly identified mechanism of cell-to-cell communication in normal physiology and in pathological conditions. This article specifically reviews evidence about the formation and release of different EVs, their definitive markers and cargo content in reproductive physiological processes, and their capacity to convey information between cells through the transfer of functional protein and genetic information to alter phenotype and function of recipient cells associated with reproductive biology. In the male reproductive tract, epididymosomes and prostasomes participate in regulating sperm motility activation, capacitation and acrosome reaction. In the female reproductive tract, follicular fluid, oviduct/tube and uterine cavity EVs are considered as vehicles to carry information during oocyte maturation, fertilization and embryo-maternal cross talk. EVs via their cargo might be also involved in the triggering, maintenance and progression of reproductive and obstetric related pathologies such as endometriosis, polycystic ovarian syndrome, pre-eclampsia, gestational diabetes, and erectile disfunction. We provide here, the current knowledge on the present and future use of EVs not only as biomarkers, but also as therapeutic targeting agents, mainly as vectors for drug/compounds delivery into target cells/tissues.

65

66

67

68

69

70

71

**Index**

73

92          ii.  Inhibition EV recognition/uptake

93   **Part III. EVs as messengers in reproductive physiology**

94          III.I. EVs in the male reproductive tract: epididymis and prostate
95              i.  Epididymosomes
96             ii.  Prostasomes

97          III.II. EVs in the female reproductive tract: follicular fluid, oviduct/tube and uterine
98              cavity
99              i.  Follicular fluid EVs
100            ii.  Oviductal/Tubal EVs
101           iii.  Uterine EVs
102            iv.  Embryonic and trophoectodemal EVs
103             v.  EVs as vehicles for embryo-maternal cross talk

104  **Part IV. Implications of EVs in reproductive pathology**

105         IV.I. Endometriosis

106         IV.II. Polycystic Ovarian Syndrome

107         IV.III. Erectile Dysfunction

108         IV.IV. Pregnancy complications
109             i.  Early pregnancy loss
110            ii.  Gestational vascular complications
111                 1.  Pre-eclampsia (PE)
112                      a.  Placental-derived EVs
113                      b.  Maternal-derived EVs
114           iii.  EVs in gestational diabetes

115  **Part V. Clinical and therapeutic applications of EVs**

116         V.I. EVs as biomarkers

117         V.II. EVs as therapeutic agents

118  **Part VI. Concluding remarks**

119  **Part VII. References**

120

121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152

153
154
155
156
157

# Part I. Introduction

159
160

Intercellular communication is an essential process both for multicellular organisms and for the relationship of unicellular organisms with the environment and hosts (1). Classically, communication has been identified as indirect as endocrine, paracrine and autocrine or direct via cell-to-cell contact, secretion, release, and uptake of chemical moieties such as hormones, growth factors or neurotransmitters (2,3). According to The Human Protein Atlas, nearly 39% of the human protein-coding genes are annotated to give rise to membrane (28%) and secreted (15%) forms of signalling protein variants, some producing both isoforms and post-translational modifications that can alter function. These molecules which constitute potential therapeutic targets include cytokines, growth factors and coagulation factors, among others, playing physiological and pathological roles in processes such as immune defense, blood coagulation, or matrix remodelling. Of note, more than 500 of these proteins are currently known as pharmacological targets with already approved druggable target available commercially.

174

A new mechanism has recently been in the spotlight for cellular communication: the release of membrane-enclosed compartments, most commonly regarded as extracellular vesicles (EVs). EVs can act to convey molecules from one cell or tissue to another. Importantly, their contents (cargo) are protected from extracellular degradation or modification. They exert their biological roles by either direct interaction with cell surface receptors or by transmission of their contents by endocytosis, phagocytosis or fusion with the membrane of the target cells. Recipient cell specificity appears to be driven by specific receptors between the target cells and EVs (4-6). EVs have been described in different body fluids including semen (9), saliva (10), plasma (11), breast milk (12), urine (13) and amniotic fluid (14), among others (reviewed).

184    EVs can be classified in different subtypes based on their biogenetic pathway, composition and

185    physical characteristics, such as size or density, giving rise to three major categories: apoptotic

186    bodies, microvesicles and exosomes (5,7,8).

187    EV content is complex as a continually progressing field with new cargo's being identified

188    continually. Regrettably, due to technical limitations in methods of isolation and differentiation

189    of the different populations of EVs, mixed, heterogeneous populations are often used making

190    interpretation of their content and functionality difficult (15-17).  This constitutes a salient

191    notion in the field at present, that populations of EV subtypes must be considered when

192    reviewing published literature. With homogeneous sample preparation and key developments

193    in characterisation of EVs, we now hold important insights into defining these select

194    communicators in far greater depth. With the implementation of high resolution and sensitive

195    instrumentation for characterisation such as mass spectrometry and next generation deep

196    sequencing, it has been possible to develop databases gathering information about protein, lipid

197    and RNA content of EVs from different sources: ExoCarta (online source: www.exocarte.org)

198    (18), EVpedia (online source: www.evpedia.info) (19) and Vesiclepedia (online source:

199    www.microvesicles.org) (20).

200    In the last years, EVs have been shown to participate in different processes committed to the

201    maintenance of the normal physiology of the organism such as tissue repair, maintenance of

202    the stem cell status of progenitor cells, platelet and immune function, nervous system

203    homeostasis. EVs potential role in the pathogenesis of different diseases has also been

204    studied, being cancer, autoimmunity, neurodegeneration, HIV-1 infection and prion diseases

205    the widest studied areas (1,6,21). In all these cases, EVs are unique as they became small

206    indicators of organism's homeostasis that can stably travel over the body fluids. The fact that

207    their content reflects cell of origin and pathophysiological states highlights their usefulness as

208    biomarkers. Importantly EVs are attributed with potential to cross tissue barriers, such as

209    blood brain barrier, possibly by transcytosis. This fact makes them appealing targets for

210    therapeutics development (22). EVs can be released in response to cell activation, pH

211    changes, hypoxia, irradiation, injury, exposure to complement proteins, and cellular stress.

212    Interestingly, EVs are also secreted by plant cells, and pathogens, including bacteria,

213    mycobacteria, archaea, and fungi, suggesting an important evolutionary conserved

214    mechanism of intercellular signaling.

215

216      In the field of reproductive biology there is a growing interest in understanding the role of EVs
217      within the male and female reproductive tracts, as they may constitute a new mechanism of
218      communication between the reproductive tract and the immature germ cells, or between the
219      mother and the developing embryo. Such developments offer great potential implications in
220      the establishment of a successful pregnancy or implications with understanding associated
221      pathological conditions (23). In the present review, we will address current knowledge on the
222      existence and functionality of EVs as cell-to-cell messengers in normal human reproductive
223      physiology, as well as their contribution in the triggering, maintenance and/or progression of
224      pathological conditions in the functionality of the reproductive tract. Further, we discuss their
225      usefulness as biomarkers of altered reproductive conditions such as pre-eclampsia,
226      spontaneous premature birth, or polycystic ovaries syndrome. We will end up gathering the
227      current knowledge on the present and future of the use of EVs as therapeutic agents, mainly as
228      vectors for drug/compounds delivery into target cells/tissues.

229

## Part II. Types, Isolation and Characterization of EVs and Cargo

**I. EV heterogeneity**

232      EVs can be classified into select subtypes according to different criteria, i.e.: cellular origin,
233      biophysical (density and size) and biochemical (biological markers) characteristics, biological
234      function, biogenetic pathway. According to their biogenetic mechanism of formation and
235      release, three main classes of EVs are defined: apoptotic bodies, microvesicles and exosomes,
236      are now known (Figure 1).

**i. Apoptotic bodies**

239      Apoptotic bodies (ABs) are EVs produced by plasma membrane blebbing in cells undergoing
240      programmed cell death. This term was coined by Kerr and colleagues (24) who defined them
241      as 'small, roughly spherical or ovoid cytoplasmatic fragments, some of which contain pyknotic
242      remnants of nuclei'. Indeed, one of the events that characterize apoptotic bodies is the
243      fragmentation and packaging of cellular organelles such as the nucleus, endoplasmic reticulum
244      or Golgi apparatus into these vesicles (25,26).

245   ABs have widely been described as 1 - 5 μm in diameter, thus overlapping with the size range

246   of platelets (27,28) although some groups extend this range to 50 nm (16,29,30). Their buoyant

247   density in a sucrose gradient is in the range of 1.16 to 1.28 g/mL (31,32).

248   This vesicle population is characterized by cytoskeletal and membrane alterations, including

249   the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the lipid

250   bilayer (33). In this way, PS serves as an 'eat me' signal for phagocytes to target and clear

251   apoptotic debris (34,35). Moreover, PS can naturally be recognized by annexin V, which is a

252   useful marker of apoptotic bodies (36). Nevertheless, care should be taken when using annexin

253   V for this purpose as PS flipping can also be triggered by other stimuli such as mechanical

254   disaggregation of tissues, enzymatic treatments for detachment of cells, electroporation,

255   chemical transfections or retroviral infections, and PS exposure has also been described in

256   healthy cells (37). PS flipping also induces microvesicle (MVs) formation, so these can also be

257   recognized by annexin V detection (38,39). Another specific feature of ABs is the oxidation of

258   surface molecules, creating sites for recognition of specific molecules such as thrombospondin

259   (40) or C3b complement protein (41), which are also useful as markers of ABs.

260   Included in newly identified potential molecular markers of ABs, VDAC1 is a protein that

261   forms ionic channels in the mitochondrial membrane and has a role in the triggering of

262   apoptosis. It proves to be a useful AB marker as its biological function and subcellular

263   localization are characteristic of this vesicular fraction (30). Calreticulin is an endoplasmic

264   reticulum (ER) protein that could also work as an AB marker due to its subcellular localization

265   (15), although it has also been observed in the smaller sized MVs fraction (30). It is possible

266   that, during the apoptosis process, the ER membrane is fragmented and forms vesicles smaller

267   in size than ABs, which would contain calreticulin and would sediment at higher centrifugal

268   forces (42,43). Indeed, proteomic studies have related calreticulin with vesicular fractions

269   across the full size range of MVs (44) and ABs (45).

270   Different functions have been attributed to ABs although most are also features of other EVs.

271   DNA can be horizontally transmitted between somatic cells, with possible integration of this

272   DNA within the receptor cell where can be functional (46). These vesicles are also a vehicle

273   for the horizontal transfer of oncogenes, which are internalized by target cells and consequently

274   increase their tumorigenic potential in vivo

275 (47,48). ABs have also been related to the immune response where they are associated with an
276 under-activation of the immune system (49), and with antigen presentation with special regard
277 to the self-tolerance (50-52).

278 ii. **Microvesicles**

279 Microvesicles (MVs) were reported for the first time by the group of Chargaff (53) as being
280 sedimented at high-speed centrifugation (31,000 x g) (not specifically at lower speeds such as
281 5,000 x g). MVs are a population of EVs that are formed and released directly from the cell
282 plasma membrane by outward budding and fission from viable cells (54,55). Plasma membrane
283 blebbing is triggered by different mechanisms that are accompanied by the remodelling of the
284 membrane proteins and lipid redistribution, which modulate membrane rigidity and curvature
285 (56). Such changes within the periphery of the plasma membrane have been associated with
286 cargo sorting in MVs (57).

287 The size range of MVs has been classically established between 100 - 1000 nm (58), thus
288 overlapping with that of bacteria (7). Some groups extend this range up to 1500 nm (59) or even
289 2000 nm (60-62). The buoyant density of MVs is not as clear as that of other vesicle populations:
290 around 1.16 g/mL in sucrose gradient (62), or 1.04-1.07 g/mL (63). The flotation density in
291 iodixanol gradient is between 1.18-1.19 g/mL (64).

292 As a proposed marker for MVs population, ARF6 is a GTP-binding protein that is implicated
293 in the regulation of cargo sorting and promotion of the budding and release of MVs through
294 the activation of phospholipase D metabolic pathway (56,65). Additionally, data coming from
295 our current knowledge on proteomic studies suggest numerous proteins (e.g: KIF23,
296 RACGAP1, exportin-2, chromosome segregation 1-like protein) as unique/enriched for MVs
297 and potentially discriminatory markers (66). Nevertheless, care should be taken with these
298 results, as different EV cell sources and techniques to selectively enrich may lead to differences
299 within EVs populations.

300 Among the functions described for MVs, are pivotal roles in cancer cell invasiveness (67,68),
301 transformation potential (69), progression (54,70,71) and drug resistance (72). MVs have also
302 been implicated in autoimmune diseases (73-75), immune system modulation and coagulation
303 (58,76,77), embryo-maternal cross-talk (78), and embryo self-regulation (79).

304 **iii. Exosomes**

The first description of exosomes in 1981, described them as a second population of vesicles that appeared in the preparations of MVs and the term exosome was coined (80). Two years later, their biogenetic pathway was formally described by transmission electron microscopy (TEM), trying to follow the pathway of uptake and trafficking of transferrin molecules within reticulocytes in an anemic mice model (81). Exosomes (EXOs) constitute a population of nano-sized EVs that arise and are trafficked through the endosomal pathway. Endosomal sorting complexes required for transport (ESCRTs) are important for multivesicular body (MVB, which include exosomes) biogenesis. During MVBs inward budding of the limiting membrane of late endosomes facilitates formation of intraluminal vesicles (ILVs) that remain enclosed inside the greater membrane compartment of MVBs. ESCRT-independent mechanisms including x, y, z may also occur (64,82). The formed MVBs can then be targeted to plasma membrane to release ILVs, now known as EXOs or otherwise fuse with lysosomes to degrade their content (83). Members of the Rab GTPase family have been shown to modulate EXOS secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. It is likely that ESCRT-dependent and ESCRT-independent MVB/exosome biogenesis machineries vary from tissue to tissue (or even cell type) depending on specific metabolic needs. There are several molecular mechanisms, both canonical and alternative, implicated in the formation, release and extracellular fate of EXOs (review: (5,66).

Most studies place EXOs in a size range of 30 to 150 nm (5,84) or even 200 nm (85), thus establishing an overlap with viruses in terms of size (8). The buoyant density of EXOs in sucrose gradients has been set in a wide range of 1.10 to 1.21 g/mL (29,86), and 1.10 to 1.12 g/mL in iodixanol gradients (87).

The classically-associated markers of EXOS are molecules mainly implicated in the biogenesis of this population, which are incorporated during this process: tetraspanins (CD63, CD9, CD81), Alix, TSG101, flotillin-1, among others (5,86). Nonetheless, with the emerging interest in studying different EV populations as isolated entities, many of these classical markers have been identified as widespread between populations, although with different relative abundances. This is the case for at least CD9, CD63, HSC70 and flotillin-1. Other molecules such as TSG101 and syntenin-1 have been ratified as markers of only this vesicle population (30). Phosphatidylserine, while being described as a broad marker of EVs, has also been reported as exposed on the surface of exosomes produced by different cell types

337   (83,88). Accumulating evidence from *in vitro* studies using cell grown in culture and ex vivo

338   body fluids indicates the existence of more than one exosome subtype (REFs) . For example,

339   EXOs contain subpopulations, including the study of EXOS derived from apical (EpCAM-

340   Exos) or basolateral (A33-Exos) surfaces of highly polarized cancer cells, indicated the

341   presence of two distinct subtypes with distinct protein (89) and RNA cargo . The biological

342   significance of these findings awaits further investigation.

343

344   Due to the high expectations and efforts dedicated to the study of the role of EXOs in different

345   biological processes, both in physiological or pathological conditions, the field of EXO biology

346   has experienced an exponential growth in recent years, with a wide range of functions identified

347   (1,90). EXOs are implicated in cancer physiology, participating in tumour progression and

348   maintenance, resistance, immune modulation and angiogenesis (91). Exosomes also transfer

349   oncogenic entities such as mutated proteins[124,125], fusion gene mRNA (EML4-ALK)[126] and

350   oncogenic lncRNA[122] to neighboring cells. As such, , EXOs are involved in diverse processes

351   that facilitate and regulate cancer progression including inflammation[41], angiogenesis[42],

352   lymphogenesis[43], cell migration[44], cell proliferation[45], immune suppression[46], invasion[47],

353   epithelial-mesenchymal transition[6,48,49] and metastasis[50]. Their function in immune regulation

354   has also been well studied in antigen presentation/modulation, immune activation and

355   suppression (92,93). Importantly knowledge of the seminal role of EXOs in reproductive

356   biology is expanding rapidly. Such studies and the molecular markers and mechanisms

357   identified have the potential for use as markers to discriminate between EV subtypes, as well

358   as various applications of EXOs in clinical diagnosis.

359

**II. Methods of isolation and purification of EVs**

361   The main experimental problem when studying EVs is to achieve a homogeneous separation

362   with appropriate yield of the EV population of interest.  Different methods of isolation and

363   purification have been developed, although to a varying extent, all carry the bias of providing

364   completely homogenous EV populations of any one vesicle type (summarized in Table 1). In

365   the field there is a pressing need to define EV surface-exposed proteins for the purpose of

366   generating mAbs that would allow – discrimination of EV class/subtype (i.e., stereotypical

367   markers). The majority of rapid/one-step approaches for isolating EVs do not take

368   consideration of the fact they are dealing with a possible mixture of vesicle classes/subtypes

369  and co-isolated contaminants such as high-$M_r$ protein oligomer and protein-RNA complexes
370  (e.g., HDL/ LDL/AGO2) complexes.

371  *i. Serial differential centrifugation*

372  Differential centrifugation is the most common and well-known method for the isolation of
373  EVs. Although each group adapts the times and centrifugal speeds depending on their samples,
374  the basic protocol is the following. (i). centrifugation at low speed for the elimination of cells
375  (300 x g, 10 minutes), (ii) centrifugation at up to 2,000 x g for 10 minutes to pellet membrane
376  debris and ABs. (iii) centrifugation at 10,000-20,000 x g for 30 minutes to pellet MVs. (iv) a
377  crude EXO preparation is pelleted by ultracentrifugation at 100,000-200,000 x g for 70
378  minutes. After steps (i)-(iii) of centrifugation, supernatants are transferred to new tubes for the
379  isolation of the subsequent EV type. Pellets (ii-iv) containing different cell populations, are
380  washed by resuspension in PBS and re-centrifugation under the same conditions. The washing
381  step removes some impurities, but also reduces EV yield.

382  Apart from vesicle size, centrifugation alone cannot achieve the separation of pure populations
383  for various reasons: sedimentation of other particles in the supernatant depending on density;
384  distance of the particles from the bottom of the tube; and vesicle/particle aggregation (94).

385  To improve EV population purity, a gradient step can be added to the centrifugation protocol.
386  This system aims to avoid as far as possible the contamination of EV pellets with large
387  protein/protein-RNA aggregates and proteins non-specifically bound to EVs (4). The essentials
388  of the technique are resuspension of the pellet from the previous serial differential
389  centrifugation in a suitable buffer (i.e., PBS), then loading on either the top or base of a
390  prepared sucrose cushion (95,96) or a sucrose gradient (97,98). Following ultracentrifugation,
391  vesicles are recovered either from the bottom of the tube (for cushions), or from a specific
392  fraction of the gradient, depending on their buoyant density. Moreover, substitution of sucrose
393  by a non-ionic density gradient medium, called iodixanol (99) offers many advantages:  better
394  separation of viral particles from EVs; low toxicity towards biological material; is clinically
395  applicable; and it forms isosmotic solutions compatible with the size and shape of EVs in a
396  wide range of densities (100-102).

397  **ii.** *Size exclusion methods: filtration and chromatography*

Filtration for isolation of EVs is often used in combination with ultracentrifugation protocols to improve separation efficiency based on size. Filtration steps using 0.8, 0.2 or even 0.1 µm filters can be inserted between the centrifugation steps depending on the size of the desired population (95,103,104). Alternatively, ultrafiltration utilizes filtration units of different molecular weight cut-off membranes which are centrifuged at moderate centrifugal forces. They allow concentration of vesicles in the interface of the filters, from which they can be recovered by washing (105-108). All these methods face several drawbacks. The pressure of the supernatant can cause the EVs to deform or break into smaller vesicles while the filter membrane may decrease the yield. Gravity filtration has been proposed to cope with the problems associated with elevated pressures (103), but this can be time consuming and filters can become saturated.

Another option for EVs isolation in conjunction with ultracentrifugation, is based on size exclusion chromatography. In brief, the medium containing the vesicles is loaded into the chromatography device, generally a gel size exclusion column, equilibrated into the column and eluted with PBS (109-111). The technique is usually coupled with previous low-speed centrifugation to remove larger debris and subsequent ultracentrifugation to wash and concentrate the vesicles from the different chromatography fractions (112,113). Its advantages are enhanced separation of EVs from proteins and high density lipoproteins (HDL), avoidance of protein and vesicle aggregate formation, reduced sensitivity to the viscosity of the vesicle media, compatibility with the biological properties and functionalities of the isolated vesicles and preservation of the vesicular structure and conformation (109). Moreover, it offers shorter isolation times and relatively low cost. As a disadvantage, this technique offers reduced EVs recovery yields in comparison to others such as ultracentrifugation or polymeric precipitation, although it is susceptible for scale-up (114,115). Nevertheless, some studies indicate that a combination of size-exclusion chromatography and ultrafiltration may produce a yield surpassing that of classical ultracentrifugation (116,117).

**iii.** *Other approaches*

Immunoaffinity uses microbeads coated with specific antibodies for the recognition of specific surface markers of EV populations. In brief, beads are incubated with the sample containing EVs, then beads linked to their epitopes on the EV surface are recovered by magnetism or low-speed centrifugation, depending on the nature of the beads (118,119). The technique can follow centrifugation and/or filtration to clear large cellular products (87,120,121). This method

differentiates EVs populations based on surface markers regardless of their size. Nevertheless, care should be taken as population specific markers are not necessarily available, and the working surface of the beads is limiting, so the EVs may not have access if they are large or present at high concentrations (99).

Aiming for a quicker and simpler method to isolate EVs, a polymeric precipitation system (ExoQuick$^{TM)}$) was commercially developed. The experimental procedure is as simple as incubating the kit reagents with the exosome-containing media and recovering the resulting polymeric complex by low-speed centrifugation. A study with human ascites samples showed that ExoQuick could provide high concentrations and purity of exosomal RNA and that the high exosomal protein concentrations from the same samples compared to other isolation methods such as ultracentrifugation, immunoaffinity isolation and chromatography (122). Even though ultracentrifugation-based protocols are preferable for exosomal protein recovery and purity, ExoQuick obtains better results in terms of exosomal mRNA and miRNA yield and quality (123). The method has a series of limitations. Impurities such as lipoproteins are possibly co-isolated along with EVs and the method is unable to provide isolation of different EVs subpopulations. It works ideally with small vesicles in the size range of 60 to 180 nm (94).

A new technology based on microfluidic devices has recently been developed for the isolation of EVs. It allows the reduction of sample volumes, processing times and costs, while maintaining high sensitivity. The chip technology can be based on different principles. The first developed systems relied on the recognition of EVs by specific antibodies on the surface of the device (124). The surface of the flow system was coated with anti-CD63 antibodies. When EV-containing media was pumped through the system, EXOs were restrained. The system allows SEM imaging and lysis of EVs for RNA isolation directly on the chip. However, it does not provide sufficient material for protein or functional analyses. Subsequently, the system was expanded with lipophilic staining of EXOs to allow simultaneous quantitation (125). A third microfluidic scheme used physical properties as the principle for EV isolation, separating microparticles based on their size within the micrometric size range (126). Clearly this method is not applicable to EV population analysis. This technology has also been combined with porous polymers, allowing purification of vesicles in the nanometric size range: the pore size can be modulated so that only EVs under a certain size can be filtered (127). A recent publication introduces the concept of using a combination of acoustics and microfluidics for a high-purity degree exosomes isolation. The platform is composed of two sequential

462    modules that remove larger components and other EVs groups (microvesicles and apoptotic

463    bodies), respectively, allowing the direct use of undiluted body fluid samples (tested in whole

464    blood) or conditioned media from cell cultures in a single step. The system is based in the

465    combination of microfluidics channels conformation and adjusted acoustic pressure, what

466    makes possible to set the cutoff particle diameter (128).

467    The demands of clinical applications involving diagnostics and therapeutics such as low cost,

468    reliability, and speed can eventually be met with modifications to existing technologies for

469    improved scalability. Isolation of EVs from blood and urine is a challenge due to the presence

470    of abundant and complex proteins and lipoproteins networks, which undoubtedly will attenuate

471    intrinsic EV protein/RNA signatures.  Distinct clinically-relevant strategies to isolate EVs are

472    currently being investigated (reviewed)

473    **III. Methods for characterization of EVs**

474    Characterization of EVs is fundamental to enable differentiation among the different

475    subpopulations within the same biological sample, between vesicles of distinct cellular origin

476    or even of the same origin in pathological vs physiologically normal conditions (summarized

477    in Table 2).

478    **i.** *Microscopy: morphology and size analysis*

479    Electron microscopy (EM) techniques are the only method available to provide the appearance

480    of EVs related to their size. Different variants offer different data to the user. Transmission EM

481    (TEM) was initially used by Raposo and colleagues, who described EXOs as cup-shaped

482    vesicles (129). Although different protocols can be used for TEM visualization, two general

483    schemes offer different views. EVs can be resuspended in fixative media and laid into grids for

484    staining and visualization. Alternatively, EV pellets from centrifugation/ultracentrifugation

485    steps can be fixed, resin embedded, cut onto ultrathin slides, which are then stained and laid in

486    grids. The first method is simpler and less time consuming, and offers a view of the exterior of

487    the EVs. The second method is more informative, shows the interior of the EVs and allows

488    immunogold staining of specific markers that are seen as electron-dense spots (130,131). Cryo-

489    EM allows direct visualization of frozen EVs without previous fixation and contrast steps. The

490    structures are seen as close as possible to their native states (not dehydrated or fixed) and

491    demonstrate variable EV morphology (132). Indeed, such analysis showed that the classical cup

492 shape attributed to exosomes was an artefact of fixation (2). Finally, scanning-EM (SEM)

493 offers three-dimensional imaging of the EVs for further morphological description (133,134).

494 Atomic force microscopy (AFM) is an alternative for the analysis of size distribution and

495 quantity of EVs within a sample and is based on the scanning of the sample by a mechanical

496 probe, which physically touches the sample providing topographical information. AFM allows

497 imaging at the sub-nanometric level. It can be adjusted to air (dry samples) or liquid modes

498 (aqueous samples), and differences in size or number measurements are negligible between

499 them. The possibility of measuring samples in aqueous media is advantageous as it permits the

500 maintenance of EVs physiological properties and structure (135-137). AFM has been efficiently

501 combined with microfluidic isolation devices to providing consecutive isolation and

502 characterization of EVs. Mica-microfluidic chips are also of interest as they provide a non-

503 conductive flat surface for in situ AFM analysis (137,138).

504 **ii.** *Size distribution analysis techniques*

505 Nanoparticle tracking analysis (NTA), a light-scattering technique is now widely used for the

506 assessment of EV size distributions and concentration in the range of 50 to 1000 nm. The

507 principle of the technique is based in the inherent Brownian motion of the particles in a

508 solution: EVs in suspension are irradiated by a laser beam thus emitting dispersed light. This

509 scattered light is captured by a microscope and NTA software tracks the movement of each

510 particle in a time lapse. Silica nanospheres have been proposed for standardization, as their

511 refraction index (RI = 1.46) is similar to that observed for most of EVs (RI around 1.39) (139).

512 Dynamic Light Scattering (DLS) is also used for the assessment of EV size distribution.

513 Although the principle for size determination is also Brownian movement of particles in

514 suspension, the way to attain this data varies from NTA technology. It has limitations when

515 measuring polydisperse samples and those containing big EVs, since the bigger particles scatter

516 more light, masking the smaller ones (140). It is also possible to calculate vesicle concentrations

517 in the samples, by direct extrapolation from the distribution representations using mathematical

518 criteria (140).

519 Tuneable Resistive Pulse Sensing (TRPS or qNano by its IZON commercial name) is a novel,

520 and cheaper technique for the analysis of particle size distributions. The system is composed

521 by a thermo-plastic polyurethane membrane containing nanopores which are selected by size

522 requirements. Currently, the system can measure individual particles in the size range of 30 nm

523 to 10 μm and in the concentration range of $10^5$ to $10^{12}$ particles/mL. Since the system analyses

524 the particles individually, multimodal populations can be studied. On the other hand, a

525 configuration of only one pore type restricts measures to a narrow size range, which is

526 particularly useful for analysis of a specific vesicle population. Combining pores of distinct

527 size and geometry allows widening of this range and analysis of a greater volume of sample

528 (141-143).

529 Flow cytometry has also been applied to the analysis of size distribution, concentration and

530 qualitative characteristics of the EVs within a sample. Light scatter flow cytometry allows the

531 analysis of vesicles with a lower size limit of usually between 300 and 500 nm (144,145), but

532 small EVs including exosomes cannot be studied by this method. However, innovative new

533 flow cytometry technology and the use of fluorescent labelling of EVs, has reduced the lower

534 limit of detection to ~100 nm, and it is possible to discriminate between vesicles 100 to 200

535 nm in size (146,147). Finally, EVs can be coupled via antibodies to their surface markers, to

536 latex beads of greater size. In this way even nano EVs can be analysed, but no quantification

537 or differentiation between vesicle populations is possible (148,149).

538 *iii. Molecular marker characterization*

539 The most effective and well accepted approach to measure EV purity is the concentration of a

540 specific EV surface-marker antigen. Approaches including western immunoblotting, ELISA

541 using surface markers can be used with adaption for the quantitation of EVs within a sample

542 (150,151), and ExoScreen have been employed (152).

543 Another approach, for the characterization and quantitation of EVs, is based on micro nuclear

544 magnetic resonance spectrometry (μNMR) (153) EV labelling with specific EV surface

545 molecular markers antibodies coupled to magnetic nanoparticles enables specific detection by

546 microfluidic μNMR. The technique offers a detection sensitivity level that greatly surpasses

547 ELISA or flow cytometry.

548 Finally, transmission surface plasmon resonance can provide an alternative method for the

549 molecular characterization and quantitation of EXOs in a system called nano-plasmonic

550 exosome assay (nPLEX). This consists of a gold film patterned with a series of nanoholes'

551 arrays, each of which is coated with specific monoclonal antibodies for the recognition of

552 EXO-specific proteins. Compared to previous systems, nPLEX is label-free, easy to

553  miniaturize and scalable for higher throughput detection and improves detection sensitivity to
554  a magnitude order lower than μNMR (154,155).

555  Over the past decade, recent studies and groups have employed developments in proteomic
556  profiling to characterise specific markers for highly purified EV subtypes (EXOs and MVs).
557  Since the emergence of the interest in studying different vesicles populations as isolated
558  entities, many of the classical markers of EXOs have been uncovered to be widespread between
559  populations, although with different relative abundances. This is the case of CD9, CD63,
560  HSC70, EpCAM, flotillin-1, among others (156). On the other hand, some new molecular
561  markers have been stated and ratified as markers of EXOs: TSG101, syntenin-1,
562  Alix/PDCD6IP (30,156). Numerous proteins found exclusively/enriched in MVs (e.g., KIF23,
563  RACGAP1, chromosome segregation 1-like protein, exportin-2 [CSE1L/CAS]) warrant
564  further study as to their potential use as discriminatory markers for MVs. Further, care should
565  be taken when analysing phosphatidylserine as a marker of ABs as it has also been reported to
566  be exposed in the surface of EXOs produced by different cell types (83,88) and also MVs
567  (38,39). An in-depth review detailing proteomic insights into EV biology and defining markers
568  for EV subtypes and understanding their trafficking and function is provided (157).

569  **IV. EV cargo**

570  Membrane receptors and cargo content are the most important feature of EVs, since they define
571  their cellular selectively, target, uptake and functionality, respectively.  EV cargo includes
572  proteins, bioactive lipids, various RNAs (including fusion gene, and splice-variant transcripts),
573  and DNAs (described below), and other cell regulatory molecules (1,4).  To date, most studies
574  have focussed on their genetic (particularly RNA and miRNA) and protein content as sensitive
575  methods exist for their comprehensive analysis and detection.

576  Protein contents in EVs has been widely studied since the application of mass spectrometry-
577  based techniques (158). EVs have been reviewed to be enriched in proteins from cytoskeleton,
578  cytosol, plasma membrane, heat-shock proteins and proteins involved in EVs biogenesis, while
579  proteins from cellular organelles are less abundant (1). From initial studies, EVs were shown
580  to carry commonly widespread EVs proteins and a specific subset of proteins, depending on
581  the cell, the type of vesicle and the method of isolation (5). Moreover, it has been observed that
582  EVs number, protein content and protein concentration varies depending on the stimuli for
583  vesiculation, even in the same subpopulation of vesicles (159).

584 Cytokines have also been described to be carried by EVs (1). IL-1β is among the examples of
585 theses soluble mediators that are secreted in EVs. In fact, it has been described that secretion
586 pathways of EVs may constitute an alternative to exocytosis for proteins that lack leader signal
587 peptide (160). Another interesting example of cytokine cargo is IL-1α, which has been reported
588 to be selectively carried by apoptotic bodies but no by smaller-in-size vesicles (<1μm) in
589 endothelial cells (161), thus confirming the cargo sorting into different populations of EVs.
590 Further examples of cytokines released into EVs are IL-18 (162), IL-32 (163), TNF-α (164) and
591 IL-6 (165), among many others. During pregnancy, EVs cytokine cargo has been shown to be
592 modified towards an increase in comparison to non-pregnancy, maybe contributing to the
593 modulation of maternal immune response against the foetus. Levels of TGF-β1 and IL-10 were
594 found to be increased in EVs from pregnant women, along with an increased ability to induce
595 caspase-3 activity in cytotoxic NK cells, thus promoting and immunosuppressive phenotype
596 through the induction of apoptosis in these cells (166).

597 Lipid content of EVs has been much less studied. However, some groups have shown that EVs
598 are enriched in certain types of lipids in comparison with their parent cells, demonstrating the
599 sorting of these molecules. Specifically, vesicles are enriched in sphingomyelin, cholesterol,
600 phosphatidylserine (167,168), ceramide and its derivate and, in general, saturated fatty acids
601 (169). It is also remarkable that the ratio lipids/proteins are higher in vesicles than in parent
602 cells. In contrast, phospatidylinositols, phospatidyglycerols, phosphatidylcholine and
603 phosphatidylethanolamines are more present in parent cells than in vesicles (167). Recently
604 using mass spectrometry quantitative lipidomics combinations of three lipid species were
605 shown to distinguish cancer patients from healthy controls.

606 RNAs in EVs were first described by Valadi's group (170) in mast cells. They found that
607 exosomes released by these cells contained mRNAs and miRNAs and were able to transfer
608 their content to other cells, where mRNA was functional and could be translated into protein.
609 More recent studies using high throughput sequencing techniques have shown that exosomes
610 contain various classes of small non-coding-RNAs in addition to mRNA, i.e: miRNA, small
611 interference RNA (siRNA), small nucleolar RNA (snoRNA), Y-RNA, vault RNA, rRNA,
612 tRNA, long non-coding RNA (lncRNA), piwi-interacting RNA (piRNA) (171-173). Ng group
613 (149) showed that endometrial epithelial cells cultured *in vitro* produced EVs containing a
614 different miRNA profile from that of parent cells, thus suggesting a sorting mechanism of this
615 miRNAs into exosomes. This could constitute a mechanism for communication between the

616    mother and the embryo with potential implications in embryo implantation. Indeed,
617    bioinformatic studies on the EVs miRNAs showed that some of the genes targeted by the
618    miRNAs are involved in implantation. More recently, our investigation group deepened in the
619    knowledge of maternal-embryo cross-talk and demonstrated that exosomes containing miR-
620    30d were actively transferred from endometrial epithelial cells to trophoblastic cells, were the
621    miRNA was subsequently internalized (133).

622    A major problem concerning RNA analysis from EVs is the variability of the results depending
623    on the methodology used for the isolation and obtaining of the data. One of the major factors
624    affecting this variability is the possibility that the RNA present in the medium, for example
625    from lysed cell, could stick to the external EVs wall, thus being isolated along with internal
626    RNA. In this sense, RNaseA treatment previous to EVs RNA isolation should be conducted
627    (174). Even with this procedure, it has been stated that extravesicular RNAs associated with
628    proteins, such as miRNAs in complex with argonaute proteins, can circumvent RNaseA
629    degradation, thus leading to bias in result interpretation. This protective role of protein
630    complexes has been reported either in extravesicular medium (175,176) and inside EVs (177).
631    In order to overcome complex protection, treatment with proteinase K has been proposed for
632    dissociation of RNA-protein complexes (178). Nevertheless, negative impact in EVs yield
633    should be investigated as proteases may provoke vesicle lysis.

634    Less has been reported regarding DNA content in EVs. Some studies have currently reported
635    the presence of double stranded DNA (dsDNA) in EVs (179,180), even distinguishing a
636    different pattern of content among EVs subpopulations (181). A previous study conducted in a
637    similar way in tumour cells, using DNase to cleave extravesicular DNA, showed that EVs DNA
638    was more abundant in microvesicles from tumour cells than from normal cells and that this
639    DNA was mainly single stranded (182). It has been shown that mitochondrial DNA (mtDNA)
640    can also be transported between cells inside exosomes, possibly constituting a pathway to
641    transmit altered mtDNA and associated pathologies (183). This may serve as an evidence of a
642    trans-acting function of DNA, being able to have functional effects on the recipient cells.

643    Of note, both the amount and content of EV genetic cargo can be hormonally-regulated in
644    exosomes from target cells: this is of particular relevance to reproductive tissues and is further
645    discussed below.

646    **V. EVs Mechanism of recognition and uptake**

647    *i. Mechanisms of EV uptake*

648    For EVs to act in cell-cell signalling, they must recognise their specific cellular target, bind to

649    that cell and undergo internalization (Figure 2).

650    *Target cell recognition.*

651    EVs may interact with recipient cells by direct signalling through ligand/receptor molecules on

652    their respective surfaces or by direct fusion of EV and recipient cell plasma membranes (184),

653    through lipid raft-, clathrin- and calveolae-dependent endocytosis, macropinocytosis and

654    phagocytosis (185-190).

655

656    Cell surface and integral membrane/ adhesion proteins on distinct EVs are important in

657    mediating associated cell recognition and adhesion. These include integrin pairs: for example,

658    distinct EXOs integrin repertoires - specifically integrins α6β4 and α6β1 - were identified as

659    associated with lung metastasis, whilst EXOs integrin αvβ5 associated with liver metastasis

660    (191). The integrin profile of each EXOs subtype permits selective cellular targeting

661    Differences in EXOs tetraspanin complexes also appear to influence target cell interaction *in*

662    *vitro* and *in vivo*, possibly by modulating the functions of associated integrin adhesion

663    molecules (192). Exosome capture by dendritic cells was reduced by 5–30% following co-

664    incubation with blocking antibodies specific for various integrins, adhesion molecules or

665    tetraspanins (185). Other membrane proteins reported as important in targeting select EVs to

666    recipient cells, include intercellular adhesion molecule 1 (ICAM-1) and milk fat globule-

667    epidermal growth factor VIII protein (MFGE-8) (193,194). Further, the delivery efficiency of

668    EXOs to cells is reported to be directly related to rigidity of cargo lipids including

669    sphingomyelin and N-acetylneuraminyl-galactosylglucosylceramide (GM3) (195).

670    Recent new data indicates that proteoglycans and lectins can participate in EXOs binding and

671    internalization. Proteoglycans are cell surface proteins while lectins, such as galectins 1, 3 and

672    5 which recognise and bind proteoglycans, are identified on EVs. Indeed proteoglycan

673    receptors along the plasma membranes of cells and proteoglycans on EXO surfaces have been

674    shown to promote docking (196).

675

676    *Exosome uptake and release of cargo.*

677 EV internalization by recipient cells is reported to occur via multiple processes such as
678 phagocytosis (170,185,186), clathrin-mediated endocytosis (197), macropinocytosis (190),
679 receptor-mediated (198), and direct fusion (195). However, much further understanding of the
680 underlying mechanisms , and importantly, whether EV subtypes have distinct mechanisms of
681 uptake their target cell specificity is required (199-203) (review(204)).

682

683 EV uptake is readily demonstrated in cell culture, using fluorescently-labelled EVs (82)).
684 Uptake and cargo release occur very rapidly, within minutes-hours. However, such techniques
685 do not absolutely prove release, as it is possible that the transfer and spread of fluorescence
686 results from the culture conditions and lipid/membrane transfer.
687 Recent developments in modification of EVs has also facilitated monitoring and tracking their
688 behaviour, interaction and transfer *in vivo* (205). Intracellular probes are utilized to
689 fluorescently label mRNA within EVs to monitor EV-borne mRNA encoding luciferase.
690 Developments in transgenic mice have also been used to visualize EV transfer to cells
691 associated with tumour stroma (206) and immune cells (207,208) while EV-mediated transfer
692 of donor genomic DNA to recipient cells supports a mechanism for genetic influence between
693 cells (209). Such *in vivo* approaches have not specifically shown whether transfer involves a
694 direct fusion of EVs with the recipient cells, formation of gap junctions or nanotubes, or
695 phagocytosis of live or apoptotic cell-derived EVs by the recipient cell.

696

697 Low pH is important for EXOs uptake. There appears to be elevated stability and
698 lipid/cholesterol content of exosomal membranes in an acidic environment (195).
699 Understanding recipient cell function and regulation by EXOs needs to focus on specific
700 mechanisms of targeting and delivery, uptake and transfer, including modulation of key
701 signalling pathways in various recipient cells both *in vitro* and *in vivo*. Processes that control
702 target cell recognition and EV uptake are not well understood.

703

704 *ii. Inhibiting EV recognition/uptake*

705 While several uptake mechanisms have been proposed for EVs, detailed knowledge regarding
706 the key steps in EV target cell definition and definitive mechanisms of uptake is required (204)
707 particularly since variability is found between cell types in vesicle internalization (210). The
708 use of inhibitors is proving useful in elucidating cell-type specific mechanisms.

709

710   As discussed, using fluorescently labelled EVs, internalization can be readily observed *in vitro*

711   within a short period of time (82,211). Treatment with inhibitory agents such as

712   chlorpromazine to examine clathrin-dependent uptake (187) and specific RGD inhibitory

713   peptides (212) to target integrin-mediated EV uptake allows identification of selective

714   processes of internalization. The efficacy of EV exchange between cells probably depends on

715   their surface antigen repertoires since partial digestion of membrane proteins exposed on EVs

716   with proteinase K can significantly decrease their uptake (187) and blockage of select integrins

717   or tetraspanins with monoclonal antibodies also has suppressive effects on EV internalization

718   (185). Further, the use of cytochalasin D, which interferes with actin polymerization and

719   endocytosis, significantly reduces the uptake of EVs (187,188). Similarly, the inhibition or

720   knockout of dynamin, a GTPase responsible for formation of endosomal vesicles, significantly

721   suppresses EV uptake (189). Further research is needed to understand the precise mechanisms

722   that underpin distinct EV entry into select target cells and importantly how to control this

723   process.

724

725

726   **Part III. EVs as messengers in reproductive physiology**

727   Normal reproductive processes are highly dynamic, with well characterized stages. The

728   considerable intercellular interactions involved at each stage have prompted the study of the

729   involvement of EVs in both the male and female reproductive tracts, from pre-conception to

730   birth. EVs associated with reproductive biology have been specifically identified and studied

731   in different fluids such as prostatic and epididymal fluid (213) seminal fluid (9,132), follicular

732   fluid (214,215) oviductal fluid (216,217), cervical mucus (218), uterine fluid (133,149), amniotic

733   fluid (98,219) and breast milk (220), and the originating tissues (reviewed in (221) (summarized

734   Table 3).

735   There is currently increasing data pointing at EVs as key regulators of different reproduction

736   processes such as sperm/ovum maturation, coordination of capacitation/acrosome reaction,

737   prevention of polyspermy, endometrial embryo cross-talk and even communication between

738   *in vitro* co-cultured embryos leading to quorum improved development (222). In these initial

739   steps of the reproductive process (e.g.: pre-conception) EVs are widely produced by different

740   organs and show specific functions. Once implantation has taken place, production of EVs

continues throughout pregnancy being the placenta the main source of EVs. During early pregnancy, EVs are released by the extravillous trophoblast. Later on, the syncytiotrophoblast (STB) is formed and establishes contact with maternal blood-flow. From here on, STB constitutes the main site of EVs generation, and these EVs get access to the maternal systemic vasculature, where they show important roles in immune modulation, either for the innate and the adaptive response (23). Of note, EVs are also found in amniotic fluid, where they are attributed inflammatory and pro-coagulant activities (223); and in maternal breast milk. In this last case, an important influence of EVs recovery procedure has been detected on subsequent analysis (224). Among attributed roles, milk EVs have been involved in bone formation, immune modulation and gene expression regulation, with special emphasis for long non-coding RNAs (220,225).

**I.** *EVs in the male reproductive tract: epididymis and prostate*

After leaving the seminiferous tubules, spermatozoa (SPZ) are still immature cells. SPZ are stored in the epididymis where they undergo a series of morphological and biochemical modifications that provide them with motility and fertilization ability in their transit from the caput to the cauda, a process called sperm maturation (226,227). During ejaculation, SPZ mix with seminal fluid from the seminal vesicles, the prostate and the bulbourethral gland to form the ejaculated semen, which is ejected into the vaginal cavity. Seminal fluid composition is crucial in promoting sperm motility and genomic stability (226,228). Moreover, it contributes to the establishment of maternal immune tolerance (229,230). Subsequently, as SPZ travel through the female reproductive tract to the upper Fallopian tube where fertilization occurs, they interact with the endometrial and tubal milieu. Finally, to achieve successful fertilization, SPZ undergo capacitation: sperm head membranes undergo a series of biochemical modifications that enable the acrosome reaction when the spermatozoon reaches the zona pellucida of the oocyte. This leads to the release of enzymes that allow SPZ to penetrate the zona pellucida and fuse with the oocyte plasma membrane (231-233). In this context, secretions from the different components of the male and female reproductive tracts have been proposed to play a sequential role in programming sperm function (234).

**i. Epididymosomes**

Epididymosomes (EVs originating from the epididymis) were first described in 1967 by Piko in the Chinese hamster (235) as having diameters between 20 and 100 nm, and being associated

with the SPZ acrosomal membrane (236). More recently, it has been shown that epididymosomes are a population of roughly spherical bilayered vesicles that display heterogeneity both in size and content that varies between the different segments of the epididymis. Their sizes range from 50 to 800 nm or even to 2-10 μm in the first segments of the caput (213). Their lipidic composition also varies: indeed, an increase in sphingomyelin and a general decrease in the other phospholipids and in the proportionof cholesterol occurs with epididymal progression from the caput to the cauda. This is in contrast to SPZ, where the proportions remain more constant. Epididymosomes also have an increased ratio of saturated/unsaturated fatty acids from the caput to the cauda, while the opposite situation is found in SPZ. Together, these data indicate that epididymosomes tend to gain membrane rigidity whilst SPZ membranes tend to become more fluid (213).

Two main classes of epididymosomes have been identified: CD9-positive epididymosomes, that preferentially bind live SPZ, and ELSPBP1-enriched epididymosomes, which present higher affinity for dead SPZ (237). CD9-positive epididymosomes are EVs of size ranging from 20 to 150 nm (238). These were recovered by ultracentrifugation from the total epididymal fluid EVs, specifically in the epididymis cauda. CD9-positive epididymal cargo transferred to SPZ includes proteins involved in sperm maturation namely P25b, GliPr1L1 and MIF (239-241), in contrast to ELSPBP1 which was widespread between all EVs. Moreover, CD9, in cooperation with CD26, plays a role in promoting this transfer (238).

ELSPBP-1-enriched epididymosomes constitute a subpopulation of vesicles obtained from the epididymal fluid by high-speed ultracentrifugation (120,000 x g) after SPZ and debris removal at 4,000 x g (242). It had been suggested that ELSPBP1 allowed distinction between dead and viable SPZ as it was only detectable in the dead SPZ population (243). Later, the same group demonstrated that epididymosomes were the only path for the transmission of molecules including ELSPBP1 to dead SPZ (238,242). Interestingly, ELSPBP and biliverdin reductase A (BLVRA) can associate and bind in tandem to dead SPZ, concurrently with the epididymal maturation of SPZ, a process during which these cells cease producing BLVRA. Therefore, BLVRA could act as a quencher of reactive oxygen species generated by dead and immature SPZ, protecting viable SPZ from oxidative stress. Moreover, BLVRA may be involved in haemic protein catabolism, changes also important in the SPZ maturation process (237,244).

Since the epididymis brings SPZ to functional maturity before they enter the vas deferens, it is not surprising that epididymosomes serve as means for protein transfer into SPZ during their

804   transit in the epididymal duct. Some epididymosomal proteins have proven roles in sperm
805   maturation: these include P25b, MIF or SPAM1, among others (245,246). Sperm adhesion
806   molecule 1 (SPAM1) is a hyaluronidase with roles in both fertilization and sperm maturation.
807   It is transferred to SPZ from epididymosomes, increasing their ability for penetrating the oocyte
808   cumulus (247). Another protein transferred to SPZ by this mechanism is ADAM7, which is
809   important for sperm motility, morphology and establishment of membrane correct protein
810   composition (248,249). Of note is the transfer of the plasma membrane ATPase 4 (PMCA4), a
811   major $Ca^{2+}$ efflux pump, into epididymosomes: this plays a pivotal role in SPZ maturation and
812   motility (247). Glutathione peroxidase 5 (GPX5) associates with SPZ during its transit through
813   the epididymis, protecting them from lipid peroxidation stress and, independently, is
814   transferred to SPZ via epididymosomes (250). Finally, components of the Notch pathway are
815   described in epididymosomes, suggesting that these vesicles transmit Notch signalling at a
816   distance between epididymal epithelial cells, but also between the epididymis and SPZ with
817   important implications for sperm motility (251).

818   Epididymosomes also convey miRNAs within the epididymal duct. As with proteins, distinct
819   regions of the epididymis produce EVs with a specific set of miRNA whose profiles differ
820   from those of parent cells, suggesting a sorting mechanism (252). Indeed, it has been proposed
821   that epididymosomes may act as modulator of gene expression between sections of the
822   epididymal duct (252). Recent analysis confirmed that they contain over 350 miRNA, showing
823   a different profile from that of parent cells and dependant on the region of the epididymis from
824   which they originate. Finally, it was demonstrated that many of these miRNAs are transported
825   into the SPZ (253).

826   An emerging concept is the transfer of traits to the offspring by epigenomic modifications. In
827   this respect, transfer (t)RNA, has been attributed a new function as a modulator of genetic
828   expression. It was initially discovered that a respiratory syncytial virus (RSV) infection of lung
829   and kidney cell lines, led to the production of specific tRNA fragments (tRFs) that are able to
830   repress the expression of specific mRNAs in the cytoplasm to favour viral replication and
831   survival (254). Subsequently, further examples of tRFs have been described with potential
832   implications in pathological processes, such as cell proliferation in cancer (255). Mature
833   molecules corresponding to tRNA fragments are highly enriched in mature sperm.
834   Interestingly, these fragments are produced by sequence specific cleavage, giving place to
835   fragments corresponding to the tRNA 5' end (256). Recently, the transfer of tRFs to maturing

836 SPZ in epididymosomes was demonstrated in mice (257), providing an explanation for the
837 scarcity of these molecules in testicular SPZ but with an increase with SPZ maturation. A tRF
838 (tRF-Gly-GCC) has been identified as transferred to SPZ by epididymosomes. This tRF,
839 represses MERVL, an endogenous retro-element, that positively regulates a set of genes that
840 are highly expressed in pre-implantation embryos. Interestingly, male mice treated with a low-
841 protein diet have a trend (non-significant) to increased tRF-Gly-GCC in mature SPZ and to
842 downregulate tRF-Gly-GCC targets in embryos at 2-cell stage. This evidence supports that
843 parental diet can affect the offspring epigenome: however, this preliminary data requires
844 confirmation (257).

845 ii. **Prostasomes**

846 Prostasomes were first described as vesicles recovered from human prostatic fluid by
847 centrifugation, that were associated with an $Mg^{2+}$ and $Ca^{2+}$-dependent ATPase activity (258).
848 They are now considered a population of EVs produced by the prostate epithelial cells that
849 interact with SPZ, epididymal and seminal secretions at the time of ejaculation. They are EVs
850 of size range 30 to 500 nm, surrounded by lipoprotein bilaminar or multilaminar membranes
851 (259,260). It is likely that a population of prostasomes is exosomal, as they originate from
852 structures resembling MVBs and exhibit classical EXO markers (261). Prostasomes' lipid
853 composition is unusual and provides them with a characteristic highly ordered structure,
854 rigidity and viscosity due to several factors: a high cholesterol/phospholipid ratio reaching
855 values of 2, which greatly surpasses the values for most of biological cholesterol-rich
856 membranes; phospholipid composition domination by sphingomyelin, which accounts for
857 almost a half of the phospholipids found in prostasomes (262); and finally prostasomes show a
858 strongly saturated fatty acid profile in comparison to SPZ membranes (263). It has been
859 reported that prostasome uptake decreases the fluidity of SPZ membranes by transfer of lipids
860 directly dependent on the prostasome/SPZ ratio (260,264). This decrement is crucial as it stands
861 as a regulator of the acrosome reaction, preventing a premature response (265).

862 Different roles have been attributed to prostasomes in sperm maturation and function, either
863 directly or indirectly. These include protection of SPZ from the female acidic environment and
864 immune surveillance modulation of SPZ motility, capacitation, acrosome reaction and
865 fertilizing ability, among others (259-261,265).

SPZ motility is vital for a successful fertilizing ability, especially for traversing the cervical mucus and zona pellucida (266). One of the first roles attributed to prostasomes was the enhancement of SPZ motility (267) in a pH-dependent manner, suggesting that prostasomes might alleviate the negative effects of vaginal acidic microenvironment on SPZ motility, thus showing a protective effect (268). $Ca^{2+}$ has been well known as the major ion promoting SPZ motility and fertility, from initial studies carried in hamster (269). Increased SPZ $Ca^{2+}$ levels have been linked to prostasomal delivery, directly depending on the extent of fusion/prostasome concentration and influenced by pH (270). However, it took a decade to identify a mechanism. Park and colleagues showed that a progesterone-triggered long-term sustained $Ca^{2+}$ stimulus is involved in SPZ motility promotion, via fusion of (acidic) pH-dependent prostasomes. Specifically, prostasomes transferred progesterone receptors and different $Ca^{2+}$ signalling cascade components to the SPZ neck region where, following progesterone stimulation, they trigger the release of $Ca^{2+}$ from SPZ internal acidic stores to promote SPZ motility (271). Other proteins involved in intracellular $Ca^{2+}$ homeostasis are also transported into SPZ in prostasomes, including PMCA4 (272), which along with nitric oxide synthases (NOSs) are delivered into SPZ by prostasomes. PMCA4 and NOS activity is stimulated by $Ca^{2+}$ ions (273) and indeed, NOS spatially interacts with PMCA4 to a degree positively related to $Ca^{2+}$ concentration levels. This supports the theory that PMCA4 expels $Ca^{2+}$ from SPZ in the presence of NOS to reduce nitric oxide production and thus oxidative stress, which could reduce SPZ viability resulting in asthenozoospermia (272). Prostasomes also carry aminopeptidase N, a protein involved in modulating sperm motility, which acts through the regulation of endogenous opioid peptides, such as enkephalins, once in SPZ (274,275).

Interestingly, EXO-like EVs found in cervical mucus have been reported to carry sialidase activity, which reaches a maximum during the ovulatory phase in healthy women. This is likely involved in modifying the properties of the highly–glycoslyated mucus to favour SPZ access to the uterine cavity and tubes (218).

There is scarce data on the prostasomes' nucleic acid cargo and its implications for male reproductive physiology. Prostasomes contain various coding and regulatory RNAs, with potential modulatory functions (172). Interestingly, mRNA and miRNA do not represent the majority of the prostasomal RNA (261), and it has been postulated that mRNA in semen is predominantly transported inside vesicles while miRNA is mostly contained in the vesicle-free

fraction of the semen, forming complexes with proteins (177). DNA inside prostasomes apparently represents random regions of the genome and is effectively transported into SPZ (276,277). Nevertheless, this DNA may be a contaminant from apoptotic bodies in the semen (278).

Capacitation is a cAMP-regulated process, whose production is in turn promoted by bicarbonate and $Ca^{2+}$ ions and influenced by membrane dynamic changes mainly due to cholesterol composition (259,261). It has been proposed that prostasomes may act as inhibitors of the capacitation process and acrosomal reaction, mainly through transfer of cholesterol (279,280). Indeed, this might represent a mechanism to avoid premature capacitation and acrosome reaction (265,281). A switch between positive and negative regulation exerted by prostasomes may be influenced by the environment or even determined by specific prostasome subpopulations. cAMP promotes capacitation through the protein kinase A (PKA) axis, by the simultaneous tyrosine-phosphorylation of specific down-stream proteins and plasma membrane protein and lipid remodelling. This remodelling breaks down plasma membrane asymmetry, exposing cholesterol molecules to external acceptors to trigger the capacitation process (282). In this context, co-incubation of equine SPZ with prostasomes led to increased cAMP levels and tyrosine phosphorylation of PKA cascade proteins, in addition to the prostasome endogenous PKA activity described in previous reports. However, these changes were not correlated with increased capacitation and acrosome-reaction rates and reverted after 3 hours of co-incubation in capacitating conditions (279). Interestingly, Aalberts and colleagues observed that at least a subpopulation of prostasomes are able to bind to live SPZ only when capacitation-inducing conditions are established, probably to promote hypermotility and acrosome reaction at the precise moment it is needed. Nonetheless, care should be taken when interpolating these results into human, as they were obtained from a stallion model, a species that deposits its ejaculate directly in the uterus (282).

Following capacitation, SPZ need to undergo an acrosome reaction to enable penetration of the zona pellucida of the oocyte and fusion of plasma membranes. The zona pellucida glycoprotein ZP3 is mandatory for this process as it facilitates sperm-binding, triggering the acrosome reaction. Nevertheless, the acrosome reaction begins before the SPZ contacts the zona pellucida, probably due to the progesterone-dependent stimulus produced by cumulus cells (283). Conversely prostasomes have been proposed as inhibitors of the acrosome reaction through the transference of cholesterol to the SPZ (280,284) or as inducers by facilitating

930 progesterone uptake by the SPZ (285), most likely by the transfer of progesterone receptors
931 (271). Other studies also supporting the promotion of the acrosome reaction via prostasomes
932 include delivery of molecules to the SPZ membrane ia a pig model (286), or progesterone
933 priming, acting via the $Ca^{2+}$ signalling axis (287). Other acrosome reaction-promoting
934 molecules in prostasomes include hydrolases (288) and lipoxygenases (289).

935 In summary, the role of prostasomes in sperm-fertilizing ability in humans is most likely the
936 result of orchestrated actions. Initially, prostasomes would attach to SPZ after mixing during
937 ejaculation, favoured by the acidic environment of the vagina, thus transferring cholesterol to
938 stabilize SPZ membranes and prevent premature capacitation and acrosome reaction. This
939 would enable prostasomes to pass the barrier of cervical mucus adhered to spermatozoa with
940 subsequent fusion and transfer of their content to the SPZ when the SPZ first contacts the
941 oocyte. At this time, the progesterone secreted by the cumulus cells would activate $Ca^{2+}$-
942 dependent pathways that promote capacitation process and acrosome reaction (259).

943 Finally, of note is the role of prostasomes in protecting SPZ from the potentially hostile female
944 genital tract They appear to exert roles as protectors from female immunity, antioxidants,
945 antibacterial, and in the process of semen liquefaction (reviews: (259,265,282)).

946 *II. EVs in the female reproductive tract: follicular fluid, oviduct/tube and uterine cavity*

947 Contemporarily to sperm maturation, a coordinate oocyte development must be taking place
948 so as both gametes can meet at the appropriate location and time inside the female reproductive
949 tract. Developing oocytes are arrested in prophase I of meiosis in primordial follicles from the
950 fetal period until female reproductive maturity. From this moment, cohorts of these oocytes
951 cyclically restart growth, forming the zona pellucida while granulosa cells proliferate in order
952 to form the cumulus, which will support posterior egg fertilization. Concomitantly, meiosis is
953 reinitiated, extruding the first polar body and arresting again at metaphase II during ovulation
954 (290). The resumption of meiosis is stimulated by the LH peak, which in turn is initiated by a
955 surge in estradiol-17β levels due to the secretion by the granulosa cells from the preovulatory
956 follicle, and results in ovulation 36 hours later (291). After ovulation, the extracellular matrix
957 of the cumulus cells serves as an adhesion dock for the Fallopian tubes, through which the eggs
958 travel as far as the ampulla where they await SPZ for fertilization (292). Following fertilization
959 embryo development to blastocyst stage, proceeds as the embryo passages through the
960 Fallopian tubes reaching the uterine cavity of about 4 days after ovulation. The blastocyst

undergoes final preparation for implantation into the maternal endometrium in the microenvironment of uterine fluid with implantation occurring 6 -10 days after ovulation (293).

The process of embryo implantation can only occur during a short period of time during the luteal phase of the menstrual cycle, which has been classically regarded as the window of implantation and that typically extends from days 5.5 and 9.5 days after ovulation in healthy normal cycling women (293,294). At this point, different factors affect and limit embryo implantation, namely: embryo quality, endometrial receptivity and embryo-endometrial cross-talk (295), where EVs stand as important potential mediators.

During all this process, EVs carry out many different supporting actions: they assist follicle and oocyte development and maturation at the initial stages, and further assist early embryo development and implantation as the conceptus reaches the uterus. Further, female tract EVs contribute in preparing endometrial vascular net, promote embryo implantation and prime the endometrium for harbouring the embryo. Moreover, these EVs also contribute to SPZ maturity, capacitation and acrosome reaction coordination, support SPZ storage while waiting for the oocyte and regulate molecule delivery into SPZ during this period. All these concepts will we discussed in the following sections.

### i. Follicular fluid EVs

Oocyte maturation occurs within the micro-environment of follicular fluid (296). The easy availability of this fluid during oocyte retrieval in assisted reproductive techniques makes it attractive in the search of biomarkers for oocyte quality (297). EVs (resembling exosomes and microvesicles) were first identified in follicular fluid by da Silveira and colleagues who demonstrated follicular fluid EV uptake by granulosa cells, both in vivo and in vitro, and their protein and miRNA cargo. EV miRNAs were also present in the surrounding granulosa and cumulus cells, thus suggesting EVs as a vehicle for biomolecule transfer within the ovary. Of particular interest, the miRNA signature of follicular EVs varied with the age of the female, suggesting EVs miRNA cargo as an indicative and possible predictor of age-related decline in oocyte quality (298). Subsequently, EV miRNAs were further evaluated and a set of four differentially expressed miRNA based on age (young/old) was defined. However, these age-related miRNAs were studied in complete follicular fluid samples and as such cannot be confidentially attributed to EVs (299).

The miRNA of bovine follicular fluid is present both in exosomes and free, each with different composition (300). The exosomes were taken up by granulosa cells in vitro, resulting in increased miRNA content and variations in mRNA profiles: some of the affected genes are involved in follicle development. Moreover, some of the miRNA within exosomes may also contribute to oocyte growth as they were differentially expressed in follicles containing oocytes at different maturation stages (300). A more exhaustive characterization of the EV content of bovine follicular fluid demonstrated variation in number, protein markers and miRNA contents depending on the developmental stage of the follicles. What is more interesting, variation in miRNA signature suggested a switch in genetic programming concurrent with the follicular maturation. As such, EVs miRNAs from small follicles preferentially promoted cell proliferation pathways while those from large follicles related to inflammatory response pathways (301). A possible role of follicular fluid-derived exosomes in follicle development and growth through the TGFB/BMP axis ACVR1 and ID2 regulation, was demonstrated when granulosa cells were exposed to follicular fluid exosomes *in vitro*. It was proposed that these effects were triggered by the direct delivery of ACVR1 and ACRV1 regulatory miRNA within follicular exosomes to granulosa cells (302).

Cumulus-oocyte complex expansion is a critical process for ovulation. In this context, in vitro co-culture experiments using bovine follicular fluid-derived exosomes and cumulus-oocyte complexes from mouse and bovine revealed that follicular EVs are taken up by cumulus cells, promoting both cumulus expansion and related genes expansion (303).

### ii. Oviduct/Tubal EVs

Fertilization of the oocyte by SPZ occurs within the Fallopian tubes/oviduct. After capacitation, SPZ must undergo an acrosome reaction and maintain hyperactivated motility in order to fuse with the oocyte, both functions being regulated by high intracellular $Ca^{2+}$ concentration levels. In this context, the major murine $Ca^{2+}$ efflux pump PMCA4, and particularly its splicing variant PMCA4a, is predominant in oviductal fluid, compared to uterine and vaginal fluids, and is totally associated with EVs. Moreover, these PMCA4a-carrying vesicles had exosomal characteristics and were taken up by SPZ, where the efflux pump was functionally relocated to their membranes. This was the first study describing the presence of exosomes in the oviducts and introduced the relevance of PMCA4 as a tool for the maintenance of $Ca^{2+}$ homeostasis and SPZ viability during SPZ storage, regulating capacitation and acrosome reaction timings and SPZ motility (216,217,304,305). Subsequently, the same authors discovered that integrins (α5β1

and αvβ3), in oviductal EVs were transferred to SPZ, and were involved in EV-SPZ fusion for cargo delivery. While the oviductal EVs, include both microvesicles and exosomes, the former appeared to be more efficient in fusing with SPZ (216).

Bovine oviductal EVs produced in vitro by cell lines, have beneficial effects on the quality and development of in vitro co-cultured bovine embryos, suggesting a functional communication between the oviduct and embryo during the early stages of embryo development (306). However, these results must be treated with caution as oviductal EVs produced in vitro have been observed to carry a differential cargo compared with in vivo produced EVs. This is the case, for example of OVGP and HSPA8, oviductal proteins known to be important in the fertilization process and early pregnancy. While HSPA8 was found in both in vitro and in vivo exosomes, OVGP was absent in exosomes of in vitro origin (307).

### iii. Uterine EVs

Endometrial fluid is a viscous liquid, secreted by the endometrial epithelial cells from the glands into the uterine cavity.  Since the endometrium is a hormonally regulated organ, the molecular composition of the fluid varies depending on the phase of the menstrual cycle (308). Uterine fluid, a biologically and clinically relevant sample source(REF) also contains contributions from the oviductal fluid and a large cohort of plasma proteins along with other factors, differentially transudated from the blood (309). It is highlighted that this uterine fluid carry information that mirrors maternal environmental exposure and possibly relay such information to the embryo, subsequently generating long-term epigenetic effects on the offspring via embryonic and placental programming.

To date, EVs have been reported throughout menstrual/estrous cycles in the endometrial fluid of different species, including humans (133,149) and sheep (310-313), and are also released by endometrial epithelial cells in culture (133,149).

Ng et al (2013) first described the production of EVs by human endometrial epithelial cells in primary culture and by the endometrial epithelial cell line ECC1. These EVs contained a specific subset of miRNAs, not detectable in the parent cells. Bioinformatic analysis revealed that some of the target genes of the EVs miRNAs are relevant to processes involved in embryo implantation. Importantly, they also verified the presence of EVs in human uterine fluid and the associated mucus (149).

1053 Greening *et al.* (388) demonstrated that the proteome of highly purified EXOs derived from
1054 human endometrial epithelial cells, is regulated by steroid hormones, and thus varies with the
1055 progression of the menstrual cycle. Under follicular phase hormonal conditions, when
1056 oestrogen constitutes the main hormonal stimulus, the EXOs proteome was enriched in proteins
1057 related to cytoskeletal reorganization and signalling cascades, coinciding with the phase of
1058 endometrial restoration. Importantly, after ovulation, when progesterone is the dominant
1059 hormone driving endometrial receptivity, the proteome altered with changes indicating
1060 enrichment in extracellular matrix reorganization and embryo implantation. As in other
1061 systems, the exosomal protein profiles were shown distinct from parental cells. Importantly,
1062 this study demonstrated that endometrial EXOs were transferred and internalized by human
1063 HTR-8 trophoblast cells, enhancing their adhesive capacity, partially through focal adhesion
1064 kinase signalling (82). This was significantly higher when the exosomes were derived from
1065 cells subjected to both estrogen and progesterone to mimic the receptive phase of the menstrual
1066 cycle.

1067 **iv. Embryonic and trophectodermal EVs.**

1068 Interestingly, murine embryonic stem cells from the inner cell mass generate microvesicles that
1069 reach the trophectodermal layer and enhance the migration ability of trophoblast cells in
1070 culture, either as isolated cells or in the whole embryo. The presence of the laminin and
1071 fibronectin in the cargo of the inner cell mass EVs, enabled attachment to the integrins on the
1072 trophoblast cell surfaces and stimulated JNK and FAK kinase cascades, increasing trophoblast
1073 migration. Further, injection of these EVs inside the blastocoele cavity of 3.5 day blastocysts
1074 increased their implantation efficiency (79). It must be noted that this mechanism may be
1075 particular to the mouse and those other species in which the ICM is distal to the site of
1076 trophectoderm attachment to the endometrial surface: in women this is the reverse with the
1077 ECM tightly aligned with the attaching trophectoderm.

1078 EVs produced by ungulate trophectoderm participate in cross-talk with the maternal
1079 endometrium (312). Bidarimath and colleagues observed that EVs from a porcine
1080 trophectodermal cell line stimulated the proliferation of endothelial cells *in vitro*, thus being
1081 potential regulators of maternal endometrial angiogenesis (314). These vesicles contained a
1082 miRNA and protein cargo likely to annotate functions in the angiogenesis process. Again, care
1083 should be taken with these data as they were retrieved from cell lines cultured *in vitro*. Further,
1084 the pig is a species with epitheliochorial placentation, and thus the *in utero* development is very

1085  different from that of the human (314). Nevertheless, study of human extravillous trophoblast

1086  cell (HTR-8/SVneo and Jeg3)-derived exosomes similarly showed that these vesicles promote

1087  vascular smooth muscle cells migration, which is important during human uterine spiral artery

1088  remodelling in successful pregnancies (315). Importantly, the two trophoblast cell lines (which

1089  are different stages along their differentiation pathway) produced differential migration results,

1090  raising the likelihood that cell origin as well as content and bioactivity of the exosomal cargo

1091  are of considerable importance, emphasising the need to keep models as close to the

1092  physiological situation as possible.

1093        **v. EVs as vehicles for embryo-maternal cross talk**

1094  The first indication that the endometrium produced EVs with unique cargo was that the human

1095  endometrial epithelial cell model ECC1 (which best represents luminal epithelium), released

1096  EVs containing a different miRNA profile from that of parent cells (149). These EVs could

1097  provide a mechanism for communication between the mother and the embryo with potential

1098  implications in embryo implantation. Indeed, bioinformatic analyses on the EV miRNAs

1099  showed predominance of the genes targeted by the miRNAs as involved in implantation.

1100  Furthermore, interrogation of the proteome of ECC1 EVs, cultured under conditions to

1101  represent the proliferative (estrogen-dominant) and secretory (estrogen plus progesterone)

1102  phases of the cycle, showed that the protein cargo of EVs is hormone-specific, enriched with

1103  254 and 126 proteins respectively (82). Importantly, 35% of the endometrial EV proteome had

1104  not been previously reported, indicating the unique cargo of endometrial EVs. These findings

1105  were validated in EVs from primary endometrial epithelial cells. Functionally the EVs were

1106  internalised by human trophoblast cells, inducing increased adhesive capacity, that was at least

1107  partially mediated through active focal adhesion kinase (FAK) signalling, indicating a likely

1108  role in promoting embryo implantation (92). Interestingly, among the implantation-related

1109  proteome of these endometrial exosomes, were the cell surface metalloproteinases ADAM10

1110  and MMP-14 (a membrane-bound MMP), for which there are abundant substrates on the

1111  trophectoderm.

1112  Another study showed that endometrial epithelial derived EVs in the uterine fluid contain has-

1113  miR-30d during the receptive phase of the cycle. This EXOs-associated hsa-miR-30d was

1114  internalized by mouse embryos via the trophectoderm, resulting in an indirect overexpression

1115  of genes encoding for certain molecules involved in the murine embryonic adhesion

1116  phenomenon—*Itgb3*, *Itga7*, and *Cdh5*. Functionally, in vitro treatment of murine embryos with

1117 miR-30d resulted in a notable increase in embryo adhesion again indicating how maternal
1118 endometrial miRNAs might act as transcriptomic modifiers of the pre-implantation embryo
1119 (133).
1120

## Part IV. Implications of EVs in Reproductive Pathology

1122 Given their seminal functional role and presence in various aspects of reproductive biology, a
1123 growing field of evidence is uncovering potential roles for EVs in regulating reproductive
1124 pathological conditions including endometriosis, polycystic ovaries syndrome, erectile
1125 dysfunction, early pregnancy loss, hypertension, pre-eclampsia or gestational diabetes mellitus
1126 (summarized Table 4). Given this importance in EVs during maternal environment and
1127 development, significant efforts are now focused on evaluating prognostic value and
1128 applicability of EVs as diagnostic and therapeutic agents (90,316).

### I. EVs in endometriosis

1130 Endometriosis is an estrogen-dependent inflammatory disease which is characterized by the
1131 deposition and growth of endometrial cells outside the uterine cavity, with the pelvic
1132 peritoneum and ovaries being the most common sites for ectopic growth (317). For this reason,
1133 endometriosis is considered a benign metastasizing disease (318).

1134 Endometriosis is characterized in part by an increase in the expression of angiogenic factors
1135 and metalloproteinases. Patients with endometriosis show higher levels of these molecules in
1136 endometriotic lesions than in eutopic endometrium and eutopic endometrium of endometriosis
1137 patients shows higher levels than healthy endometrial controls (319). Indeed, by inhibiting
1138 metalloproteinases it is possible to avoid the establishment of ectopic endometriotic cysts (320).
1139 In this context, EMMPRIN, a metalloproteinase inducer, is carried in EVs produced by uterine
1140 epithelial cells and stimulates the expression of metalloproteinases in stromal fibroblasts. The
1141 secretion of both EMMPRIN and metalloproteinases, is positively regulated by IL-1β/α, whose
1142 secretion is increased in women under endometriosis conditions in whom there is a pro-
1143 inflammatory peritoneal environment. This would allow the increase of metalloproteinases
1144 production by fibroblasts to trigger endometriotic lesions invasion (321).

1145 In terms of EV RNA cargo, EVs from endometrial stromal cells from women with
1146 endometriosis versus women without the disorder, showed different profiles of exosomal

1147     miRNA content between EVs derived from eutopic and ectopic endometrium from
1148     endometriosis subjects and between eutopic endometrium from women without or with disease
1149     (322). Moreover, there was a differential miRNA signature, between eutopic endometriotic and
1150     control exosomes. Among these miRNAs, miR21, is already known for a role in angiogenesis.
1151     It remains to be established whether miR-21 can promote angiogenesis following EV uptake
1152     (322).

1153     Ectonucleotidases are enzymes involved in inflammatory processes and previously reported as
1154     expressed in the endometrium. Teixidó and colleagues investigated ectonucleotidase activity
1155     from endometriotic cysts (endometriomas) on the ovary, one of the common sites for
1156     endometriotic lesion development. Ectonucleotidases were highly enriched in endometriomas
1157     compared to simple cysts. Interestingly, the ectonucleotidase activity was also contained by
1158     exosomes derived from endometriomas and simple cyst fluids, but and was significantly higher
1159     for exosomes from endometriomas (323).

1160     **II. Polycystic Ovarian Syndrome**

1161     Polycystic ovarian syndrome (PCOS) is one of the most common hormonal disorders affecting
1162     women, characterized by androgen excess and insulin resistance, leading to androgenism, high
1163     risk of glucose intolerance, diabetes and lipid abnormalities (324). Its complex phenotypic
1164     manifestation was formally described nearly a century ago as the concurrence in women of
1165     amenorrhea, hirsutism, obesity and typical polycystic appearance of the ovaries (325).

1166     Koiou and collaborators observed that platelet microvesicles in plasma from PCOS affected
1167     women (defined by elevated circulating androgens and insulin resistance markers) were at
1168     higher levels than in healthy controls. Moreover, there was a significant positive correlation
1169     between microvesicles and numbers of follicles in the ovaries of these women (326).
1170     Subsequent confirmation of the increase in EVs levels (mainly of exosomal size) in PCOS also
1171     demonstrated a direct correlation with insulin resistance markers. Furthermore, PCO-derived
1172     EVs showed a higher content in annexin-V along with 16 miRNA that are normally expressed
1173     at low levels, being increased with PCOS, (327).

1174     Sang and colleagues described EVs in the human follicular fluid and identified 120 miRNAs
1175     within their cargo, 11 of which were highly expressed and with target genes in pathways
1176     involved in reproduction, endocrine and metabolic processes. Two of these miRNAs, miR-132
1177     and -320, were significantly decreased in the follicular fluid EVs from PCOS patients

compared to non-affected controls (328). Of note, miR-132 and -320 have HMGA2 and RAB5B respectively as target genes: these were associated with key roles in the etiology of PCOS in a previous genome-wide association study (329).

DENND1A is a PCOS candidate locus, characterized in a number of genome-wide association studies (329,330). DENND1A variant 2 levels, both at protein and mRNA levels, were increased in theca cells of PCOS patients compared to healthy controls. In agreement with these results, mRNA for this locus was significantly increased in exosomes extracted from urine of PCOS-affected women in comparison to normal-cycling controls. In this sense, the exosomal miRNA profile is proposed to reflect the physiological status of the source cells, providing a potential biomarker of PCOS (331). Further studies are needed to uncover the roles of EVs in the triggering and development of PCOS.

### III. Erectile dysfunction

Erectile dysfunction (ED) is the most studied sexual problem worldwide and mainly affects men over 40 years of age. It costs up to £7 million in the UK and $15 billion in the USA. The prevalence of this condition varies greatly throughout the world, highlighting the Middle East (45.1%), United States (37.7%) and specially mainland China (varying from 17.1 to 92.3%), according to a retrospective study carried on men of different ages (332).

Microparticles have been proposed as involved in endothelial dysfunction and atherogenesis, with special regard to ED. Initially, microparticles defined as membrane vesicles, apoptotic or not, smaller than 1.5 µm, were recovered from plasma after platelet depletion at 900 x g and measured by flow cytometry using specific markers (333). These circulating endothelial-derived microparticles were increased in type 2 diabetic men with ED, compared with controls and a positive correlation between microparticle counts and ED severity, determined by the International Index of Erectile Function (IIEF), was shown. However, diabetes risk factors did not influence microparticle levels and so, these were postulated to be independently linked to ED severity. Finally, microparticles were proposed as possible links between endothelial dysfunction and ED (333). Retrospectively, a molecular signature identified in microparticles enabled discrimination between diabetes and ED. The marker CD31 in microparticles was mainly related to diabetes, whereas CD62E, was directly linked to ED, without diabetes. The ratio CD31/CD62 could be used to evaluate endothelial function, with a high ratio being related to endothelial activation and a low ratio associated with apoptosis. In the study, diabetic men

1209 with ED men showed lower ratios, maybe indicating a cooperative effect of the two disorders.

1210 Finally, levels of CD31+ microparticles were directly correlated with ED aggressiveness (334).

1211 La Vignera and colleagues, increased the centrifugal force to achieve a better clearance of
1212 platelets from serum (13.000 x g). They confirmed an increase in endothelial-derived
1213 microparticles levels in ED patients with arterial etiology, in comparison to patients with ED
1214 of psychogenic origin. Since a positive correlation was observed with typical ED metabolic
1215 parameters they proposed endothelial dysfunction as the cause underlying ED and reasserted
1216 microparticles as predictors of the condition (335). Furthermore, their levels were directly
1217 related to the aggressiveness of arterial ED (336): a combination of disorders leading to a greater
1218 vascular damage was associated with more severe ED and endothelial dysfunction, and
1219 correlated with increasing levels of endothelial microparticles (337).

1220 ED is associated with increased endothelial apoptosis, and both can be in part, reverted by
1221 treatment with a type 5 phosphodiesterase inhibitor such as tadalafil (338). Treatment benefits
1222 were maintained for 4 weeks after the cessation of a 1-year treatment in almost half of the
1223 analysed cases (339). Subsequently, the effect of tadalafil treatment and discontinuation on the
1224 production of apoptotic endothelial-derived microparticles was examined. ED patients had
1225 increased levels of apoptotic microparticles compared to controls before the start of the
1226 treatment. 90-days of tadalafil administration improved IIEF, endothelial parameters and
1227 reduced apoptotic microparticle levels, although not to control levels. These improvements
1228 reverted by six months after treatment discontinuation (340). Interestingly, complementation of
1229 tadalafil treatment with an antioxidant, maintained the tadalafil effects at least until 6 months
1230 after treatment cessation, prolonging the duration of the antiapoptotic effect within
1231 endothelium (341). This is in accord with other studies implicating oxidative stress in
1232 endothelial dysfunction (342,343). Patients with greater severity and duration of ED, associated
1233 with the concurrence of high cardiovascular risk profiles, were non-responders to sildenafil,
1234 another type 5 phosphodiesterase inhibitor.

1235 Androgen deficiency has also been proposed to contribute to the development of cardiovascular
1236 disease and endothelial function impairment (344). Six months of androgen replacement
1237 therapy (Tostrex) improved endothelial and erectile dysfunction features and decreased
1238 endothelial derived microparticle levels in patients of ED and late onset hypogonadism (LOH;
1239 a new vascular risk factor) (345). Indeed, LOH worsened metabolic parameters and increased
1240 the already high endothelial microparticle levels in ED patients (346).

## IV. Pregnancy complications

EVs from a variety of sources (epididymis, prostate, cervical mucus, ovarian follicle, embryo, and endometrium) have potential roles in both the establishment and development of a successful pregnancy. However, from the sixth week of gestation (347), placental-derived EVs mainly of syncytiotrophoblast origin, represent the main source of vesicles with potential implication in feto-maternal communication (23,78). Their concentrations in maternal plasma increase gradually as pregnancy progresses (348). Their release and bioactivity are favoured by both low oxygen tensions (349) and high D-glucose concentrations (350). Changes in concentration, composition and bioactivity of placental and non-placental EVs have been reported in pregnancy disorders (351). Notably, the secretion of EVs is increased in the two main EVs-related pregnancy complications: gestational diabetes (352) and preeclampsia (353).

### i. EVs in early pregnancy loss

Early pregnancy loss (PL) is a common complication that affects around 15% of the gestations and shows recurrence rates of 2-3%. Importantly, up to 50% of these cases are usually of idiopathic etiology (354). Interestingly, the levels of plasma endothelial microparticles are decreased in pregnancy loss, especially in cases with recurrent miscarriage, compared to controls (355). However, these results should be viewed with caution, as in healthy pregnancy (their controls), there is also an increase in EV levels, mainly due to the contribution of placental-derived EVs (347).

In pregnancy, the haemostatic balance shifts towards upregulated pro-coagulant activity, with increased clotting factors and fibrinogen, and concurrently decreased anticoagulant factors and fibrinolytic activity (356). An excessive pro-coagulant response leading to thrombosis of the uteroplacental vasculature and subsequent hypoxia, has been proposed as a factor accounting for an important part of the fetal loss cases (357). In this regard, blood microparticles with pro-coagulant activity are increased in miscarriage cases, in parallel with the enhanced coagulation-promoting activity. These microparticles may play a role in this outcome by favouring the thrombotic phenomena (358,359). Furthermore, PL-affected women present with lower levels of platelet microparticles and higher levels of endothelial microparticles than controls, although this could not be directly related to the hypercoagulation phenotype, it was suggested to reflect endothelial dysfunction (360). In contrast, plasma platelet-derived microparticles were increased in women with recurrent miscarriage compared to controls (326): However, these

1272 results may be biased by the small size of the study population (361) and the controls may be
1273 inappropriate due to the contribution of the placenta to the total EV content.

1274 ### *ii. EVs in gestational vascular complications*

1275 Gestational vascular complications which include hypertension (HT) and pre-eclampsia (PE),
1276 are prevalent causes of maternal and fetal morbidity and mortality. HT may appear as a
1277 consequence of abnormal placentation into the maternal uterus, and may lead to the
1278 development of impaired liver function, progressive renal insufficiency, pulmonary edema and
1279 the new onset of cerebral or visual disturbances that might end in HELLP syndrome
1280 (hemolysis, elevated liver enzymes and low platelet count) and/ or eclampsia (362). PE is a
1281 complex disorder causing preterm birth, intra-uterine growth-restriction and maternal death
1282 (363). In general, different studies point towards an increase in endothelial microparticle
1283 shedding within GVC conditions, thus suggesting vascular injury (364).

1284

1285 ### *1*. **Pre-eclampsia (PE)**

1286 PE is a pregnancy-related syndrome affecting between 2 and 8% of pregnancies and
1287 characterized by a variety of systemic symptoms. It is detected by new onset hypertension and
1288 proteinuria after the $20^{th}$ week of gestation. Its etiology is not well known, but the pathogenesis
1289 of PE is conceptualized in a two-stage model with the placental defect precipitating an
1290 abnormal vascular maternal response that manifests as the signs of this pathological condition
1291 Early PE appears before 34 weeks of gestation and involves the fetus, showing reduced
1292 placental perfusion, possibly due to abnormal trophoblast invasion and/or uterine spiral arteries
1293 remodeling. Late PE appears after 34 weeks, and the maternal manifestations appear, a series
1294 of inflammatory, metabolic and thrombotic responses compromise vascular function up to the
1295 point of producing systemic organ damage (365).

1296 Several published studies have attempted to elucidate the relevance of EVs of both maternal
1297 and placental syncytiotrophoblast origin, in the pathophysiology of PE. Changes in EV
1298 concentration and cargo, affect PE development via pro-inflammatory and pro-coagulatory
1299 activities enhancement. Here we summarize current knowledge of EVs in relation to PE.

1300 #### a. *Placental-derived EVs*

The placenta plays a critical role and is undoubtedly the source of PE development. PE can develop even in the absence of a fetus, provided that trophoblast tissues are established, forming the characteristic mass known as a hydatidiform mole, a tissue abnormality formed by the distension of some or all of the chorionic villi (366).

Syncytiotrophoblast-derived exosomes and microvesicles (STMBs) are increased in PE compared to normal pregnancies (367), maybe in part due to the hypoxia resulting from abnormal placentation (315). This increase occurs specifically in early-onset PE cases but not in late-onset PE or normotensive intrauterine growth restriction (368,369). Importantly, early-onset pre-eclampsia is established in the first trimester when trophoblast invasion and vascular remodelling occurs (315), emphasising the importance of STMBs in these processes. Furthermore, variations in protein (353,370,371), lipid (372) and miRNA (353) cargo of STMBs may explain the specific roles of STMB in PE including immune response, coagulation, oxidative stress and apoptosis.

One of the main characteristics of PE is abnormal remodelling of the uterine spiral arteries, which in normal pregnancies ensures enough maternal blood flow to support fetal growth and development. Thus, a role for extravillous trophoblast (EVT)-derived EVs has been proposed in PE development. Variations in concentration, cargo and bioactivity of EVT-derived EVs as indicated above, may result from a pro-inflammatory environment, inducing these changes, impairing their physiological roles in vascular/smooth muscle tissue remodelling, and thus stimulating the emergence of PE (315,373). In PE, increased amounts of pro-inflammatory cytokines (IL-18, IL12, TNF-α) are released by monocytes and lymphocytes. PE-increased STMBs can bind monocytes to promote the production of more inflammatory cytokines, perpetuating the pro-inflammatory environment and hence stimulation of EV alterations and endothelial cell damage (367). Furthermore, villous cytotrophoblast-derived exosomes carry syncytins 1 and 2, which are involved in exosome fusion with the target cells. Importantly syncytin-2 content was reduced in exosomes derived from serum of PE patients (374).

Antiangiogenic factors, such as sFlt1 and sEng, appear to participate in PE through a series of mechanisms that lead to the imbalance of angiogenic factors and finally to the generation of endothelial dysfunction and the maternal syndrome of PE. Increasing levels of sFLT and sEng can predict PE and directly correlate with the aggressiveness of this syndrome (375). PAI-1 and, to a lesser extent PAI-2, which is predominant in placenta, are important inhibitors of fibrinolysis. Their overactivation results in the establishment of fibrin deposits that occlude

placental vasculature and spiral arteries, leading to hypertension and endothelial damage causing PE. Moreover, increasing levels of PAI-1 in plasma directly correlate with PE severity (376). Eng and PAI-2 are highly expressed and localized to the surface of STMB microvesicles and exosomes, and thus can readily influence the development of PE (377). In addition, STMB from PE patients possess increased tissue factor activity compared to normotensive patients (378) and this could increase fibrin deposition. Coagulation may be enhanced by STMB action directly by direct association with platelets leading to activation: such activity is increased in PE-derived STMBs and correlates with PE-associated thrombotic risk. Moreover, treatment with aspirin, which is usually prescribed for PE women to reduce platelet aggregation, also inhibits STMB-induced platelet aggregation (379).

Cell-free haemoglobin (HbF) is released by the placenta and increased haemoglobin (Hb) expression as well as HbF accumulation in the vascular lumen of PE placentas has been reported (380). Indeed, HbF has been proposed as an important factor marking the transition between the first and second stages of PE. HbF causes placental damage similar to that observed in PE by inducing oxidative stress, which affects the blood-placenta barrier (BPB) integrity (381). BPB disruption may lead to the release of placental factors, including HbF which leak into the maternal circulation contributing to the maternal affectations of PE. Moreover, levels of HbF correlate with PE severity symptoms (382). Placental HbF can provoke differential alterations in STBM miRNA cargo between EVs populations: three miRNAs were specifically downregulated in microvesicle populations under HbF influence. STBMs may also transport HbF itself, although these data may be an artifact of the external HbF perfusion (383). Furthermore, STBMs from PE pregnancies exacerbated the production of superoxide radicals by neutrophils in a dose-dependent manner, also correlating with PE severity. In this way, STBMs display multiple mechanisms to cause vascular damage and dysfunction in women with PE (384).

*b. Maternally-derived EVs*

Even before pregnancy, maternal risk factors for PE are obesity, diabetes mellitus, hypertension and Systemic Lupus Erythematous (SLE). Pro-PE EVs have altered concentrations and modified molecular contents that may alter the functioning of maternal tissues prior to pregnancy. In particular, changes in endothelial cells, leukocyte and platelet-derived EVs are associated with the risk of PE. All share the common feature of a general increase in endothelial and platelet-derived EV levels (for review see (385)).

1365 Once pregnancy is established, maternal EVs of different cellular origin interact with
1366 embryonic tissues with potential implications in PE pathogenesis. Platelets have crucial roles
1367 in PE development and several studies report decreased platelet-derived EV levels in
1368 pregnancy compared to non-pregnancy, with further decrease in PE (385). EVs of maternal
1369 endothelial and platelet origin appear to unleash a thrombo-inflammatory response in the
1370 placenta. EVs cause activated platelet aggregation and inflammasome activation within the
1371 placental vascular and trophoblastic cells, triggering a PE-like phenotype. Further, inhibition
1372 of inflammasome or platelet activation components within the placenta abrogated the PE-like
1373 phenotype (386).

1374 In contrast to platelets, leukocytes and certain derived EVs populations are increased in PE in
1375 comparison to normotensive pregnancies, mainly those EVs of granulocyte and monocyte
1376 origin (387). Interestingly, low levels of NK cell-derived EVs are observed in PE, linking with
1377 PE-associated maternal immune tolerance disorders (NK cell death activity dysfunction) (388).
1378 Of interest, Holder and collaborators showed that human placenta is able to internalize
1379 exosomes from macrophages via endocytosis. Importantly, macrophage exosomes uptake
1380 induced the release of proinflammatory cytokines by the placenta (389). Previously, the same
1381 group had reported that exosomes from PE placenta can activate peripheral blood mononuclear
1382 cells (PBMCs), inducing a pro-inflammatory response to a greater extent than EVs from normal
1383 placenta, and related to their cytokine content, mainly IL1β. Moreover, PE-derived EVs
1384 stimulated an enhanced response of PMBC to external PAMPs such as LPS (389). Such
1385 outcomes may be triggered by direct stimulation by EVs of TLR, the signal subsequently
1386 internalized via NF-κB (390). Taken together, these studies indicate a potential positive feed-
1387 back loop by which an inflammatory response is overstimulated under PE conditions via EVs.
1388 Endothelial-derived EVs levels correlate with the increment of the anti-angiogenic factor sFLt1
1389 and the ratio sFLt1:P1GF. This combined evidence suggests that apoptosis of endothelia occurs
1390 along with inhibition of angiogenesis, and correlates with PE-characteristic endothelial damage
1391 which persists between <1 week (391) to 72 hours postpartum (392).

1392 Regarding obesity, a link between exosomes release and the progression of PE is emerging. A
1393 recent study has observed that the levels of exosomes in maternal blood are correlated with
1394 maternal BMI. A positive correlation of BMI with exosomes levels was established, leading to
1395 the decrease of placental-derived exosomes proportions throughout gestation. These increased
1396 exosomes levels contributed to a further exacerbated release of IL-6, IL-8 and TFN-α from

1397 endothelial cells thus leading to a worsen systemic inflammation in a BMI-dependent manner
1398 (393).

1399 Finally, it has been observed that serum microvesicles from healthy pregnant women can
1400 reduce caspase activity and stimulate migration and tube formation in endothelial cells, while
1401 this is abrogated when the microvesicles are derived from patients with gestational vascular
1402 complications such as PE and hypertension. Further, similar opposing actions on early-stage
1403 trophoblast of these vesicles was observed (394).

1404       *iii. EVs in gestational diabetes*

1405 Gestational diabetes (GD) is defined as a carbohydrate intolerance of variable severity that
1406 appears or is first recognized during pregnancy. Along with PE, GD represents the most
1407 common metabolic complication of pregnancy, affecting between 1 and 15% of all pregnancies
1408 and increasing concurrently with obesity rates. It is characterized by pancreatic beta cell
1409 insufficient insulin production, usually due to pregnancy and characteristic insulin resistance,
1410 and is associated with maternal and fetal morbidity. Moreover, women with GD have increased
1411 risks of developing type II diabetes in the future (395,396).

1412 To date, little is known about the contribution of EVs in this pathophysiology. Salomon and
1413 colleagues showed increased serum placenta-derived exosomes in GD pregnancies compared
1414 to control pregnancies, regardless of gestational age. In vitro, GD exosomes increased the
1415 release of proinflammatory cytokines from endothelial cells contributing to the enhanced
1416 proinflammatory state in pregnancy under GD conditions (397).

1417 # **Part V. Clinical and therapeutic applications of EVs**

1418

1419 The involvement of EVs in a wide variety of pathophysiological processes has made them
1420 appealing players as biomarkers and to carry therapeutic agents. This may also be the case
1421 when considering reproductive disorders.

1422

1423 ## **I. EVs as biomarkers**

1424

1425 EVs have been proposed as potential biomarkers of disorders of reproductive organs. The
1426 placenta releases EVs from the sixth week of pregnancy with steady increase as pregnancy

1427 proceeds, peaking at term (379). Importantly, their release is modulated by a number of factors
1428 that arise from the placenta; hence EVs may provide mirrors of placental/foetal health and
1429 evolution (351). Since maternal blood is the primary source of placental exosomes it will
1430 contain both maternal and placenta-specific EV populations and thus placental alkaline
1431 phosphatase (PLAP) has been proposed as a marker for the placental EVs, since it is restricted
1432 to placental cell linages (347).

1433

1434 Alterations in both, the levels and cargo of placental-derived exosomes during pregnancy are
1435 associated with different pregnancy complications. A proteomic signature of 62 proteins in
1436 microparticles was developed from plasma samples of women at 10-12 weeks of gestation
1437 (385). This signature was able to predict and differentiate spontaneous premature births (SPB)
1438 from normal term births. Functional enrichment analyses showed processes related with
1439 preterm birth, such as inflammation, fibrinolysis, immune modulation, the coagulation cascade
1440 or steroid metabolism. Currently, the only tool for evaluation of risk of spontaneous preterm
1441 birth is measurement of cervical length by ultrasound (386). A retrospective study on plasma
1442 samples of women at early gestational age (prior to 18 weeks), demonstrated potential for
1443 exosomes in the diagnosis of PE and SPB with higher (but not significant) levels of exosomes
1444 in both pathological conditions versus normal pregnancies. More interestingly, a specific
1445 exosomal miRNA signature could differentiate between the three conditions, being more
1446 similar between normal pregnancy and SPB compared with that of PE. When these miRNA
1447 profiles were compared with those from the extravillous trophoblast HTR-8/SVneo cell line
1448 cultured under normal and low-oxygen tension (LOT) conditions there was a strong correlation
1449 between the SPB and LOT conditions, with a common variation in >45% of the SPB condition
1450 miRNA profile. Placental-exosomal miRNA cargo was related to cell migration potential and
1451 inflammatory cytokine production. Particularly, LOT-exosomes decreased endothelial cell
1452 migration potential and increased their TNF-α production, which could negatively impact
1453 spiral artery remodelling during placentation. Thus, under circumstances that favour a pro-
1454 inflammatory environment or a reduction of oxygen tension such as advanced gestational age,
1455 placental EVs may be negatively altered, impacting spiral artery remodelling and resulting in
1456 development of pathologies such as PE or SPB (387). In this sense, placental EVs may be
1457 potential early biomarkers of PE/SPB or as targets for directed therapy. Finally, both total and
1458 placental-derived EVs are increased in women delivering low-birthweight babies compared to
1459 those with normal-birthweight deliveries (398).

1460

EVs have been further investigated as biomarkers of PE. Recent publications debate the usefulness of EVs content for their predictive value in the diagnosis of PE. As an example, Tan and colleagues analysed three candidate biomarkers, TIMP-1, PAI-1 and P1GF, for their predictive ability in a large cohort of low-risk PE women from bank plasma samples. They concluded that the use of TIMP-1 and PAI-1 reinforced the value of the classical P1GF for PE prediction (399). In fact, TIMP-1 and PAI-1 were analysed in specific subgroups of EVs which can be retrived thanks to their affinity to cholera toxin B and annexin V, the interest of both of which had been described in a previous work for the study of PE biomarkers. In this study, they purified EVs from plasma of women at ~32 week of pregnancy, using immunoadsorption to the surface proteins, GM1 ganglioside (binds to cholera toxin B chain) and phosphatidylserine (binds to Annexin V). They isolated two populations of EVs (one from each marker) and a specific protein signature was identified for PE compared to healthy pregnant controls. It is important to highlight that such biomarker discovery is highly dependent on the selected conditions providing a possible limitation. Indeed, in this study, large cellular debris were not removed from samples prior to the immunoadsorption step, providing a major potential source of error (400). In another study, different subtypes of microvesicles were evaluated in plasma, compared with cord blood from normal women and those with PE. Microparticles were more abundant, and had altered coagulation–related factors in cord blood in PE compared with no PE (401). Recently, Salomon and colleagues (2017) investigated whether exosomes and their miRNA cargo might provide early biomarkers of PE. Over 300miRNAs were identified in total and placenta-derived exosomes in maternal plasma across gestation with has-miR-486-1-5p and has-miR-486-2-5 being identified as candidates for further study. Functional analysis showed that these miRNAs are involved in migration, placental development and angiogenesis (402). Since PLAP is a marker of serum placental-derived exosomes, which trend upwards with gestational age, exosomal content of PLAP has been proposed as a potential biomarker of PE in saliva and gingival cervical fluid (403). Finally, reduced EV-associated endothelial nitric oxide synthase expression and activity, a common feature of PE, was elevated in EVs from PE placentas (defined by PLAP), in both serum and placental perfusates, compared with healthy controls (404). Considering the previous information, it is important to notice that current biomarkers of pregnancy complications, such as PE or GDM, allow us to diagnose these states once the pathologies are established and the clinical management is limited. In this sense, in order to evolve in the field, efforts should be conducted to discover new biomarkers during early gestation.

1495     EVs have also been proposed as biomarkers of peripartum cardiomyopathy (PPCM). PPCM is

1496     an idiopathic form of cardiomyopathy characterized by left ventricular systolic dysfunction

1497     (the ejaculation fraction is reduced normally bellow 45%) and subsequent heart failure. It

1498     usually appears around the end of pregnancy and in the next few months and, it is currently

1499     only diagnosed by exclusion of other heart failure causes (405) making a search for new

1500     biomarkers of considerable importance. Initially, Walenta and collaborators (2012) reported

1501     increased levels of blood-derived activated endothelial microparticles in PPCM when

1502     compared with healthy post-partum, pregnant and non-pregnant control but also with patients

1503     of ischemic cardiomyopathy (ICM) and stable coronary arterial disease (CAD). These

1504     microparticles in PPCM were mainly platelet-derived and monocyte microparticles. Treatment

1505     with bromocriptine, a therapy proven to work in animal models and human patients,

1506     significantly reduced endothelial and platelet-derived microparticles in PPCM compared to

1507     patients treated with standard undirected heart failure therapy. Thus, specific microparticle

1508     profiles may provide biomarkers that can distinguish PPCM from normal pregnancy, vascular

1509     diseases and heart failure of different origin (406). MiR146a has also been identified as a

1510     possible exosome-associated biomarker for PPCM. The 16-kDa N-terminal prolactin fragment,

1511     the primary known trigger of PPCM, stimulates the packaging of miR-146a into exosomes

1512     from HUVECs, which then are able to reach cardiomyocytes and trigger PPCM. Thus miR-

1513     146a may provide a biomarker and therapeutic target for PPCM (407).

1514

1515     Placental EVs may provide indicators of infectious diseases during pregnancy. Both total and

1516     placental-derived EVs are increased in plasma from pregnant women with HIV infection

1517     compared with non-infected controls. In contrast, there were no changes in the level of plasma

1518     EVs due to malaria infection, neither for placental malaria nor for its peripheral variant.

1519     Nonetheless, miR-517c was found to be increased in microparticles from plasma of women

1520     with active placental malaria compared with non-infected controls (398).

1521

1522     **II. Clinical and therapeutic aspects of EVs in reproductive biology**

1523

1524     Intercellular transfer of genetic and protein material mediated by EVs could facilitate new

1525     diagnostic and therapeutic tools in the field of reproductive biology. As discussed, EVs are

1526     stable, versatile, cell-derived nanovesicles with target-homing specificity and the ability to

1527     transfer through *in vivo* biological barriers and hold promise for the development of new

1528     approaches in drug delivery (66). Specifically, bioengineered EVs are being successfully

1529 deployed to deliver potent drugs and the capacity for select cellular reprograming capacity

1530 (6,32). Recently, members of the International Society for Extracellular Vesicles (ISEV) and

1531 the Society for Clinical Research and Translation of Extracellular Vesicles presented a

1532 framework for challenges associated with development of EV-based therapeutics at the

1533 preclinical and clinical levels (408). This discussion addresses development of best-practice

1534 models and current outlook for EV therapies.

1535

1536 Engineered or modified EVs can be designed for cell-specific targeting and delivery (409,410).

1537 A seminal study has demonstrated the selective cellular uptake of EVs surpasses that of more

1538 traditional carriers such as liposomes or nanoparticles, taking advantage of EVs' natural

1539 characteristics to deliver molecules to target cells (411). Such insights provide future

1540 possibilities for clinical applications of EVs based on their ability to circumvent the limitations

1541 of various drug delivery systems of mucosal and blood brain barrier traversal. The

1542 physicochemical configuration of EVs can also be modified to enable extended clearance

1543 compared with synthetic nanoparticles, and spatio temporal localization (ligand and cell-type

1544 specific targeting) and controlled release (212,412-414). With respect to modifying EV cargo,

1545 a recent, comprehensive study compared various passive and active drug-loading methods

1546 including electroporation, saponin treatment, extrusion and dialysis, and used porphyrins of

1547 various hydrophobicities as model drugs (415). A comprehensive overview of EV cargo

1548 loading strategies, including electroporation, sonication, direct transfection, and cellular

1549 engineering is reviewed (416,417).

1550

1551 The potential functional roles of EVs in human embryo development have only recently been

1552 demonstrated. Embryos may generate their own microenvironment by secreting soluble factors

1553 and membrane vesicles, which constitute a secretome with select autocrine and paracrine

1554 signaling (82,418-422). In reproductive biology, nanoparticles have been used experimentally

1555 to load sperm with exogenous genetic material that is subsequently transferred to the oocyte

1556 during fertilization (423,424). EVs have been identified in uterine fluid during the estrous/

1557 menstrual cycles including humans, sheep and mice (66,133,149,311,425). Indeed, EVs

1558 derived from the maternal endometrium contain multiple subtypes including mixtures of EVs,

1559 exosomes and packaged different proteins, miRNAs and endogenous retrovirus mRNA

1560 (82,133,311-313,321,426). In the broader context of trophectodermal preparation for

1561 implantation, EVs have been shown to mediate communication between the inner cell mass

1562 (ICM) and the trophectoderm (79). EV-encapsulated cargo is protected from degradation and

1563 are highly stable in biological fluids. Such unique properties may greatly facilitate the

1564 translation of EVs and their select bioactive cargo and surface ligands into clinical applications.

1565 The study of EVs in reproduction has the potential for expanding our current understanding of

1566 the normal physiology of reproduction and pathological conditions such as implantation failure

1567 (421). Recent studies have provided key insights into the functional capacity of maternal EVs

1568 and how the protein cargo is directly modulated by uterine hormones during implantation to

1569 subsequently modulate trophoblast adhesive capacity (82). This study further validated select

1570 components in primary human endometrial cells under hormonal control.

1571 Select works have observed the ability of EVs to undergo cell-selective fusion (427) and tissue-

1572 specific tropism (202,428-430), as well as their capacity to transverse the blood-brain barrier

1573 (431) and penetrate structural tissue (432). Importantly, based on their surface composition,

1574 EVs may be directed to specific tissues and organs (202,428-430). Imaging of EVs in select

1575 targeted organs has indeed demonstrated that the interactions of EVs with target cells are highly

1576 dynamic (206,433). Such unique properties of circulating EVs make them promising

1577 applications for the delivery of therapeutic cargo. Several studies support the utility of EVs as

1578 a novel path for drug delivery and as new drug targets. Alvarez-Erviti et al., used an in vivo

1579 study to demonstrate that systemically injected neuron-targeted exosomes loaded with BACE1

1580 siRNAs (small interfering RNA) were able to significantly reduce BACE mRNA and protein,

1581 specifically in neurons (434). Further, exosomes loaded with artificial siRNA against MAPK

1582 were able to efficiently knockdown MAPK1 upon their delivery into monocytes and

1583 lymphocytes in vitro (435). Similarly, exosomes from iPSCs have been shown to deliver

1584 siRNA to attenuate expression of ICAM1 and neutrophils adhesion in pulmonary

1585 microvascular endothelial cells (436). Exosomes have further been applied for drug delivery

1586 to target a small-molecule, anti-inflammatory drug to select organs and immune cells . These

1587 studies have demonstrated the capacity for EV-mediated targeted and delivery capacity and

1588 importantly the ability for exosomes to deliver and modulate multiple pathways simultaneously

1589 in the targeted cells. All these studies are examples showing how EVs cargo can be manipulated

1590 in a way that may be useful for target-based drug development for successful in vivo drug

1591 delivery.

1592

1593 Recent reviews have discussed the rationale to aim for selective silencing of EVs that promote

1594 unwanted functional effects, however this is still an emerging concept in the field. Some of the

1595 strategies for specific silencing of EV subtypes (cell-specific) are likely to require careful and

1596 detailed mechanistic studies. This attends to the inherent difficulties to avoid the blocking of

1597 all EV types indiscriminately, which may interfere and perturb with physiological intercellular
1598 communication. Some examples of systems for abrogating EV formation and
1599 targeting/recipient cell uptake (reviewed include: (i) Inhibition of exosome formation,
1600 including treatment with dimethyl amiloride, (ii) Inhibition of the endolysosomal compartment
1601 functions, including proton pump inhibitors (PPI), (iii) Blocking of exosomes release, for
1602 example silencing GTPase Rab11/27A/35 using siRNA or targeting ESCRT proteins and/or
1603 GTPases involved in trafficking of exosomes, and (iv) Prevention of fusion or uptake of
1604 exosomes by target cells, what can be done by different reagents for blocking phosphatidyl
1605 serine such as diannexin, heparin to inhibit endocytosis (heparan sulphate proteoglycans),
1606 cytochalasin D to inhibit endocytosis and micropinocytosis, chlorpromazine to inhibit clathrin-
1607 dependent endocytosis, EIPA and LY294002 to block micropinocytosis, annexin-V to inhibit
1608 phagocytosis and macropinocytosis, methyl-β-cyclodextrin (MβCD), simvastatin and filipin
1609 III to target lipid raft–mediated endocytosis, nystatin to target caveolae-mediated endocytosis,
1610 dynasore to inhibit clathrin-independent endocytosis (calveolae), and nystatin to perturb lipid
1611 raft–mediated endocytosis.

1612

1613 Future studies are required towards investigating EVs from primary tissues and biofluids and
1614 incorporate state-of-the-art quantitative analyses, including quantitative proteomics (157,437)
1615 and sequencing technology could be exploited to study protein and gene regulation during
1616 pregnancy and allow identification and monitoring of functional or low-abundant EV cargo,
1617 and cellular drivers of implantation and signaling, that hitherto, have been unreported or
1618 functionally masked. Unlike small molecule pharmaceutical compounds, there are no defined
1619 parameters or assays for current safety testing of EV-based therapeutics (438). Understanding
1620 biodistribution patterns and circulating timeframe of locally and systemically administered
1621 EVs is important to assessing safety, in addition to techniques which enable reproducible
1622 mionitoring and safety testing of select EV marker cargo. Targeted studies using EVs (modified
1623 or engineered) will hold the potential to develop novel nanodiagnostics and nanotherapeutics
1624 to increase the success of pregnancy rates during ART or IVF. Recent work on targetable
1625 biodegradable delivery platforms for transporting biological cargo into gametes and embryos
1626 (reviewed (439)), emphasizes the need to understand how EVs enter cells. We anticipate that
1627 future investigations into the use of EVs for the intentional targeted delivery of molecular
1628 compounds will provide new horizons for reproductive science and clinical ART, ultimately
1629 leading to improvements in pregnancy success.

1630

## Part VI. Concluding remarks

Considering all the body of evidence treated in the present review, there is no doubt that the field of EVs and its implication in reproduction is rapidly evolving and promises a further understanding of the processes that lead to a successful pregnancy, as well as markers of correct or compromised reproductive function. Nonetheless, there is still a harsh way to go through. First of all, there is an unavoidable need to firmly define standard methods for EVs isolation, since they define the fractions we consider as different EVs populations and, as such, may lead to ambiguous results that cannot be compared among studies. New challenges associated with standardization of methods for isolation, quantification and analysis of EVs from complex tissues such as blood, and the stability of EVs within such biofluid samples, is still significant aspects in the field of EV biology.

It is undoubtfully necessary to get to know to how extent EVs are important and participate in the reproductive events that lead to the delivery of healthy normal newborns, as this knowledge will lead to new therapies and clinical test to ensure good pregnancy outcomes. Sample availability is maybe one of the main limiting factors that hinder science progress. In this sense, much more is known about epidydimal and prostatic EVs regulation of sperm compared with embryo maternal cross-talk through EVs. Anyway, EVs communication may constitute the cornerstone that will allow us to better understand the conception process. This is important as it paves the way to deal with those patients in which the current assisted reproductive techniques fail.

Finally, the data about the involvement of EVs in the triggering, maintenance and progression of reproductive and obstetric related disorders is still in its infancy and further key investigations utilizing homogeneous and human-specific matierial is needed. The use of EVs as disease biomarkers have the opportunity for diagnostic potential with reduced invasiveness, as they can be retrieved from body fluid instead of tissue biopsies. This is vital for embryo diagnosing, where the possibility of getting STMBs from mother blood-flow appears as an interesting alternative to invasive amniocentesis and chorionic villi sampling, further offering the possibility of an earlier diagnostic. Regarding EVs use as therapeutic agents, many different variants could be exploited. EVs could be used as vectors to deliver drugs and biological compounds in a targeted manner. Nevertheless, we could think of using them as therapeutic targets, when they are produced by affected cells and present disease promoting characteristics. This way can be achieved by inhibiting their biosynthesis, capturing them once produced or

1663    blocking their uptake by target cells, and may be interesting in diseases such as pre-eclampsia.

1664    Further, they could be used as natural therapeutic agents when experimental strategies rely on

1665    their natural features. Understanding cell-type specificity and the long-term effects of EV

1666    remodelling, and their potential to impart transgenerational consequences on the offspring's

1667    health, ranging from metabolism to sex determination, and potential epigenetic changes

1668    affecting the mother's fertility can alter the offspring's fertility, are key factors to be addressed

1669    in the field moving forward. Advances in research on noncoding RNAs Therefore,

1670    understanding molecular signaling networks, utilising advances in quantitative proteomics and

1671    sequencing technology, mediated by EVs that coordinate strategies for successful implantation

1672    may lead to approaches to improve the outcome of natural pregnancy and pregnancy conceived

1673    from in vitro fertilization.

1677

1678    **Figures and Tables Legends:**

1679    **Figure 1. Main types of extracellular vesicles present in body fluids and culture media.**

1680    EVs are classified in three groups according to their biogenetic pathways. Exosomes are

1681    produced in the endosomal pathway by invagination of the membrane of late endosomes to

1682    form intraluminal vesicles (ILV) enclosed in multivesicular bodies (MVB). MVBs can then

1683    fuse with lysosomes and degrade its content, or fuse with cell plasma membrane to release

1684    ILV, now regarded as exosomes. Microvesicles are produced directly from the cell plasma

1685    membrane by outward budding. Apoptotic bodies are generated as blebs in cells undergoing

1686    programmed cell death. Abbreviations: **E.E.:** Early Endosome, **Ex.V.:** Exocytic vesicle, **L.E.:**

1687    Late Endosome, **M.V.B.:** Multivesicular Body, **I.L.V.:** Intraluminal Vesicle, **EXO:** Exosome.

1688

1689    **Figure 2. Pathways shown to participate in EV uptake by target cells.** EVs transport signals

1690    between cells and facilitate selective reprogramming. EVs have been shown to be internalized

1691    by cells through **(1)** phagocytosis, **(2)** clathrin- and **(3)** caveolin-mediated endocytosis. There

1692    is also evidence to support their interaction with **(4)** lipid rafts resulting in EV uptake. Lipid

1693    rafts are involved in both clathrin- and caveolin-mediated endocytosis. EVs may also deliver

1694    their protein, mRNA and miRNA content by **(5)** fusion with the plasma membrane. EVs can

1695    be internalized by **(6)** macropinocytosis where membrane protrusions or blebs extend from the

1696 cell, fold backwards around the EVs and enclose them into the lumen of a macropinosome; **(7)**
1697 alternatively EVs are macropinocytosed after becoming caught in membrane ruffles. On the
1698 other hand, **(8)** intraluminal EVs may fuse with the endosomal limiting membrane following
1699 endocytosis to deliver their protein, mRNA and miRNA cargo and elicit a phenotypic response.
1700

1701 **Table 1. Classification of the methods of isolation of extracellular vesicles based on their**
1702 **principle.**
1703

1704 **Table 2. Classification of the methods of characterization of extracellular vesicles based**
1705 **on their principle.**
1706

1707 **Table 3. Main functions of extracellular vesicles in reproductive physiology classified by**
1708 **their origin.**
1709

1710 **Table 4. Involvement of extracellular vesicles in reproductive-related pathologies.**
1711

1712

1713 **Abbreviations**
1714

1715 AB: Apoptotic body
1716 AFM: Atomic force microscopy
1717 BLVRA: Biliverdin reductase A
1718 BPB: Blood-placenta barrier
1719 CAD: Coronary arterial disease
1720 cAMP: Cyclic adenosine monophosphate
1721 DLS: Dynamic Light Scattering
1722 dsDNA: double stranded DNA
1723 ED: Erectile dysfunction
1724 ELISA: Enzyme-linked immunosorbent assay
1725 EM: Electron microscopy
1726 ER: Endoplasmic reticulum
1727 ESCRT: Endosomal sorting complexes required for transport
1728 EV: Extracellular vesicle

1729    EVT: Extravillous trophoblast

1730    EXO: Exosomes

1731    FAK: focal adhesion kinase

1732    GD: Gestational diabetes

1733    GVC: Gestational vascular complications

1734    Hb: Haemoglobin

1735    HbF: Cell-free haemoglobin

1736    HDL: High density lipoproteins

1737    HIV: Human Immunodeficiency Virus

1738    HT: Hypertension

1739    ICM: Ischemic cardiomyopathy / Inner cell mass

1740    IIEF: International Index of Erectile Function

1741    ILV: Intraluminal vesicle

1742    ISEV: International Society for Extracellular Vesicles

1743    lncRNA: long non-coding RNA

1744    LOH: Late onset hypogonadism

1745    LOT: Low-oxygen tension

1746    MERVL: Endogenous retrovirus-like element

1747    miRNA: microRNA

1748    MMP: Matrix Metalloproteinase

1749    mtDNA: mitochondrial DNA

1750    MV: Microvesicle

1751    MVB: Multivesicular body

1752    μNMR: Micro nuclear magnetic resonance spectrometry

1753    NK: Natural Killer cells

1754    NOS: Nitric oxide synthase

1755    nPLEX: nano-plasmonic exosome assay

1756    NTA: Nanoparticle tracking analysis

1757    PBS: Phosphate buffer solution

1758    PBMC: Peripheral blood mononuclear cells

1759    PCOS: Polycystic ovarian syndrome

1760    PE: Pre-eclampsia

1761    piRNA: piwi-interacting RNA

1762    PKA: Protein kinase A

1763     PL: Pregnancy loss

1764     PLAP: Placental alkaline phosphatase

1765     PPCM: Peripartum cardiomyopathy

1766     PS: Phosphatidylserine

1767     RI: Refraction index

1768     RSV: Respiratory syncytial virus

1769     SEM: Scanning electron microscopy

1770     siRNA: small interference RNA

1771     SLE: Systemic Lupus Erythematous

1772     snoRNA: small nucleolar RNA

1773     SPB: Spontaneous premature births

1774     SPZ: spermatozoa

1775     STB: syncytiotrophoblast

1776     STMB: Syncytiotrophoblast-derived exosomes and microvesicles

1777     TEM: Transmission electron microscopy

1778     tRFs: tRNA fragments

1779     tRNA: Transfer RNA

1780     TRPS: Tuneable Resistive Pulse Sensing

1781

1782     **References:**

1783     1.     **Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borràs FE, Buzás EI,**

1784        **Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S,**

1785        **Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I,**

1786        **Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M,**

1787        **Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E,**

1788        **Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A,**

1789        **Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L,**

1790        **Olivan M, Oliveira C, Pállinger É, del Portillo HA, Reventós J, Rigau M, Rohde**

1791        **E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostenfeld MS,**

1792        **Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MHM,**

1793        **De Wever O.** Biological properties of extracellular vesicles and their physiological

1794        functions. *Journal of Extracellular Vesicles* 2015;4(0):27066.

1795        doi:10.3402/jev.v4.27066.

1796    2.    **Harding CV, Heuser JE, Stahl PD.** Extracellular vesicles: exosomes,
1797          microvesicles, and friends. *The Journal of Cell Biology* 2013;200(4):373–383.
1798          doi:10.1083/jcb.201211138.

1799    3.    **Hardie DG.** *Biochemical Messengers: Hormones, Neurotransmitters and Growth*
1800          *Factors - D.G. Hardie - Google Books*. 1991.

1801    4.    **Zaborowski MP, Balaj L, Breakefield XO, Lai CP.** Extracellular Vesicles:
1802          Composition, Biological Relevance, and Methods of Study. *Bioscience*
1803          2015;65(8):783–797. doi:10.1093/biosci/biv084.

1804    5.    **Colombo M, Raposo G, Théry C.** Biogenesis, Secretion, and Intercellular
1805          Interactions of Exosomes and Other Extracellular Vesicles. *Annu. Rev. Cell Dev.*
1806          *Biol.* 2014;30(1):255–289. doi:10.1146/annurev-cellbio-101512-122326.

1807    6.    **Andaloussi El S, Mäger I, Breakefield XO, Wood MJA.** Extracellular vesicles:
1808          biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*
1809          2013;12(5):347–357. doi:10.1038/nrd3978.

1810    7.    **Akers JC, Gonda D, Kim R, Carter BS, Chen CC.** Biogenesis of extracellular
1811          vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic
1812          bodies. *J. Neurooncol.* 2013;113(1):1–11. doi:10.1007/s11060-013-1084-8.

1813    8.    **György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B, László V, Pállinger**
1814          **É, Pap E, Kittel Á, Nagy G, Falus A, Buzás EI.** Membrane vesicles, current state-
1815          of-the-art: emerging role of extracellular vesicles. *Cell. Mol. Life Sci.*
1816          2011;68(16):2667–2688. doi:10.1007/s00018-011-0689-3.

1817    9.    **Ronquist G, Brody I.** The prostasome: its secretion and function in man. *Biochim.*
1818          *Biophys. Acta* 1985;822(2):203–218.

1819    10.   **Ogawa Y, Kanai-Azuma M, Akimoto Y, Kawakami H, Yanoshita R.** Exosome-
1820          like vesicles with dipeptidyl peptidase IV in human saliva. *Biol. Pharm. Bull.*
1821          2008;31(6):1059–1062.

1822    11.   **Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C.**
1823          Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.*

1824   2005;17(7):879–887. doi:10.1093/intimm/dxh267.

1825   12.   **Lässer C, Alikhani VS, Ekström K, Eldh M, Paredes PT, Bossios A, Sjöstrand**
1826        **M, Gabrielsson S, Lötvall J, Valadi H.** Human saliva, plasma and breast milk
1827        exosomes contain RNA: uptake by macrophages. *J Transl Med* 2011;9:9.
1828        doi:10.1186/1479-5876-9-9.

1829   13.   **Pisitkun T, Shen R-F, Knepper MA.** Identification and proteomic profiling of
1830        exosomes in human urine. *Proc. Natl. Acad. Sci. U.S.A.* 2004;101(36):13368–13373.
1831        doi:10.1073/pnas.0403453101.

1832   14.   **Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D, Witkin SS.**
1833        Heat shock protein-containing exosomes in mid-trimester amniotic fluids. *J. Reprod.*
1834        *Immunol.* 2008;79(1):12–17. doi:10.1016/j.jri.2008.06.001.

1835   15.   **Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K,**
1836        **Vandesompele J, Bracke M, De Wever O, Hendrix A.** The impact of disparate
1837        isolation methods for extracellular vesicles on downstream RNA profiling. *Journal*
1838        *of Extracellular Vesicles* 2014;3. doi:10.3402/jev.v3.24858.

1839   16.   **Szatanek R, Baran J, Siedlar M, Baj-Krzyworzeka M.** Isolation of extracellular
1840        vesicles: Determining the correct approach (Review). *Int J Mol Med* 2015;36(1):11–
1841        17. doi:10.3892/ijmm.2015.2194.

1842   17.   **Tkach M, Théry C.** Communication by Extracellular Vesicles: Where We Are and
1843        Where We Need to Go. *Cell* 2016;164(6):1226–1232.
1844        doi:10.1016/j.cell.2016.01.043.

1845   18.   **Simpson RJ, Kalra H, Mathivanan S.** ExoCarta as a resource for exosomal
1846        research. *Journal of Extracellular Vesicles* 2012;1. doi:10.3402/jev.v1i0.18374.

1847   19.   **Kim D-K, Kang B, Kim OY, Choi D-S, Lee J, Kim SR, Go G, Yoon YJ, Kim JH,**
1848        **Jang SC, Park K-S, Choi E-J, Kim KP, Desiderio DM, Kim Y-K, Lötvall J,**
1849        **Hwang D, Gho YS.** EVpedia: an integrated database of high-throughput data for
1850        systemic analyses of extracellular vesicles. *Journal of Extracellular Vesicles* 2013;2.
1851        doi:10.3402/jev.v2i0.20384.

1852 20. **Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC,**
1853 **Borràs FE, Breakefield X, Budnik V, Buzas E, Camussi G, Clayton A, Cocucci**
1854 **E, Falcon-Perez JM, Gabrielsson S, Gho YS, Gupta D, Harsha HC, Hendrix A,**
1855 **Hill AF, Inal JM, Jenster G, Krämer-Albers E-M, Lim SK, Llorente A, Lötvall**
1856 **J, Marcilla A, Mincheva-Nilsson L, Nazarenko I, Nieuwland R, Nolte-'t Hoen**
1857 **ENM, Pandey A, Patel T, Piper MG, Pluchino S, Prasad TSK, Rajendran L,**
1858 **Raposo G, Record M, Reid GE, Sánchez-Madrid F, Schiffelers RM, Siljander P,**
1859 **Stensballe A, Stoorvogel W, Taylor D, Théry C, Valadi H, van Balkom BWM,**
1860 **Vázquez J, Vidal M, Wauben MHM, Yáñez-Mó M, Zoeller M, Mathivanan S.**
1861 Vesiclepedia: a compendium for extracellular vesicles with continuous community
1862 annotation. *Plos Biol* 2012;10(12):e1001450. doi:10.1371/journal.pbio.1001450.

1863 21. **Budnik V, Ruiz-Cañada C, Wendler F.** Extracellular vesicles round off
1864 communication in the nervous system. *Nat. Rev. Neurosci.* 2016;17(3):160–172.
1865 doi:10.1038/nrn.2015.29.

1866 22. **Maas SLN, Breakefield XO, Weaver AM.** Extracellular Vesicles: Unique
1867 Intercellular Delivery Vehicles. *Trends Cell Biol* 2017;27(3):172–188.
1868 doi:10.1016/j.tcb.2016.11.003.

1869 23. **Tannetta D, Dragovic R, Alyahyaei Z, Southcombe J.** Extracellular vesicles and
1870 reproduction–promotion of successful pregnancy. *Cell Mol Immunol*
1871 2014;11(6):548–563. doi:10.1038/cmi.2014.42.

1872 24. **Kerr JF, Wyllie AH, Currie AR.** Apoptosis: a basic biological phenomenon with
1873 wide-ranging implications in tissue kinetics. *Br. J. Cancer* 1972;26(4):239–257.

1874 25. **Taylor RC, Cullen SP, Martin SJ.** Apoptosis: controlled demolition at the cellular
1875 level. *Nat Rev Mol Cell Biol* 2008;9(3):231–241. doi:10.1038/nrm2312.

1876 26. **Elmore S.** Apoptosis: a review of programmed cell death. *Toxicol Pathol*
1877 2007;35(4):495–516. doi:10.1080/01926230701320337.

1878 27. **Atkin-Smith GK, Tixeira R, Paone S, Mathivanan S, Collins C, Liem M,**
1879 **Goodall KJ, Ravichandran KS, Hulett MD, Poon IKH.** A novel mechanism of
1880 generating extracellular vesicles during apoptosis via a beads-on-a-string membrane

1881        structure. *Nature Communications* 2015;6:7439. doi:10.1038/ncomms8439.

1882  28.    **Hristov M, Erl W, Linder S, Weber PC.** Apoptotic bodies from endothelial cells
1883        enhance the number and initiate the differentiation of human endothelial progenitor
1884        cells in vitro. *Blood* 2004;104(9):2761–2766. doi:10.1182/blood-2003-10-3614.

1885  29.    **Willms E, Johansson HJ, Mäger I, Lee Y, Blomberg KEM, Sadik M, Alaarg A,**
1886        **Smith CIE, Lehtiö J, Andaloussi El S, Wood MJA, Vader P.** Cells release
1887        subpopulations of exosomes with distinct molecular and biological properties.
1888        *Nature Publishing Group* 2016;6:22519. doi:10.1038/srep22519.

1889  30.    **Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B,**
1890        **Dyrskjøt L, Orntoft TF, Howard KA, Ostenfeld MS.** Comparative analysis of
1891        discrete exosome fractions obtained by differential centrifugation. *Journal of*
1892        *Extracellular Vesicles* 2014;3:25011.

1893  31.    **Osteikoetxea X, Németh A, Sódar BW, Vukman KV, Buzás EI.** Extracellular
1894        vesicles in cardiovascular disease: are they Jedi or Sith? *J. Physiol. (Lond.)*
1895        2016;594(11):2881–2894. doi:10.1113/JP271336.

1896  32.    **van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R.** Classification,
1897        functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.*
1898        2012;64(3):676–705. doi:10.1124/pr.112.005983.

1899  33.    **van Engeland M, Kuijpers HJ, Ramaekers FC, Reutelingsperger CP, Schutte B.**
1900        Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp. Cell Res.*
1901        1997;235(2):421–430. doi:10.1006/excr.1997.3738.

1902  34.    **Hochreiter-Hufford A, Ravichandran KS.** Clearing the dead: apoptotic cell
1903        sensing, recognition, engulfment, and digestion. *Cold Spring Harb Perspect Biol*
1904        2013;5(1):a008748. doi:10.1101/cshperspect.a008748.

1905  35.    **Wu Y, Tibrewal N, Birge RB.** Phosphatidylserine recognition by phagocytes: a
1906        view to a kill. *Trends Cell Biol* 2006;16(4):189–197. doi:10.1016/j.tcb.2006.02.003.

1907  36.    **Fadok VA, Bratton DL, Henson PM.** Phagocyte receptors for apoptotic cells:
1908        recognition, uptake, and consequences. *J. Clin. Invest.* 2001;108(7):957–962.

1909        doi:10.1172/JCI14122.

1910   37.   **Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z.** Apoptosis and beyond:
1911        cytometry in studies of programmed cell death. *Methods Cell Biol.* 2011;103:55–98.
1912        doi:10.1016/B978-0-12-385493-3.00004-8.

1913   38.   **Bailey RW, Nguyen T, Robertson L, Gibbons E, Nelson J, Christensen RE, Bell**
1914        **JP, Judd AM, Bell JD.** Sequence of physical changes to the cell membrane during
1915        glucocorticoid-induced apoptosis in S49 lymphoma cells. *Biophys. J.*
1916        2009;96(7):2709–2718. doi:10.1016/j.bpj.2008.12.3925.

1917   39.   **Hugel B, Martínez MC, Kunzelmann C, Freyssinet J-M.** Membrane
1918        microparticles: two sides of the coin. *Physiology (Bethesda)* 2005;20:22–27.
1919        doi:10.1152/physiol.00029.2004.

1920   40.   **Friedl P, Vischer P, Freyberg MA.** The role of thrombospondin-1 in apoptosis.
1921        *Cell. Mol. Life Sci.* 2002;59(8):1347–1357.

1922   41.   **Takizawa F, Tsuji S, Nagasawa S.** Enhancement of macrophage phagocytosis upon
1923        iC3b deposition on apoptotic cells. *FEBS Lett.* 1996;397(2-3):269–272.

1924   42.   **Abas L, Luschnig C.** Maximum yields of microsomal-type membranes from small
1925        amounts of plant material without requiring ultracentrifugation. *Analytical*
1926        *Biochemistry* 2010;401(2):217–227. doi:10.1016/j.ab.2010.02.030.

1927   43.   **Lavoie C, Lanoix J, Kan FW, Paiement J.** Cell-free assembly of rough and smooth
1928        endoplasmic reticulum. *J. Cell. Sci.* 1996;109 ( Pt 6):1415–1425.

1929   44.   **Tong M, Kleffmann T, Pradhan S, Johansson CL, DeSousa J, Stone PR, James**
1930        **JL, Chen Q, Chamley LW.** Proteomic characterization of macro-, micro- and nano-
1931        extracellular vesicles derived from the same first trimester placenta: relevance for
1932        feto-maternal communication. *Human Reproduction* 2016;31(4):687–699.
1933        doi:10.1093/humrep/dew004.

1934   45.   **Pantham P, Viall CA, Chen Q, Kleffmann T, Print CG, Chamley LW.**
1935        Antiphospholipid antibodies bind syncytiotrophoblast mitochondria and alter the
1936        proteome of extruded syncytial nuclear aggregates. *Placenta* 2015;36(12):1463–

1937          1473. doi:10.1016/j.placenta.2015.10.006.

1938  46.  **Holmgren L, Szeles A, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, Falk KI.**
1939       Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood*
1940       1999;93(11):3956–3963.

1941  47.  **Ehnfors J, Kost-Alimova M, Persson NL, Bergsmedh A, Castro J, Levchenko-**
1942       **Tegnebratt T, Yang L, Panaretakis T, Holmgren L.** Horizontal transfer of tumor
1943       DNA to endothelial cellsin vivo. 2009;16(5):749–757. doi:10.1038/cdd.2009.7.

1944  48.  **Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL,**
1945       **Holmgren L.** Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc.*
1946       *Natl. Acad. Sci. U.S.A.* 2001;98(11):6407–6411. doi:10.1073/pnas.101129998.

1947  49.  **Savill J, Dransfield I, Gregory C, Haslett C.** A blast from the past: clearance of
1948       apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2002;2(12):965–
1949       975. doi:10.1038/nri957.

1950  50.  **Bellone M, Iezzi G, Rovere P, Galati G, Ronchetti A, Protti MP, Davoust J,**
1951       **Rugarli C, Manfredi AA.** Processing of engulfed apoptotic bodies yields T cell
1952       epitopes. *J. Immunol.* 1997;159(11):5391–5399.

1953  51.  **Cocca BA, Cline AM, Radic MZ.** Blebs and apoptotic bodies are B cell
1954       autoantigens. *J. Immunol.* 2002;169(1):159–166.

1955  52.  **Bellone M.** Apoptosis, cross-presentation, and the fate of the antigen specific
1956       immune response. *Apoptosis* 2000;5(4):307–314.

1957  53.  **CHARGAFF E, WEST R.** The biological significance of the thromboplastic protein
1958       of blood. *J. Biol. Chem.* 1946;166(1):189–197.

1959  54.  **Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C.**
1960       Microvesicles: mediators of extracellular communication during cancer progression.
1961       *J. Cell. Sci.* 2010;123(Pt 10):1603–1611. doi:10.1242/jcs.064386.

1962  55.  **Cocucci E, Racchetti G, Meldolesi J.** Shedding microvesicles: artefacts no more.
1963       *Trends Cell Biol* 2009;19(2):43–51. doi:10.1016/j.tcb.2008.11.003.

1964  56.  **Tricarico C, Clancy J, D'Souza-Schorey C.** Biology and biogenesis of shed
1965      microvesicles. *Small GTPases* 2016:1–13. doi:10.1080/21541248.2016.1215283.

1966  57.  **D'Souza-Schorey C, Clancy JW.** Tumor-derived microvesicles: shedding light on
1967      novel microenvironment modulators and prospective cancer biomarkers. *Genes &*
1968      *Development* 2012;26(12):1287–1299. doi:10.1101/gad.192351.112.

1969  58.  **Théry C, Ostrowski M, Segura E.** Membrane vesicles as conveyors of immune
1970      responses. *Nat. Rev. Immunol.* 2009;9(8):581–593. doi:10.1038/nri2567.

1971  59.  **Antonyak MA, Cerione RA.** Emerging picture of the distinct traits and functions of
1972      microvesicles and exosomes. *Proceedings of the National Academy of Sciences*
1973      2015;112(12):3589–3590. doi:10.1073/pnas.1502590112.

1974  60.  **Lawson C, Vicencio JM, Yellon DM, Davidson SM.** Microvesicles and exosomes:
1975      new players in metabolic and cardiovascular disease. *J. Endocrinol.*
1976      2016;228(2):R57–71. doi:10.1530/JOE-15-0201.

1977  61.  **Vader P, Breakefield XO, Wood MJA.** Extracellular vesicles: emerging targets for
1978      cancer therapy. *Trends Mol Med* 2014;20(7):385–393.
1979      doi:10.1016/j.molmed.2014.03.002.

1980  62.  **Kreimer S, Belov AM, Ghiran I, Murthy SK, Frank DA, Ivanov AR.** Mass-
1981      spectrometry-based molecular characterization of extracellular vesicles: lipidomics
1982      and proteomics. *J. Proteome Res.* 2015;14(6):2367–2384. doi:10.1021/pr501279t.

1983  63.  **Sluijter JPG, Verhage V, Deddens JC, van den Akker F, Doevendans PA.**
1984      Microvesicles and exosomes for intracardiac communication. *Cardiovasc. Res.*
1985      2014;102(2):302–311. doi:10.1093/cvr/cvu022.

1986  64.  **Xu R, Greening DW, Rai A, Ji H, Simpson RJ.** Highly-purified exosomes and
1987      shed microvesicles isolated from the human colon cancer cell line LIM1863 by
1988      sequential centrifugal ultrafiltration are biochemically and functionally distinct.
1989      *Methods* 2015;87:11–25. doi:10.1016/j.ymeth.2015.04.008.

1990  65.  **Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G,**
1991      **D'Souza-Schorey C.** ARF6-regulated shedding of tumor cell-derived plasma

1992    membrane microvesicles. *Curr. Biol.* 2009;19(22):1875–1885.

1993    doi:10.1016/j.cub.2009.09.059.

1994  66.  **Xu R, Greening DW, Zhu H-J, Takahashi N, Simpson RJ.** Extracellular vesicle

1995    isolation and characterization: toward clinical application. *Journal of Clinical*

1996    *Investigation* 2016;126(4):1152–1162. doi:10.1172/JCI81129.

1997  67.  **Clancy JW, Sedgwick A, Rosse C, Muralidharan-Chari V, Raposo G, Method**

1998    **M, Chavrier P, D'Souza-Schorey C.** Regulated delivery of molecular cargo to

1999    invasive tumour-derived microvesicles. *Nature Communications* 2015;6:6919.

2000    doi:10.1126/science.1102026.

2001  68.  **Menck K, Scharf C, Bleckmann A, Dyck L, Rost U, Wenzel D, Dhople VM,**

2002    **Siam L, Pukrop T, Binder C, Klemm F.** Tumor-derived microvesicles mediate

2003    human breast cancer invasion through differentially glycosylated EMMPRIN. *J Mol*

2004    *Cell Biol* 2015;7(2):143–153. doi:10.1093/jmcb/mju047.

2005  69.  **Antonyak MA, Li B, Boroughs LK, Johnson JL, Druso JE, Bryant KL,**

2006    **Holowka DA, Cerione RA.** Cancer cell-derived microvesicles induce

2007    transformation by transferring tissue transglutaminase and fibronectin to recipient

2008    cells. *Proceedings of the National Academy of Sciences* 2011;108(12):4852–4857.

2009    doi:10.1073/pnas.1017667108.

2010  70.  **Arshad Malik MF.** Influence of microvesicles in breast cancer metastasis and their

2011    therapeutic implications. *Arch Iran Med* 2015;18(3):189–192.

2012  71.  **McDaniel K, Correa R, Zhou T, Johnson C, Francis H, Glaser S, Venter J,**

2013    **Alpini G, Meng F.** Functional role of microvesicles in gastrointestinal malignancies.

2014    *Ann Transl Med* 2013;1(1):4. doi:10.3978/j.issn.2305-5839.2012.10.01.

2015  72.  **Jorfi S, Inal JM.** The role of microvesicles in cancer progression and drug

2016    resistance. *Biochem. Soc. Trans.* 2013;41(1):293–298. doi:10.1042/BST20120273.

2017  73.  **Dye JR, Ullal AJ, Pisetsky DS.** The role of microparticles in the pathogenesis of

2018    rheumatoid arthritis and systemic lupus erythematosus. *Scand. J. Immunol.*

2019    2013;78(2):140–148. doi:10.1111/sji.12068.

2020   74.    **Cicero Lo A, Majkowska I, Nagase H, Di Liegro I, Troeberg L.** Microvesicles
2021       shed by oligodendroglioma cells and rheumatoid synovial fibroblasts contain
2022       aggrecanase activity. *Matrix Biol.* 2012;31(4):229–233.
2023       doi:10.1016/j.matbio.2012.02.005.

2024   75.    **Sellam J, Proulle V, Jüngel A, Ittah M, Richard CM, Gottenberg J-E, Toti F,**
2025       **Benessiano J, Gay S, Freyssinet J-M, Mariette X.** Increased levels of circulating
2026       microparticles in primary Sjögren's syndrome, systemic lupus erythematosus and
2027       rheumatoid arthritis and relation with disease activity. *Arthritis Res. Ther.*
2028       2009;11(5):R156. doi:10.1186/ar2833.

2029   76.    **Nomura S, Shimizu M.** Clinical significance of procoagulant microparticles. *J*
2030       *Intensive Care* 2015;3(1):2. doi:10.1186/s40560-014-0066-z.

2031   77.    **Xiong J, Miller VM, Li Y, Jayachandran M.** Microvesicles at the crossroads
2032       between infection and cardiovascular diseases. *J. Cardiovasc. Pharmacol.*
2033       2012;59(2):124–132. doi:10.1097/FJC.0b013e31820c6254.

2034   78.    **Tong M, Chamley LW.** Placental extracellular vesicles and feto-maternal
2035       communication. *Cold Spring Harb Perspect Med* 2015;5(3):a023028.
2036       doi:10.1101/cshperspect.a023028.

2037   79.    **Desrochers LM, Bordeleau FCO, Reinhart-King CA, Antonyak MA, Cerione**
2038       **RA.** Microvesicles provide a mechanism for intercellular communication by
2039       embryonic stem cells during embryo implantation. *Nature Communications*
2040       2016;7:1–11. doi:10.1038/ncomms11958.

2041   80.    **Trams EG, Lauter CJ, Salem N, Heine U.** Exfoliation of membrane ecto-enzymes
2042       in the form of micro-vesicles. *Biochim. Biophys. Acta* 1981;645(1):63–70.

2043   81.    **Harding C, Heuser J, Stahl P.** Receptor-mediated endocytosis of transferrin and
2044       recycling of the transferrin receptor in rat reticulocytes. *The Journal of Cell Biology*
2045       1983;97(2):329–339.

2046   82.    **Greening DW, Nguyen HPT, Elgass K, Simpson RJ, Salamonsen LA.** Human
2047       Endometrial Exosomes Contain Hormone-Specific Cargo Modulating Trophoblast
2048       Adhesive Capacity: Insights into Endometrial-Embryo Interactions. *Biol. Reprod.*

2049        2016;94(2):38. doi:10.1095/biolreprod.115.134890.

2050   83.   **Théry C, Zitvogel L, Amigorena S.** Exosomes: composition, biogenesis and
2051        function. *Nat. Rev. Immunol.* 2002;2(8):569–579. doi:10.1038/nri855.

2052   84.   **Lane RE, Korbie D, Anderson W, Vaidyanathan R, Trau M.** Analysis of
2053        exosome purification methods using a model liposome system and tunable-resistive
2054        pulse sensing. *Nature Publishing Group* 2015;5:7639. doi:10.1038/srep07639.

2055   85.   **Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, Manel N,**
2056        **Moita LF, Théry C, Raposo G.** Analysis of ESCRT functions in exosome
2057        biogenesis, composition and secretion highlights the heterogeneity of extracellular
2058        vesicles. *J. Cell. Sci.* 2013;126(Pt 24):5553–5565. doi:10.1242/jcs.128868.

2059   86.   **Mathivanan S, Ji H, Simpson RJ.** Exosomes: extracellular organelles important in
2060        intercellular communication. *J Proteomics* 2010;73(10):1907–1920.
2061        doi:10.1016/j.jprot.2010.06.006.

2062   87.   **Mathivanan S, Lim JWE, Tauro BJ, Ji H, Moritz RL, Simpson RJ.** Proteomics
2063        analysis of A33 immunoaffinity-purified exosomes released from the human colon
2064        tumor cell line LIM1215 reveals a tissue-specific protein signature. *Mol. Cell*
2065        *Proteomics* 2010;9(2):197–208. doi:10.1074/mcp.M900152-MCP200.

2066   88.   **Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S.**
2067        Identification of Tim4 as a phosphatidylserine receptor. *Nature* 2007;450(7168):435–
2068        439. doi:10.1038/nature06307.

2069   89.   **Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ.** Two
2070        Distinct Populations of Exosomes Are Released from LIM1863 Colon Carcinoma
2071        Cell-derived Organoids. *Mol. Cell Proteomics* 2013;12(3):587–598.
2072        doi:10.1074/mcp.M112.021303.

2073   90.   **De Toro J, Herschlik L, Waldner C, Mongini C.** Emerging roles of exosomes in
2074        normal and pathological conditions: new insights for diagnosis and therapeutic
2075        applications. *Front Immunol* 2015;6:203. doi:10.3389/fimmu.2015.00203.

2076   91.   **Suchorska WM, Lach MS.** The role of exosomes in tumor progression and

2077    metastasis (Review). *Oncol. Rep.* 2016;35(3):1237–1244. doi:10.3892/or.2015.4507.

2078    92.    **Greening DW, Gopal SK, Xu R, Simpson RJ, Chen W.** Exosomes and their roles
2079          in immune regulation and cancer. *Semin. Cell Dev. Biol.* 2015;40:72–81.
2080          doi:10.1016/j.semcdb.2015.02.009.

2081    93.    **Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL.** Tumor-derived
2082          exosomes regulate expression of immune function-related genes in human T cell
2083          subsets. *Nature Publishing Group* 2016;6:20254. doi:10.1038/srep20254.

2084    94.    **Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-'t Hoen
2085          EN, Piper MG, Sivaraman S, Skog J, Théry C, Wauben MH, Hochberg F.**
2086          Standardization of sample collection, isolation and analysis methods in extracellular
2087          vesicle research. *Journal of Extracellular Vesicles* 2013;2.
2088          doi:10.3402/jev.v2i0.20360.

2089    95.    **Théry C, Amigorena S, Raposo G, Clayton A.** Isolation and characterization of
2090          exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*
2091          2006;Chapter 3:Unit 3.22. doi:10.1002/0471143030.cb0322s30.

2092    96.    **Lamparski HG, Metha-Damani A, Yao J-Y, Patel S, Hsu D-H, Ruegg C, Le
2093          Pecq J-B.** Production and characterization of clinical grade exosomes derived from
2094          dendritic cells. *J. Immunol. Methods* 2002;270(2):211–226.

2095    97.    **Chiou N-T, Ansel KM.** Improved exosome isolation by sucrose gradient
2096          fractionation of ultracentrifuged crude exosome pellets. 2016.
2097          doi:10.1038/protex.2016.057.

2098    98.    **Keller S, Ridinger J, Rupp A-K, Janssen JWG, Altevogt P.** Body fluid derived
2099          exosomes as a novel template for clinical diagnostics. *J Transl Med* 2011;9:86.
2100          doi:10.1186/1479-5876-9-86.

2101    99.    **Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ.** A protocol for exosome
2102          isolation and characterization: evaluation of ultracentrifugation, density-gradient
2103          separation, and immunoaffinity capture methods. *Methods Mol. Biol.*
2104          2015;1295:179–209. doi:10.1007/978-1-4939-2550-6_15.

2105 100. **Cantin R, Diou J, Bélanger D, Tremblay AM, Gilbert C.** Discrimination between
2106 exosomes and HIV-1: purification of both vesicles from cell-free supernatants. *J.*
2107 *Immunol. Methods* 2008;338(1-2):21–30. doi:10.1016/j.jim.2008.07.007.

2108 101. **Klimentová J, Stulík J.** Methods of isolation and purification of outer membrane
2109 vesicles from gram-negative bacteria. *Microbiol. Res.* 2015;170:1–9.
2110 doi:10.1016/j.micres.2014.09.006.

2111 102. **Ford T, Graham J, Rickwood D.** Iodixanol: a nonionic iso-osmotic centrifugation
2112 medium for the formation of self-generated gradients. *Analytical Biochemistry*
2113 1994;220(2):360–366. doi:10.1006/abio.1994.1350.

2114 103. **György B, Módos K, Pállinger É, Pálóczi K, Pásztói M, Misják P, Deli MA,**
2115 **Sipos A, Szalai A, Voszka I, Polgár A, Tóth K, Csete M, Nagy G, Gay S, Falus**
2116 **A, Kittel Á, Buzás EI.** Detection and isolation of cell-derived microparticles are
2117 compromised by protein complexes resulting from shared biophysical parameters.
2118 *Blood* 2011;117(4):e39–48. doi:10.1182/blood-2010-09-307595.

2119 104. **Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigor'eva AE,**
2120 **Zaporozhchenko IA, Morozkin ES, Ryabchikova EI, Yurchenko YB, Voitsitskiy**
2121 **VE, Laktionov PP.** Comparative Study of Extracellular Vesicles from the Urine of
2122 Healthy Individuals and Prostate Cancer Patients. *PLoS ONE* 2016;11(6):e0157566.
2123 doi:10.1371/journal.pone.0157566.

2124 105. **Lobb RJ, Becker M, Wen SW, Wong CSF, Wiegmans AP, Leimgruber A,**
2125 **Möller A.** Optimized exosome isolation protocol for cell culture supernatant and
2126 human plasma. *Journal of Extracellular Vesicles* 2015;4:27031.

2127 106. **Klein-Scory S, Tehrani MM, Eilert-Micus C, Adamczyk KA, Wojtalewicz N,**
2128 **Schnölzer M, Hahn SA, Schmiegel W, Schwarte-Waldhoff I.** New insights in the
2129 composition of extracellular vesicles from pancreatic cancer cells: implications for
2130 biomarkers and functions. *Proteome Sci* 2014;12(1):50. doi:10.1186/s12953-014-
2131 0050-5.

2132 107. **Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PST, Star**
2133 **RA.** Rapid isolation of urinary exosomal biomarkers using a nanomembrane

2134   ultrafiltration concentrator. *Am. J. Physiol. Renal Physiol.* 2007;292(5):F1657–61.
2135   doi:10.1152/ajprenal.00434.2006.

2136 108. **Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM,**
2137   **McAfee KJ, Fleischer C, Klein E, Klein JB.** Microfiltration isolation of human
2138   urinary exosomes for characterization by MS. *Proteomics Clin Appl* 2010;4(1):84–
2139   96. doi:10.1002/prca.200800093.

2140 109. **Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland**
2141   **R.** Single-step isolation of extracellular vesicles by size-exclusion chromatography.
2142   *Journal of Extracellular Vesicles* 2014;3. doi:10.3402/jev.v3.23430.

2143 110. **de Menezes-Neto A, Sáez MJF, Lozano-Ramos I, Segui-Barber J, Martin-Jaular**
2144   **L, Ullate JME, Fernandez-Becerra C, Borràs FE, del Portillo HA.** Size-exclusion
2145   chromatography as a stand-alone methodology identifies novel markers in mass
2146   spectrometry analyses of plasma-derived vesicles from healthy individuals. *Journal*
2147   *of Extracellular Vesicles* 2015;4:27378.

2148 111. **Lozano-Ramos I, Bancu I, Oliveira-Tercero A, Armengol MP, Menezes-Neto A,**
2149   **del Portillo HA, Lauzurica-Valdemoros R, Borràs FE.** Size-exclusion
2150   chromatography-based enrichment of extracellular vesicles from urine samples.
2151   *Journal of Extracellular Vesicles* 2015;4:27369.

2152 112. **Muller G.** Novel Tools for the Study of Cell Type-Specific Exosomes and
2153   Microvesicles. *J Bioanal Biomed* 2015;04(04). doi:10.4172/1948-593X.1000063.

2154 113. **Taylor DD, Lyons KS, Gerçel-Taylor Ç.** Shed Membrane Fragment-Associated
2155   Markers for Endometrial and Ovarian Cancers - ScienceDirect. *Gynecol. Oncol.*
2156   2002.

2157 114. **Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa ML,**
2158   **Beyer K, Borràs FE.** Size-Exclusion Chromatography-based isolation minimally
2159   alters Extracellular Vesicles' characteristics compared to precipitating agents. *Nature*
2160   *Publishing Group* 2016;6:33641. doi:10.1038/srep33641.

2161 115. **Corso G, Mäger I, Lee Y, Görgens A, Bultema J, Giebel B, Wood MJA, Nordin**
2162   **JZ, Andaloussi SE.** Reproducible and scalable purification of extracellular vesicles

2163     using combined bind-elute and size exclusion chromatography. *Nature Publishing*
2164     *Group* 2017;7(1):11561. doi:10.1038/s41598-017-10646-x.

2165 116. **Benedikter BJ, Bouwman FG, Vajen T, Heinzmann ACA, Grauls G, Mariman**
2166     **EC, Wouters EFM, Savelkoul PH, Lopez-Iglesias C, Koenen RR, Rohde GGU,**
2167     **Stassen FRM.** Ultrafiltration combined with size exclusion chromatography
2168     efficiently isolates extracellular vesicles from cell culture media for compositional
2169     and functional studies. *Nature Publishing Group* 2017;7(1):15297.
2170     doi:10.1038/s41598-017-15717-7.

2171 117. **Nordin JZ, Lee Y, Vader P, Mäger I, Johansson HJ, Heusermann W,**
2172     **Wiklander OPB, Hällbrink M, Seow Y, Bultema JJ, Gilthorpe J, Davies T,**
2173     **Fairchild PJ, Gabrielsson S, Meisner-Kober NC, Lehtiö J, Smith CIE, Wood**
2174     **MJA, Andaloussi SE.** Ultrafiltration with size-exclusion liquid chromatography for
2175     high yield isolation of extracellular vesicles preserving intact biophysical and
2176     functional properties. *Nanomedicine* 2015;11(4):879–883.
2177     doi:10.1016/j.nano.2015.01.003.

2178 118. **Hong CS, Muller L, Boyiadzis M, Whiteside TL.** Isolation and characterization of
2179     CD34+ blast-derived exosomes in acute myeloid leukemia. *PLoS ONE*
2180     2014;9(8):e103310. doi:10.1371/journal.pone.0103310.

2181 119. **Yoo CE, Kim G, Kim M, Park D, Kang HJ, Lee M, Huh N.** A direct extraction
2182     method for microRNAs from exosomes captured by immunoaffinity beads.
2183     *Analytical Biochemistry* 2012;431(2):96–98. doi:10.1016/j.ab.2012.09.008.

2184 120. **Ji H, Chen M, Greening DW, He W, Rai A, Zhang W, Simpson RJ.** Deep
2185     sequencing of RNA from three different extracellular vesicle (EV) subtypes released
2186     from the human LIM1863 colon cancer cell line uncovers distinct miRNA-
2187     enrichment signatures. *PLoS ONE* 2014;9(10):e110314.
2188     doi:10.1371/journal.pone.0110314.

2189 121. **Clayton A, Court J, Navabi H, Adams M.** Analysis of antigen presenting cell
2190     derived exosomes, based on immuno-magnetic isolation and flow cytometry -
2191     ScienceDirect. *Journal of ...* 2001.

2192    122.    **Taylor DD, Zacharias W, Gercel-Taylor C.** Exosome isolation for proteomic
2193            analyses and RNA profiling. *Methods Mol. Biol.* 2011;728:235–246.
2194            doi:10.1007/978-1-61779-068-3_15.

2195    123.    **Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK.** Comparison of
2196            protein, microRNA, and mRNA yields using different methods of urinary exosome
2197            isolation for the discovery of kidney disease biomarkers. *Kidney International*
2198            2012;82(9):1024–1032. doi:10.1038/ki.2012.256.

2199    124.    **Chen C, Skog J, Hsu C-H, Lessard RT, Balaj L, Wurdinger T, Carter BS,**
2200            **Breakefield XO, Toner M, Irimia D.** Microfluidic isolation and transcriptome
2201            analysis of serum microvesicles. *Lab Chip* 2010;10(4):505. doi:10.1039/b916199f.

2202    125.    **Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S.** Microfluidic device (ExoChip)
2203            for on-chip isolation, quantification and characterization of circulating exosomes.
2204            *Lab Chip* 2014;14(11):1891–1900. doi:10.1039/c4lc00136b.

2205    126.    **Bhagat AAS, Kuntaegowdanahalli SS, Papautsky I.** Continuous particle
2206            separation in spiral microchannels using Dean flows and differential migration. *Lab*
2207            *Chip* 2008;8(11):1906–1914. doi:10.1039/b807107a.

2208    127.    **Davies RT, Kim J, Jang SC, Choi E-J, Gho YS, Park J.** Microfluidic filtration
2209            system to isolate extracellular vesicles from blood. *Lab Chip* 2012;12(24):5202–
2210            5210. doi:10.1039/c2lc41006k.

2211    128.    **Wu M, Ouyang Y, Wang Z, Zhang R, Huang P-H, Chen C, Li H, Li P, Quinn D,**
2212            **Dao M, Suresh S, Sadovsky Y, Huang TJ.** Isolation of exosomes from whole blood
2213            by integrating acoustics and microfluidics. *Proc. Natl. Acad. Sci. U.S.A.*
2214            2017;114(40):10584–10589. doi:10.1073/pnas.1709210114.

2215    129.    **Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ,**
2216            **Geuze HJ.** B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.*
2217            1996;183(3):1161–1172.

2218    130.    **Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, LeBleu**
2219            **VS, Mittendorf EA, Weitz J, Rahbari N, Reissfelder C, Pilarsky C, Fraga MF,**
2220            **Piwnica-Worms D, Kalluri R.** Glypican-1 identifies cancer exosomes and detects

2221    early pancreatic cancer. *Nature* 2015;523(7559):177–182. doi:10.1038/nature14581.

2222    131.    **Roccaro AM, Sacco A, Maiso P, Azab AK, Tai Y-T, Reagan M, Azab F, Flores**
2223            **LM, Campigotto F, Weller E, Anderson KC, Scadden DT, Ghobrial IM.** BM
2224            mesenchymal stromal cell-derived exosomes facilitate multiple myeloma
2225            progression. *Journal of Clinical Investigation* 2013;123(4):1542–1555.
2226            doi:10.1172/JCI66517.

2227    132.    **Höög JL, Lötvall J.** Diversity of extracellular vesicles in human ejaculates revealed
2228            by cryo-electron microscopy. *Journal of Extracellular Vesicles* 2015;4:28680.

2229    133.    **Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martinez S,**
2230            **Marcilla A, Simón C.** Hsa-miR-30d, secreted by the human endometrium, is taken
2231            up by the pre-implantation embryo and might modify its transcriptome. *Development*
2232            2015;142(18):3210–3221. doi:10.1242/dev.124289.

2233    134.    **Wu Y, Deng W, Klinke DJ.** Exosomes: improved methods to characterize their
2234            morphology, RNA content, and surface protein biomarkers. *Analyst*
2235            2015;140(19):6631–6642. doi:10.1039/c5an00688k.

2236    135.    **Iwai K, Minamisawa T, Suga K, Yajima Y, Shiba K.** Isolation of human salivary
2237            extracellular vesicles by iodixanol density gradient ultracentrifugation and their
2238            characterizations. *Journal of Extracellular Vesicles* 2016;5:30829.

2239    136.    **Hardij J, Cecchet F, Berquand A, Gheldof D, Chatelain C, Mullier F, Chatelain**
2240            **B, Dogné J-M.** Characterisation of tissue factor-bearing extracellular vesicles with
2241            AFM: comparison of air-tapping-mode AFM and liquid Peak Force AFM. *Journal of*
2242            *Extracellular Vesicles* 2013;2. doi:10.3402/jev.v2i0.21045.

2243    137.    **Yuana Y, Oosterkamp TH, Bahatyrova S, Ashcroft B, Garcia Rodriguez P,**
2244            **Bertina RM, Osanto S.** Atomic force microscopy: a novel approach to the detection
2245            of nanosized blood microparticles. *J. Thromb. Haemost.* 2010;8(2):315–323.
2246            doi:10.1111/j.1538-7836.2009.03654.x.

2247    138.    **Ashcroft BA, de Sonneville J, Yuana Y, Osanto S, Bertina R, Kuil ME,**
2248            **Oosterkamp TH.** Determination of the size distribution of blood microparticles
2249            directly in plasma using atomic force microscopy and microfluidics. *Biomed*

2250       *Microdevices* 2012;14(4):641–649. doi:10.1007/s10544-012-9642-y.

2251 139. **Gardiner C, Ferreira YJ, Dragovic RA, Redman CWG, Sargent IL.**
2252       Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis.
2253       *Journal of Extracellular Vesicles* 2013;2. doi:10.3402/jev.v2i0.19671.

2254 140. **Filipe V, Hawe A, Jiskoot W.** Critical evaluation of Nanoparticle Tracking Analysis
2255       (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates.
2256       *Pharm. Res.* 2010;27(5):796–810. doi:10.1007/s11095-010-0073-2.

2257 141. **van der Pol E, Coumans FAW, Grootemaat AE, Gardiner C, Sargent IL,**
2258       **Harrison P, Sturk A, van Leeuwen TG, Nieuwland R.** Particle size distribution of
2259       exosomes and microvesicles determined by transmission electron microscopy, flow
2260       cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J. Thromb.*
2261       *Haemost.* 2014;12(7):1182–1192. doi:10.1111/jth.12602.

2262 142. **Momen-Heravi F, Balaj L, Alian S, Tigges J, Toxavidis V, Ericsson M, Distel**
2263       **RJ, Ivanov AR, Skog J, Kuo WP.** Alternative methods for characterization of
2264       extracellular vesicles. *Front Physiol* 2012;3:354. doi:10.3389/fphys.2012.00354.

2265 143. **Garza-Licudine E, Deo D, Yu S, Uz-Zaman A, Dunbar WB.** Portable nanoparticle
2266       quantization using a resizable nanopore instrument - the IZON qNano™. *Conf Proc*
2267       *IEEE Eng Med Biol Soc* 2010;2010:5736–5739. doi:10.1109/IEMBS.2010.5627861.

2268 144. **van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R.**
2269       Optical and non-optical methods for detection and characterization of microparticles
2270       and exosomes. *J. Thromb. Haemost.* 2010;8(12):2596–2607. doi:10.1111/j.1538-
2271       7836.2010.04074.x.

2272 145. **Lacroix R, Robert S, Poncelet P, Dignat-George F.** Overcoming limitations of
2273       microparticle measurement by flow cytometry. *Semin. Thromb. Hemost.*
2274       2010;36(8):807–818. doi:10.1055/s-0030-1267034.

2275 146. **van der Vlist EJ, Nolte-'t Hoen ENM, Stoorvogel W, Arkesteijn GJA, Wauben**
2276       **MHM.** Fluorescent labeling of nano-sized vesicles released by cells and subsequent
2277       quantitative and qualitative analysis by high-resolution flow cytometry. *Nat Protoc*
2278       2012;7(7):1311–1326. doi:10.1038/nprot.2012.065.

2279 147. **Nolte-'t Hoen ENM, van der Vlist EJ, Aalberts M, Mertens HCH, Bosch BJ,**
2280 **Bartelink W, Mastrobattista E, van Gaal EVB, Stoorvogel W, Arkesteijn GJA,**
2281 **Wauben MHM.** Quantitative and qualitative flow cytometric analysis of nanosized
2282 cell-derived membrane vesicles. *Nanomedicine* 2012;8(5):712–720.
2283 doi:10.1016/j.nano.2011.09.006.

2284 148. **Osteikoetxea X, Balogh A, Szabó-Taylor K, Németh A, Szabó TG, Pálóczi K,**
2285 **Sódar B, Kittel Á, György B, Pállinger É, Matkó J, Buzás EI.** Improved
2286 characterization of EV preparations based on protein to lipid ratio and lipid
2287 properties. *PLoS ONE* 2015;10(3):e0121184. doi:10.1371/journal.pone.0121184.

2288 149. **Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, Salamonsen LA.**
2289 Endometrial exosomes/microvesicles in the uterine microenvironment: a new
2290 paradigm for embryo-endometrial cross talk at implantation. *PLoS ONE*
2291 2013;8(3):e58502. doi:10.1371/journal.pone.0058502.

2292 150. **Nakai W, Yoshida T, Diez D, Miyatake Y, Nishibu T, Imawaka N, Naruse K,**
2293 **Sadamura Y, Hanayama R.** A novel affinity-based method for the isolation of
2294 highly purified extracellular vesicles. *Nature Publishing Group* 2016;6:33935.
2295 doi:10.1038/srep33935.

2296 151. **Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, Perdicchio**
2297 **M, Marino ML, Federici C, Iessi E, Brambilla D, Venturi G, Lozupone F,**
2298 **Santinami M, Huber V, Maio M, Rivoltini L, Fais S.** High levels of exosomes
2299 expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS ONE*
2300 2009;4(4):e5219. doi:10.1371/journal.pone.0005219.

2301 152. **Yoshioka Y, Kosaka N, Konishi Y, Ohta H, Okamoto H, Sonoda H, Nonaka R,**
2302 **Yamamoto H, Ishii H, Mori M, Furuta K, Nakajima T, Hayashi H, Sugisaki H,**
2303 **Higashimoto H, Kato T, Takeshita F, Ochiya T.** Ultra-sensitive liquid biopsy of
2304 circulating extracellular vesicles using ExoScreen. *Nature Communications*
2305 2014;5:3591. doi:10.1038/ncomms4591.

2306 153. **Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, Hochberg FH,**
2307 **Breakefield XO, Weissleder R, Lee H.** Protein typing of circulating microvesicles
2308 allows real-time monitoring of glioblastoma therapy. *Nat. Med.* 2012;18(12):1835–

2309       1840. doi:10.1038/nm.2994.

2310   154.   **Im H, Shao H, Weissleder R, Castro CM, Lee H.** Nano-plasmonic exosome
2311       diagnostics. *Expert Review of Molecular Diagnostics* 2015.

2312   155.   **Im H, Shao H, Park YI, Peterson VM, Castro CM, Weissleder R, Lee H.** Label-
2313       free detection and molecular profiling of exosomes with a nano-plasmonic sensor.
2314       *Nat. Biotechnol.* 2014;32(5):490–495. doi:10.1038/nbt.2886.

2315   156.   **Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B,**
2316       **Dingli F, Loew D, Tkach M, Théry C.** Proteomic comparison defines novel
2317       markers to characterize heterogeneous populations of extracellular vesicle subtypes.
2318       *Proceedings of the National Academy of Sciences* 2016;113(8):E968–77.
2319       doi:10.1073/pnas.1521230113.

2320   157.   **Greening DW, Xu R, Gopal SK, Rai A, Simpson RJ.** Proteomic insights into
2321       extracellular vesicle biology - defining exosomes and shed microvesicles. *Expert Rev*
2322       *Proteomics* 2017;14(1):69–95. doi:10.1080/14789450.2017.1260450.

2323   158.   **Théry C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P,**
2324       **Raposo G, Amigorena S.** Molecular characterization of dendritic cell-derived
2325       exosomes. Selective accumulation of the heat shock protein hsc73. *The Journal of*
2326       *Cell Biology* 1999;147(3):599–610.

2327   159.   **Aatonen MT, Ohman T, Nyman TA, Laitinen S, Grönholm M, Siljander PRM.**
2328       Isolation and characterization of platelet-derived extracellular vesicles. *Journal of*
2329       *Extracellular Vesicles* 2014;3. doi:10.3402/jev.v3.24692.

2330   160.   **MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, Surprenant A.**
2331       Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity*
2332       2001;15(5):825–835.

2333   161.   **Berda-Haddad Y, Robert S, Salers P, Zekraoui L, Farnarier C, Dinarello CA,**
2334       **Dignat-George F, Kaplanski G.** Sterile inflammation of endothelial cell-derived
2335       apoptotic bodies is mediated by interleukin-1α. *Proceedings of the National*
2336       *Academy of Sciences* 2011;108(51):20684–20689. doi:10.1073/pnas.1116848108.

2337   162.   **Gulinelli S, Salaro E, Vuerich M, Bozzato D, Pizzirani C, Bolognesi G, Idzko M,**
2338         **Virgilio FD, Ferrari D.** IL-18 associates to microvesicles shed from human
2339         macrophages by a LPS/TLR-4 independent mechanism in response to P2X receptor
2340         stimulation. *Eur J Immunol* 2012;42(12):3334–3345. doi:10.1016/S1074-
2341         7613(00)80111-9.

2342   163.   **Hasegawa H, Thomas HJ, Schooley K, Born TL.** Native IL-32 is released from
2343         intestinal epithelial cells via a non-classical secretory pathway as a membrane-
2344         associated protein. *Cytokine* 2011;53(1):74–83. doi:10.1016/j.cyto.2010.09.002.

2345   164.   **Zhang H-G, Liu C, Su K, Su K, Yu S, Zhang L, Zhang S, Wang J, Cao X,**
2346         **Grizzle W, Kimberly RP.** A membrane form of TNF-alpha presented by exosomes
2347         delays T cell activation-induced cell death. *J. Immunol.* 2006;176(12):7385–7393.

2348   165.   **Kandere-Grzybowska K, Letourneau R, Kempuraj D, Donelan J, Poplawski S,**
2349         **Boucher W, Athanassiou A, Theoharides TC.** IL-1 induces vesicular secretion of
2350         IL-6 without degranulation from human mast cells. *J. Immunol.* 2003;171(9):4830–
2351         4836.

2352   166.   **Nardi FDS, Michelon TF, Neumann J, Manvailer LFS, Wagner B, Horn PA,**
2353         **Bicalho MDG, Rebmann V.** High levels of circulating extracellular vesicles with
2354         altered expression and function during pregnancy. *Immunobiology* 2016;221(7):753–
2355         760. doi:10.1016/j.imbio.2016.03.001.

2356   167.   **Llorente A, Skotland T, Sylvänne T, Kauhanen D, Róg T, Orłowski A,**
2357         **Vattulainen I, Ekroos K, Sandvig K.** Molecular lipidomics of exosomes released
2358         by PC-3 prostate cancer cells. *Biochim. Biophys. Acta* 2013;1831(7):1302–1309.

2359   168.   **Brouwers JF, Aalberts M, Jansen JWA, van Niel G, Wauben MH, Stout TAE,**
2360         **Helms JB, Stoorvogel W.** Distinct lipid compositions of two types of human
2361         prostasomes. *Proteomics* 2013;13(10-11):1660–1666. doi:10.1002/pmic.201200348.

2362   169.   **Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille**
2363         **P, Brügger B, Simons M.** Ceramide triggers budding of exosome vesicles into
2364         multivesicular endosomes. *Science* 2008;319(5867):1244–1247.
2365         doi:10.1126/science.1153124.

2366  170.  **Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO.** Exosome-
2367        mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic
2368        exchange between cells. *Nat. Cell Biol.* 2007;9(6):654–659. doi:10.1038/ncb1596.

2369  171.  **Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL,**
2370        **Liu Y, Liang M, Kohli M, Thibodeau SN, Boardman L, Wang L.**
2371        Characterization of human plasma-derived exosomal RNAs by deep sequencing.
2372        *BMC Genomics* 2013;14:319. doi:10.1186/1471-2164-14-319.

2373  172.  **Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, Strobl J,**
2374        **Westerberg K, Gottardo R, Tewari M, Hladik F.** Exosomes in human semen carry
2375        a distinctive repertoire of small non-coding RNAs with potential regulatory
2376        functions. *Nucleic Acids Research* 2014;42(11):7290–7304. doi:10.1093/nar/gku347.

2377  173.  **van Balkom BWM, Eisele AS, Pegtel DM, Bervoets S, Verhaar MC.** Quantitative
2378        and qualitative analysis of small RNAs in human endothelial cells and exosomes
2379        provides insights into localized RNA processing, degradation and sorting. *Journal of*
2380        *Extracellular Vesicles* 2015;4:26760.

2381  174.  **Cheng L, Sharples RA, Scicluna BJ, Hill AF.** Exosomes provide a protective and
2382        enriched source of miRNA for biomarker profiling compared to intracellular and
2383        cell-free blood. *Journal of Extracellular Vesicles* 2014;3. doi:10.3402/jev.v3.23743.

2384  175.  **Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell**
2385        **PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M.**
2386        Argonaute2 complexes carry a population of circulating microRNAs independent of
2387        vesicles in human plasma. *Proc. Natl. Acad. Sci. U.S.A.* 2011;108(12):5003–5008.
2388        doi:10.1073/pnas.1019055108.

2389  176.  **Turchinovich A, Weiz L, Langheinz A, Burwinkel B.** Characterization of
2390        extracellular circulating microRNA. *Nucleic Acids Research* 2011;39(16):7223–
2391        7233. doi:10.1093/nar/gkr254.

2392  177.  **Li H, Huang S, Guo C, Guan H, Xiong C.** Cell-free seminal mRNA and
2393        microRNA exist in different forms. *PLoS ONE* 2012;7(4):e34566.
2394        doi:10.1371/journal.pone.0034566.

2395 178. **Shelke GV, Lässer C, Gho YS, Lötvall J.** Importance of exosome depletion
2396 protocols to eliminate functional and RNA-containing extracellular vesicles from
2397 fetal bovine serum. *Journal of Extracellular Vesicles* 2014;3.
2398 doi:10.3402/jev.v3.24783.

2399 179. **Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, Zheng Y,**
2400 **Hoshino A, Brazier H, Xiang J, Williams C, Rodriguez-Barrueco R, Silva JM,**
2401 **Zhang W, Hearn S, Elemento O, Paknejad N, Manova-Todorova K, Welte K,**
2402 **Bromberg J, Peinado H, Lyden D.** Double-stranded DNA in exosomes: a novel
2403 biomarker in cancer detection. *Cell Res.* 2014;24(6):766–769.
2404 doi:10.1038/cr.2014.44.

2405 180. **Waldenström A, Gennebäck N, Hellman U, Ronquist G.** Cardiomyocyte
2406 microvesicles contain DNA/RNA and convey biological messages to target cells.
2407 *PLoS ONE* 2012;7(4):e34653. doi:10.1371/journal.pone.0034653.

2408 181. **Lázaro-Ibáñez E, Sanz-Garcia A, Visakorpi T, Escobedo-Lucea C, Siljander P,**
2409 **Ayuso-Sacido Á, Yliperttula M.** Different gDNA content in the subpopulations of
2410 prostate cancer extracellular vesicles: Apoptotic bodies, microvesicles, and
2411 exosomes. *Prostate* 2014;74(14):1379–1390. doi:10.1002/pros.22853.

2412 182. **Balaj L, Lessard R, Dai L, Cho Y-J, Pomeroy SL, Breakefield XO, Skog J.**
2413 Tumour microvesicles contain retrotransposon elements and amplified oncogene
2414 sequences. *Nature Communications* 2011;2:180. doi:10.1038/ncomms1180.

2415 183. **Guescini M, Genedani S, Stocchi V, Agnati LF.** Astrocytes and Glioblastoma cells
2416 release exosomes carrying mtDNA. *J Neural Transm (Vienna)* 2010;117(1):1–4.
2417 doi:10.1007/s00702-009-0288-8.

2418 184. **Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson**
2419 **JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito**
2420 **SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE.** Mechanism of transfer of
2421 functional microRNAs between mouse dendritic cells via exosomes. *Blood*
2422 2012;119(3):756–766. doi:10.1182/blood-2011-02-338004.

2423 185. **Morelli AE, Larregina AT, Shufesky WJ, Sullivan MLG, Stolz DB, Papworth**

GD, Zahorchak AF, Logar AJ, Wang Z, Watkins SC, Falo LD, Thomson AW.
Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells.
*Blood* 2004;104(10):3257–3266. doi:10.1182/blood-2004-03-0824.

186.   **Feng D, Zhao W-L, Ye Y-Y, Bai X-C, Liu R-Q, Chang L-F, Zhou Q, Sui S-F.**
Cellular internalization of exosomes occurs through phagocytosis. *Traffic*
2010;11(5):675–687. doi:10.1111/j.1600-0854.2010.01041.x.

187.   **Escrevente C, Keller S, Altevogt P, Costa J.** Interaction and uptake of exosomes by
ovarian cancer cells. *BMC Cancer* 2011;11:108. doi:10.1186/1471-2407-11-108.

188.   **Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E,
Svensson LM, Mörgelin M, Belting M.** Exosome uptake depends on ERK1/2-heat
shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated
by caveolin-1. *Journal of Biological Chemistry* 2013;288(24):17713–17724.
doi:10.1074/jbc.M112.445403.

189.   **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.* 2013;87(18):10334–
10347. doi:10.1128/JVI.01310-13.

190.   **Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M,
Regen T, Hanisch U-K, Simons M.** Selective transfer of exosomes from
oligodendrocytes to microglia by macropinocytosis. *J. Cell. Sci.* 2011;124(Pt 3):447–
458. doi:10.1242/jcs.074088.

191.   **Paolillo M, Schinelli S.** Integrins and Exosomes, a Dangerous Liaison in Cancer
Progression. *Cancers (Basel)* 2017;9(8). doi:10.3390/cancers9080095.

192.   **Rana S, Yue S, Stadel D, Zöller M.** Toward tailored exosomes: the exosomal
tetraspanin web contributes to target cell selection. *Int. J. Biochem. Cell Biol.*
2012;44(9):1574–1584. doi:10.1016/j.biocel.2012.06.018.

193.   **Segura E, Guérin C, Hogg N, Amigorena S, Théry C.** CD8+ dendritic cells use
LFA-1 to capture MHC-peptide complexes from exosomes in vivo. *J. Immunol.*
2007;179(3):1489–1496.

2453 194. **Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S.**
2454 Identification of a factor that links apoptotic cells to phagocytes. *Nature*
2455 2002;417(6885):182–187. doi:10.1038/417182a.

2456 195. **Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C,**
2457 **Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S.**
2458 Microenvironmental pH is a key factor for exosome traffic in tumor cells. *Journal of*
2459 *Biological Chemistry* 2009;284(49):34211–34222. doi:10.1074/jbc.M109.041152.

2460 196. **French KC, Antonyak MA, Cerione RA.** Seminars in Cell & Developmental
2461 Biology. *Semin. Cell Dev. Biol.* 2017;67:48–55. doi:10.1016/j.semcdb.2017.01.002.

2462 197. **Tian T, Zhu Y-L, Zhou Y-Y, Liang G-F, Wang Y-Y, Hu F-H, Xiao Z-D.**
2463 Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and
2464 mediating miR-21 delivery. *Journal of Biological Chemistry* 2014;289(32):22258–
2465 22267. doi:10.1074/jbc.M114.588046.

2466 198. **Christianson HC, Svensson KJ, van Kuppevelt TH, Li J-P, Belting M.** Cancer
2467 cell exosomes depend on cell-surface heparan sulfate proteoglycans for their
2468 internalization and functional activity. *Proceedings of the National Academy of*
2469 *Sciences* 2013;110(43):17380–17385. doi:10.1073/pnas.1304266110.

2470 199. **Camussi G, Deregibus M-C, Bruno S, Grange C, Fonsato V, Tetta C.**
2471 Exosome/microvesicle-mediated epigenetic reprogramming of cells. *Am J Cancer*
2472 *Res* 2011;1(1):98–110.

2473 200. **Hong BS, Cho J-H, Kim H, Choi E-J, Rho S, Kim J, Kim JH, Choi D-S, Kim Y-**
2474 **K, Hwang D, Gho YS.** Colorectal cancer cell-derived microvesicles are enriched in
2475 cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC*
2476 *Genomics* 2009;10:556. doi:10.1186/1471-2164-10-556.

2477 201. **Hood JL, San RS, Wickline SA.** Exosomes released by melanoma cells prepare
2478 sentinel lymph nodes for tumor metastasis. *Cancer Res.* 2011;71(11):3792–3801.
2479 doi:10.1158/0008-5472.CAN-10-4455.

2480 202. **Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno**
2481 **G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar C, Nitadori-**

2482 **Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins**
2483 **VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y,**
2484 **Bromberg J, Lyden D.** Melanoma exosomes educate bone marrow progenitor cells
2485 toward a pro-metastatic phenotype through MET. *Nat. Med.* 2012;18(6):883–891.
2486 doi:10.1038/nm.2753.

2487 203. **Sheldon H, Heikamp E, Turley H, Dragovic R, Thomas P, Oon CE, Leek R,**
2488 **Edelmann M, Kessler B, Sainson RCA, Sargent I, Li J-L, Harris AL.** New
2489 mechanism for Notch signaling to endothelium at a distance by Delta-like 4
2490 incorporation into exosomes. *Blood* 2010;116(13):2385–2394. doi:10.1182/blood-
2491 2009-08-239228.

2492 204. **Mulcahy LA, Pink RC, Carter DRF.** Routes and mechanisms of extracellular
2493 vesicle uptake. *Journal of Extracellular Vesicles* 2014;3. doi:10.3402/jev.v3.24641.

2494 205. **Lai CP, Kim EY, Badr CE, Weissleder R, Mempel TR, Tannous BA,**
2495 **Breakefield XO.** Visualization and tracking of tumour extracellular vesicle delivery
2496 and RNA translation using multiplexed reporters. *Nature Communications*
2497 2015;6:7029. doi:10.1038/ncomms8029.

2498 206. **Zomer A, Maynard C, Verweij FJ, Kamermans A, Schäfer R, Beerling E,**
2499 **Schiffelers RM, de Wit E, Berenguer J, Ellenbroek SIJ, Wurdinger T, Pegtel**
2500 **DM, van Rheenen J.** In Vivo imaging reveals extracellular vesicle-mediated
2501 phenocopying of metastatic behavior. *Cell* 2015;161(5):1046–1057.
2502 doi:10.1016/j.cell.2015.04.042.

2503 207. **Ridder K, Sevko A, Heide J, Dams M, Rupp A-K, Macas J, Starmann J, Tjwa**
2504 **M, Plate KH, Sültmann H, Altevogt P, Umansky V, Momma S.** Extracellular
2505 vesicle-mediated transfer of functional RNA in the tumor microenvironment.
2506 *Oncoimmunology* 2015;4(6):e1008371. doi:10.1080/2162402X.2015.1008371.

2507 208. **Ridder K, Keller S, Dams M, Rupp A-K, Schlaudraff J, Del Turco D, Starmann**
2508 **J, Macas J, Karpova D, Devraj K, Depboylu C, Landfried B, Arnold B, Plate**
2509 **KH, Höglinger G, Sültmann H, Altevogt P, Momma S.** Extracellular vesicle-
2510 mediated transfer of genetic information between the hematopoietic system and the
2511 brain in response to inflammation. *Plos Biol* 2014;12(6):e1001874.

2512    doi:10.1371/journal.pbio.1001874.

2513    209.    **Cai J, Han Y, Ren H, Chen C, He D, Zhou L, Eisner GM, Asico LD, Jose PA,**
2514    **Zeng C.** Extracellular vesicle-mediated transfer of donor genomic DNA to recipient
2515    cells is a novel mechanism for genetic influence between cells. *J Mol Cell Biol*
2516    2013;5(4):227–238. doi:10.1093/jmcb/mjt011.

2517    210.    **Keller S, König A-K, Marmé F, Runz S, Wolterink S, Koensgen D, Mustea A,**
2518    **Sehouli J, Altevogt P.** Systemic presence and tumor-growth promoting effect of
2519    ovarian carcinoma released exosomes. *Cancer Letters* 2009;278(1):73–81.
2520    doi:10.1016/j.canlet.2008.12.028.

2521    211.    **Christianson HC, Belting M.** Heparan sulfate proteoglycan as a cell-surface
2522    endocytosis receptor. *Matrix Biol.* 2014;35:51–55.
2523    doi:10.1016/j.matbio.2013.10.004.

2524    212.    **Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Mark MT,**
2525    **Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop**
2526    **N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T,**
2527    **Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH,**
2528    **Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH,**
2529    **Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K,**
2530    **Batra SK, Jarnagin WR, Brady MS, Fodstad O, Müller V, Pantel K, Minn AJ,**
2531    **Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado**
2532    **H, Bromberg J, Lyden D.** Tumour exosome integrins determine organotropic
2533    metastasis. *Nature* 2015;527(7578):329–335. doi:10.1038/nature15756.

2534    213.    **Rejraji H, Sion B, Prensier G, Carreras M, Motta C, Frenoux J-M, Vericel E,**
2535    **Grizard G, Vernet P, Drevet JR.** Lipid remodeling of murine epididymosomes and
2536    spermatozoa during epididymal maturation. *Biol. Reprod.* 2006;74(6):1104–1113.
2537    doi:10.1095/biolreprod.105.049304.

2538    214.    **Franz C, Böing AN, Montag M, Strowitzki T, Markert UR, Mastenbroek S,**
2539    **Nieuwland R, Toth B.** Extracellular vesicles in human follicular fluid do not
2540    promote coagulation. *Reprod. Biomed. Online* 2016;33(5):652–655.
2541    doi:10.1016/j.rbmo.2016.08.005.

2542    215.    **Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M,**
2543            **Barbagallo D, Borzì P, Rizzari S, Maugeri M, Scollo P, Tatone C, Valadi H,**
2544            **Purrello M, Di Pietro C.** Molecular characterization of exosomes and their
2545            microRNA cargo in human follicular fluid: bioinformatic analysis reveals that
2546            exosomal microRNAs control pathways involved in follicular maturation. *Fertil.*
2547            *Steril.* 2014;102(6):1751–61.e1. doi:10.1016/j.fertnstert.2014.08.005.

2548    216.    **Al-Dossary AA, Bathala P, Caplan JL, Martin-DeLeon PA.** Oviductosome-
2549            Sperm Membrane Interaction in Cargo Delivery: DETECTION OF FUSION AND
2550            UNDERLYING MOLECULAR PLAYERS USING THREE-DIMENSIONAL
2551            SUPER-RESOLUTION STRUCTURED ILLUMINATION MICROSCOPY (SR-
2552            SIM). *Journal of Biological Chemistry* 2015;290(29):17710–17723.
2553            doi:10.1074/jbc.M114.633156.

2554    217.    **Al-Dossary AA, Strehler EE, Martin-DeLeon PA.** Expression and secretion of
2555            plasma membrane Ca2+-ATPase 4a (PMCA4a) during murine estrus: association
2556            with oviductal exosomes and uptake in sperm. Al-Dossary AA, Strehler EE, Martin-
2557            DeLeon PA, eds. *PLoS ONE* 2013;8(11):e80181.
2558            doi:10.1371/journal.pone.0080181.g008.

2559    218.    **Flori F, Secciani F, Capone A, Paccagnini E, Caruso S, Ricci MG, Focarelli R.**
2560            Menstrual cycle-related sialidase activity of the female cervical mucus is associated
2561            with exosome-like vesicles. *Fertil. Steril.* 2007;88(4 Suppl):1212–1219.
2562            doi:10.1016/j.fertnstert.2007.01.209.

2563    219.    **Uszyński W, Zekanowska E, Uszyński M, Zyliński A, Kuczyński J.** New
2564            observations on procoagulant properties of amniotic fluid: microparticles (MPs) and
2565            tissue factor-bearing MPs (MPs-TF), comparison with maternal blood plasma.
2566            *Thrombosis Research* 2013;132(6):757–760. doi:10.1016/j.thromres.2013.10.001.

2567    220.    **Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, Neve**
2568            **EPA, Scheynius A, Gabrielsson S.** Exosomes with immune modulatory features are
2569            present in human breast milk. *J. Immunol.* 2007;179(3):1969–1978.
2570            doi:10.4049/jimmunol.179.3.1969.

2571    221.    **Foster BP, Balassa T, Benen TD, Dominovic M, Elmadjian GK, Florova V,**

Fransolet MD, Kestlerova A, Kmiecik G, Kostadinova IA, Kyvelidou C, Meggyes M, Mincheva MN, Moro L, Pastuschek J, Spoldi V, Wandernoth P, Weber M, Toth B, Markert UR. Extracellular vesicles in blood, milk and body fluids of the female and male urogenital tract and with special regard to reproduction. *Crit Rev Clin Lab Sci* 2016;53(6):379–395. doi:10.1080/10408363.2016.1190682.

222.  Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Hum. Reprod. Update* 2015:dmv055. doi:10.1093/humupd/dmv055.

223.  Hell L, Wisgrill L, Ay C, Spittler A, Schwameis M, Jilma B, Pabinger I, Altevogt P, Thaler J. Procoagulant extracellular vesicles in amniotic fluid. *Transl Res* 2017;184:12–20.e1. doi:10.1016/j.trsl.2017.01.003.

224.  Wang X. Isolation of Extracellular Vesicles from Breast Milk. *Methods Mol. Biol.* 2017;1660:351–353. doi:10.1007/978-1-4939-7253-1_28.

225.  Karlsson O, Rodosthenous RS, Jara C, Brennan KJ, Wright RO, Baccarelli AA, Wright RJ. Detection of long non-coding RNAs in human breastmilk extracellular vesicles: Implications for early child development. *epigenetics* 2016:0. doi:10.1080/15592294.2016.1216285.

226.  Sullivan R, Saez F. Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction* 2013;146(1):R21–35. doi:10.1530/REP-13-0058.

227.  Schoysman RJ, Bedford JM. The role of the human epididymis in sperm maturation and sperm storage as reflected in the consequences of epididymovasostomy. *Fertil. Steril.* 1986;46(2):293–299.

228.  Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. *J. Androl.* 2005;26(4):459–469. doi:10.2164/jandrol.04104.

229.  Sisti G, Kanninen TT, Witkin SS. Maternal immunity and pregnancy outcome: focus on preconception and autophagy. *Genes Immun.* 2016;17(1):1–7. doi:10.1038/gene.2015.57.

2601 230. **Robertson SA, Sharkey DJ.** The role of semen in induction of maternal immune
2602      tolerance to pregnancy. *Semin. Immunol.* 2001;13(4):243–254.
2603      doi:10.1006/smim.2000.0320.

2604 231. **Ickowicz D, Finkelstein M, Breitbart H.** Mechanism of sperm capacitation and the
2605      acrosome reaction: role of protein kinases. *Asian J. Androl.* 2012;14(6):816–821.
2606      doi:10.1038/aja.2012.81.

2607 232. **Fraser LR.** Sperm capacitation and the acrosome reaction. *Human Reproduction*
2608      1998;13(suppl 1):9–19. doi:10.1093/humrep/13.suppl_1.9.

2609 233. **Zaneveld LJD, De Jonge CJ, Anderson RA, Mack SR.** Human sperm capacitation
2610      and the acrosome reaction. *Human Reproduction* 1991;6(9):1265–1274.
2611      doi:10.1093/oxfordjournals.humrep.a137524.

2612 234. **Zhu J, Barratt CL, Lippes J, Pacey AA, Cooke ID.** The sequential effects of
2613      human cervical mucus, oviductal fluid, and follicular fluid on sperm function. *Fertil.*
2614      *Steril.* 1994;61(6):1129–1135.

2615 235. **Pikó L.** Immunological phenomena in the reproductive process. *Int. J. Fertil.*
2616      1967;12(4):377–383.

2617 236. **Yanagimachi R, Kamiguchi Y, Mikamo K, Suzuki F, Yanagimachi H.**
2618      Maturation of spermatozoa in the epididymis of the Chinese hamster. *Am. J. Anat.*
2619      1985;172(4):317–330. doi:10.1002/aja.1001720406.

2620 237. **Sullivan R.** Epididymosomes: a heterogeneous population of microvesicles with
2621      multiple functions in sperm maturation and storage. *Asian J. Androl.*
2622      2015;17(5):726–729. doi:10.4103/1008-682X.155255.

2623 238. **Caballero JN, Frenette G, Belleannée C, Sullivan R.** CD9-positive microvesicles
2624      mediate the transfer of molecules to Bovine Spermatozoa during epididymal
2625      maturation. *PLoS ONE* 2013;8(6):e65364. doi:10.1371/journal.pone.0065364.

2626 239. **Frenette G, Sullivan R.** Prostasome-like particles are involved in the transfer of
2627      P25b from the bovine epididymal fluid to the sperm surface. *Mol. Reprod. Dev.*
2628      2001;59(1):115–121. doi:10.1002/mrd.1013.

2629  240.  **Caballero J, Frenette G, D'Amours O, Belleannée C, Lacroix-Pépin N, Robert C, Sullivan R.** Bovine sperm raft membrane associated Glioma Pathogenesis-Related 1-like protein 1 (GliPr1L1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida. *J. Cell. Physiol.* 2012;227(12):3876–3886. doi:10.1002/jcp.24099.

2634  241.  **Frenette G, Lessard C, Madore E, Fortier MA, Sullivan R.** Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. *Biol. Reprod.* 2003;69(5):1586–1592. doi:10.1095/biolreprod.103.019216.

2638  242.  **D'Amours O, Frenette G, Bordeleau L-J, Allard N, Leclerc P, Blondin P, Sullivan R.** Epididymosomes transfer epididymal sperm binding protein 1 (ELSPBP1) to dead spermatozoa during epididymal transit in bovine. *Biol. Reprod.* 2012;87(4):94. doi:10.1095/biolreprod.112.100990.

2642  243.  **D'Amours O, Bordeleau L-J, Frenette G, Blondin P, Leclerc P, Sullivan R.** Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. *Reproduction* 2012;143(6):759–771. doi:10.1530/REP-11-0392.

2646  244.  **D'Amours O, Frenette G, Caron P, Belleannée C, Guillemette C, Sullivan R.** Evidences of Biological Functions of Biliverdin Reductase A in the Bovine Epididymis. *J. Cell. Physiol.* 2016;231(5):1077–1089. doi:10.1002/jcp.25200.

2649  245.  **Sullivan R, Frenette G, Girouard J.** Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J. Androl.* 2007;9(4):483–491. doi:10.1111/j.1745-7262.2007.00281.x.

2652  246.  **Thimon V, Frenette G, Saez F, Thabet M, Sullivan R.** Protein composition of human epididymosomes collected during surgical vasectomy reversal: a proteomic and genomic approach. *Human Reproduction* 2008;23(8):1698–1707. doi:10.1093/humrep/den181.

2656  247.  **Martin-DeLeon PA.** Epididymosomes: transfer of fertility-modulating proteins to the sperm surface. *Asian J. Androl.* 2015;17(5):720–725. doi:10.4103/1008-

2658    682X.155538.

2659    248.    **Oh JS, Han C, Cho C.** ADAM7 is associated with epididymosomes and integrated
2660    into sperm plasma membrane. *Mol. Cells* 2009;28(5):441–446. doi:10.1007/s10059-
2661    009-0140-x.

2662    249.    **Choi H, Han C, Jin S, Kwon JT, Kim J, Jeong J, Kim J, Ham S, Jeon S, Yoo YJ,**
2663    **Cho C.** Reduced Fertility and Altered Epididymal and Sperm Integrity in Mice
2664    Lacking ADAM7. *Biol. Reprod.* 2015;93(3):70. doi:10.1095/biolreprod.115.130252.

2665    250.    **Taylor A, Robson A, Houghton BC, Jepson CA, Ford WCL, Frayne J.**
2666    Epididymal specific, selenium-independent GPX5 protects cells from oxidative
2667    stress-induced lipid peroxidation and DNA mutation. *Human Reproduction*
2668    2013;28(9):2332–2342. doi:10.1093/humrep/det237.

2669    251.    **Murta D, Batista M, Silva E, Trindade A, Henrique D, Duarte A, Lopes-da-**
2670    **Costa L.** Notch signaling in the epididymal epithelium regulates sperm motility and
2671    is transferred at a distance within epididymosomes. *Andrology* 2016;4(2):314–327.
2672    doi:10.1111/andr.12144.

2673    252.    **Belleannée C, Calvo É, Caballero J, Sullivan R.** Epididymosomes convey different
2674    repertoires of microRNAs throughout the bovine epididymis. *Biol. Reprod.*
2675    2013;89(2):30. doi:10.1095/biolreprod.113.110486.

2676    253.    **Reilly JN, McLaughlin EA, Stanger SJ, Anderson AL, Hutcheon K, Church K,**
2677    **Mihalas BP, Tyagi S, Holt JE, Eamens AL, Nixon B.** Characterisation of mouse
2678    epididymosomes reveals a complex profile of microRNAs and a potential
2679    mechanism for modification of the sperm epigenome. *Nature Publishing Group*
2680    2016;6:31794. doi:10.1038/srep31794.

2681    254.    **Wang Q, Lee I, Ren J, Ajay SS, Lee YS, Bao X.** Identification and functional
2682    characterization of tRNA-derived RNA fragments (tRFs) in respiratory syncytial
2683    virus infection. *Mol. Ther.* 2013;21(2):368–379. doi:10.1038/mt.2012.237.

2684    255.    **Lee YS, Shibata Y, Malhotra A, Dutta A.** A novel class of small RNAs: tRNA-
2685    derived RNA fragments (tRFs). *Genes & Development* 2009;23(22):2639–2649.
2686    doi:10.1101/gad.1837609.

2687    256.    **Peng H, Shi J, Zhang Y, Zhang H, Liao S, Li W, Lei L, Han C, Ning L, Cao Y,**
2688            **Zhou Q, Chen Q, Duan E.** A novel class of tRNA-derived small RNAs extremely
2689            enriched in mature mouse sperm. *Cell Res.* 2012;22(11):1609–1612.
2690            doi:10.1038/cr.2012.141.

2691    257.    **Sharma U, Conine CC, Shea JM, Boskovic A, Derr AG, Bing XY, Belleannée C,**
2692            **Kucukural A, Serra RW, Sun F, Song L, Carone BR, Ricci EP, Li XZ, Fauquier**
2693            **L, Moore MJ, Sullivan R, Mello CC, Garber M, Rando OJ.** Biogenesis and
2694            function of tRNA fragments during sperm maturation and fertilization in mammals.
2695            *Science* 2016;351(6271):391–396. doi:10.1126/science.aad6780.

2696    258.    **Ronquist G, Brody I, Gottfries A, Stegmayr B.** An Mg2+ and Ca2+-stimulated
2697            adenosine triphosphatase in human prostatic fluid: part I. *Andrologia*
2698            1978;10(4):261–272.

2699    259.    **Saez F, Sullivan R.** Prostasomes, post-testicular sperm maturation and fertility.
2700            *Front Biosci (Landmark Ed)* 2016;21:1464–1473.

2701    260.    **Kravets FG, Lee J, Singh B, Trocchia A, Pentyala SN, Khan SA.** Prostasomes:
2702            current concepts. *Prostate* 2000;43(3):169–174.

2703    261.    **Aalberts M, Stout TAE, Stoorvogel W.** Prostasomes: extracellular vesicles from
2704            the prostate. *Reproduction* 2014;147(1):R1–14. doi:10.1530/REP-13-0358.

2705    262.    **Arvidson G, Ronquist G, Wikander G, Ojteg AC.** Human prostasome membranes
2706            exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering.
2707            *Biochim. Biophys. Acta* 1989;984(2):167–173.

2708    263.    **Arienti G, Carlini E, Polci A, Cosmi EV, Palmerini CA.** Fatty acid pattern of
2709            human prostasome lipid. *Arch. Biochem. Biophys.* 1998;358(2):391–395.
2710            doi:10.1006/abbi.1998.0876.

2711    264.    **Carlini E, Palmerini CA, Cosmi EV, Arienti G.** Fusion of sperm with
2712            prostasomes: effects on membrane fluidity. *Arch. Biochem. Biophys.* 1997;343(1):6–
2713            12. doi:10.1006/abbi.1997.9999.

2714    265.    **Burden HP.** Prostasomes--their effects on human male reproduction and fertility.

2715    *Hum. Reprod. Update* 2006;12(3):283–292. doi:10.1093/humupd/dmi052.

2716  266.  **Saez F, Frenette G, Sullivan R.** Epididymosomes and prostasomes: their roles in
2717         posttesticular maturation of the sperm cells. *J. Androl.* 2003;24(2):149–154.

2718  267.  **Stegmayr B, Ronquist G.** Promotive effect on human sperm progressive motility by
2719         prostasomes. *Urol. Res.* 1982;10(5):253–257.

2720  268.  **Arienti G, Carlini E, Nicolucci A, Cosmi EV, Santi F, Palmerini CA.** The
2721         motility of human spermatozoa as influenced by prostasomes at various pH levels.
2722         *Biol. Cell* 1999;91(1):51–54.

2723  269.  **Suarez SS, Dai X.** Intracellular calcium reaches different levels of elevation in
2724         hyperactivated and acrosome-reacted hamster sperm. *Mol. Reprod. Dev.*
2725         1995;42(3):325–333. doi:10.1002/mrd.1080420310.

2726  270.  **Palmerini CA, Carlini E, Nicolucci A, Arienti G.** Increase of human spermatozoa
2727         intracellular Ca2+ concentration after fusion with prostasomes. *Cell Calcium*
2728         1999;25(4):291–296. doi:10.1054/ceca.1999.0031.

2729  271.  **Park K-H, Kim B-J, Kang J, Nam T-S, Lim JM, Kim HT, Park JK, Kim YG,**
2730         **Chae S-W, Kim U-H.** Ca2+ signaling tools acquired from prostasomes are required
2731         for progesterone-induced sperm motility. *Sci Signal* 2011;4(173):ra31.
2732         doi:10.1126/scisignal.2001595.

2733  272.  **Andrews RE, Galileo DS, Martin-DeLeon PA.** Plasma membrane Ca2+-ATPase 4:
2734         interaction with constitutive nitric oxide synthases in human sperm and prostasomes
2735         which carry Ca2+/CaM-dependent serine kinase. *Mol. Hum. Reprod.*
2736         2015;21(11):832–843. doi:10.1093/molehr/gav049.

2737  273.  **R G Knowles SM.** Nitric oxide synthases in mammals. *Biochemical Journal*
2738         1994;298(Pt 2):249.

2739  274.  **Subirán N, Agirregoitia E, Valdivia A, Ochoa C, Casis L, Irazusta J.** Expression
2740         of enkephalin-degrading enzymes in human semen and implications for sperm
2741         motility. *Fertil. Steril.* 2008;89(5 Suppl):1571–1577.
2742         doi:10.1016/j.fertnstert.2007.06.056.

275. **Arienti G, Carlini E, Verdacchi R, Cosmi EV, Palmerini CA.** Prostasome to sperm transfer of CD13/aminopeptidase N (EC 3.4.11.2). *Biochim. Biophys. Acta* 1997;1336(3):533–538.

276. **Ronquist GK, Larsson A, Ronquist G, Isaksson A, Hreinsson J, Carlsson L, Stavreus-Evers A.** Prostasomal DNA characterization and transfer into human sperm. *Mol. Reprod. Dev.* 2011;78(7):467–476. doi:10.1002/mrd.21327.

277. **Ronquist KG, Ronquist G, Carlsson L, Larsson A.** Human prostasomes contain chromosomal DNA. *Prostate* 2009;69(7):737–743. doi:10.1002/pros.20921.

278. **Ronquist G.** Prostasomes: Their Characterisation: Implications for Human Reproduction: Prostasomes and Human Reproduction. *Adv. Exp. Med. Biol.* 2015;868:191–209. doi:10.1007/978-3-319-18881-2_9.

279. **Pons-Rejraji H, Artonne C, Sion B, Brugnon F, Canis M, Janny L, Grizard G.** Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. *Int. J. Androl.* 2011;34(6 Pt 1):568–580. doi:10.1111/j.1365-2605.2010.01116.x.

280. **Cross NL, Mahasreshti P.** Prostasome fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. *Arch. Androl.* 1997;39(1):39–44.

281. **Bechoua S, Rieu I, Sion B, Grizard G.** Prostasomes as potential modulators of tyrosine phosphorylation in human spermatozoa. *Syst Biol Reprod Med* 2011;57(3):139–148. doi:10.3109/19396368.2010.549538.

282. **Aalberts M, Sostaric E, Wubbolts R, Wauben MWM, Nolte-'t Hoen ENM, Gadella BM, Stout TAE, Stoorvogel W.** Spermatozoa recruit prostasomes in response to capacitation induction. *Biochim. Biophys. Acta* 2013;1834(11):2326–2335. doi:10.1016/j.bbapap.2012.08.008.

283. **Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N.** Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proceedings of the National Academy of Sciences* 2011;108(12):4892–4896.

2772    doi:10.1073/pnas.1018202108.

2773    284.    **Cross NL.** Effect of cholesterol and other sterols on human sperm acrosomal
2774    responsiveness. *Mol. Reprod. Dev.* 1996;45(2):212–217. doi:10.1002/(SICI)1098-
2775    2795(199610)45:2<212::AID-MRD14>3.0.CO;2-2.

2776    285.    **Palmerini CA, Saccardi C, Carlini E, Fabiani R, Arienti G.** Fusion of
2777    prostasomes to human spermatozoa stimulates the acrosome reaction. *Fertil. Steril.*
2778    2003;80(5):1181–1184.

2779    286.    **Siciliano L, Marcianò V, Carpino A.** Prostasome-like vesicles stimulate acrosome
2780    reaction of pig spermatozoa. *Reprod. Biol. Endocrinol.* 2008;6:5. doi:10.1186/1477-
2781    7827-6-5.

2782    287.    **Arienti G, Carlini E, Saccardi C, Palmerini CA.** Nitric oxide and fusion with
2783    prostasomes increase cytosolic calcium in progesterone-stimulated sperm. *Arch.*
2784    *Biochem. Biophys.* 2002;402(2):255–258. doi:10.1016/S0003-9861(02)00090-5.

2785    288.    **Minelli A, Allegrucci C, Liguori L, Ronquist G.** Ecto-diadenosine polyphosphates
2786    hydrolase activity on human prostasomes. *Prostate* 2002;51(1):1–9.

2787    289.    **Oliw EH, Fabiani R, Johansson L, Ronquist G.** Arachidonic acid 15-lipoxygenase
2788    and traces of E prostaglandins in purified human prostasomes. *J. Reprod. Fertil.*
2789    1993;99(1):195–199.

2790    290.    **Cha K-Y.** Maturation in vitro of immature human oocytes for clinical use. *Hum.*
2791    *Reprod. Update* 1998;4(2):103–120. doi:10.1093/humupd/4.2.103.

2792    291.    **Reed BG, Carr BR.** The Normal Menstrual Cycle and the Control of Ovulation.
2793    2015.

2794    292.    **Okabe M.** The cell biology of mammalian fertilization. *Development*
2795    2013;140(22):4471–4479. doi:10.1242/dev.090613.

2796    293.    **Hertig AT, Rock J, ADAMS EC.** A description of 34 human ova within the first 17
2797    days of development. *Am. J. Anat.* 1956;98(3):435–493.

2798    294.    **Aplin JD.** The cell biological basis of human implantation - ScienceDirect. *Best*

2799      *Practice & Research Clinical Obstetrics & ...* 2000.

2800  295.  **Wang H, Dey SK.** Roadmap to embryo implantation: clues from mouse models.
2801      *Nat. Rev. Genet.* 2006;7(3):185–199. doi:10.1038/nrg1808.

2802  296.  **Zamah AM, Hassis ME, Albertolle ME, Williams KE.** Proteomic analysis of
2803      human follicular fluid from fertile women. *Clin Proteomics* 2015;12(1):5.
2804      doi:10.1186/s12014-015-9077-6.

2805  297.  **Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P.**
2806      Follicular fluid content and oocyte quality: from single biochemical markers to
2807      metabolomics. *Reprod. Biol. Endocrinol.* 2009;7:40. doi:10.1186/1477-7827-7-40.

2808  298.  **da Silveira JC, Veeramachaneni DNR, Winger QA, Carnevale EM, Bouma GJ.**
2809      Cell-Secreted Vesicles in Equine Ovarian Follicular Fluid Contain miRNAs and
2810      Proteins: A Possible New Form of Cell Communication Within the Ovarian Follicle.
2811      *Biol. Reprod.* 2012;86(3):71–71. doi:10.1095/biolreprod.111.093252.

2812  299.  **Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter
2813      P, Benoit Y, Espeel M, D'Herde K.** Age-associated differential microRNA levels in
2814      human follicular fluid reveal pathways potentially determining fertility and success
2815      of in vitro fertilization. *Hum Fertil (Camb)* 2014;17(2):90–98.
2816      doi:10.3109/14647273.2014.897006.

2817  300.  **Sohel MMH, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C,
2818      Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D.** Exosomal and Non-
2819      Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications
2820      for Bovine Oocyte Developmental Competence. *PLoS ONE* 2013;8(11):e78505.
2821      doi:10.1371/journal.pone.0078505.

2822  301.  **Navakanitworakul R, Hung W-T, Gunewardena S, Davis JS, Chotigeat W,
2823      Christenson LK.** Characterization and Small RNA Content of Extracellular Vesicles
2824      in Follicular Fluid of Developing Bovine Antral Follicles. *Nature Publishing Group*
2825      2016;6:25486. doi:10.1038/srep25486.

2826  302.  **da Silveira JC, Carnevale EM, Winger QA, Bouma GJ.** Regulation of ACVR1
2827      and ID2 by cell-secreted exosomes during follicle maturation in the mare. *Reprod.*

2828    *Biol. Endocrinol.* 2014;12:44. doi:10.1186/1477-7827-12-44.

2829    303.    **Hung W-T, Hong X, Christenson LK, McGinnis LK.** Extracellular Vesicles from
2830            Bovine Follicular Fluid Support Cumulus Expansion. *Biol. Reprod.* 2015;93(5):117.
2831            doi:10.1095/biolreprod.115.132977.

2832    304.    **Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams**
2833            **JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch K-P, Neyses L.** Plasma
2834            membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. *J. Biol.*
2835            *Chem.* 2004;279(27):28220–28226. doi:10.1074/jbc.M312599200.

2836    305.    **Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC,**
2837            **Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE.** Targeted
2838            ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major
2839            housekeeping function for PMCA1 and a critical role in hyperactivated sperm
2840            motility and male fertility for PMCA4. *J. Biol. Chem.* 2004;279(32):33742–33750.
2841            doi:10.1074/jbc.M404628200.

2842    306.    **Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P,**
2843            **Calle A, Redruello A, López-Martín S, Gutierrez-Adán A, Yáñez-Mó M,**
2844            **Ramírez MÁ, Rizos D.** Extracellular Vesicles from BOEC in In Vitro Embryo
2845            Development and Quality. *PLoS ONE* 2016;11(2):e0148083.
2846            doi:10.1371/journal.pone.0148083.

2847    307.    **Almiñana C.** Snooping on a private conversation between the oviduct and
2848            gametes/embryos. *Animal Reproduction* 2015;12(3):366–374.

2849    308.    **Li M-Q, Jin L-P.** Ovarian stimulation for in vitro fertilization alters the protein
2850            profile expression in endometrial secretion. *Int J Clin Exp Pathol* 2013;6(10):1964–
2851            1971.

2852    309.    **Salamonsen LA, Nie G, Hannan NJ, Dimitriadis E.** Society for Reproductive
2853            Biology Founders' Lecture 2009. Preparing fertile soil: the importance of
2854            endometrial receptivity. *Reprod Fertil Dev* 2009;21(7):923–934.
2855            doi:10.1071/RD09145.

2856    310.    **Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Pérez-**

2857         **Benavente A, Garcia A, Borrós S, Santamaria A, Ponce J, Matias-Guiu X,**
2858         **Reventós J, Gil-Moreno A, Rigau M, Colás E.** Exosome-like vesicles in uterine
2859         aspirates: a comparison of ultracentrifugation-based isolation protocols. *J Transl*
2860         *Med* 2016;14(1):180. doi:10.1186/s12967-016-0935-4.

2861  311.  **Burns GW, Brooks KE, Spencer TE.** Extracellular Vesicles Originate from the
2862         Conceptus and Uterus During Early Pregnancy in Sheep. *Biol. Reprod.*
2863         2016;94(3):56. doi:10.1095/biolreprod.115.134973.

2864  312.  **Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK,**
2865         **Spencer TE.** Extracellular vesicles in luminal fluid of the ovine uterus. Burns G,
2866         Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE, eds.
2867         *PLoS ONE* 2014;9(3):e90913. doi:10.1371/journal.pone.0090913.s004.

2868  313.  **Ruiz-González I, Xu J, Wang X, Burghardt RC, Dunlap KA, Bazer FW.**
2869         Exosomes, endogenous retroviruses and toll-like receptors: pregnancy recognition in
2870         ewes. *Reproduction* 2015;149(3):281–291. doi:10.1530/REP-14-0538.

2871  314.  **Bidarimath M, Khalaj K, Kridli RT, Kan FWK, Koti M, Tayade C.** Extracellular
2872         vesicle mediated intercellular communication at the porcine maternal-fetal interface:
2873         A new paradigm for conceptus-endometrial cross-talk. *Nature Publishing Group*
2874         2017;7:40476. doi:10.1038/srep40476.

2875  315.  **Salomon C, Yee SW, Mitchell MD, Rice GE.** The possible role of extravillous
2876         trophoblast-derived exosomes on the uterine spiral arterial remodeling under both
2877         normal and pathological conditions. *BioMed Research International*
2878         2014;2014:693157. doi:10.1155/2014/693157.

2879  316.  **Yuana Y, Sturk A, Nieuwland R.** Extracellular vesicles in physiological and
2880         pathological conditions - ScienceDirect. *Blood reviews* 2013.

2881  317.  **Bulun SE.** Endometriosis. *N. Engl. J. Med.* 2009;360(3):268–279.
2882         doi:10.1056/NEJMra0804690.

2883  318.  **Tarin D.** Cell and tissue interactions in carcinogenesis and metastasis and their
2884         clinical significance. *Semin. Cancer Biol.* 2011;21(2):72–82.
2885         doi:10.1016/j.semcancer.2010.12.006.

319. **Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G, Nappi C.** Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. *Fertil. Steril.* 2009;91(6):2315–2323. doi:10.1016/j.fertnstert.2008.03.079.

320. **Bruner-Tran KL, Eisenberg E, Yeaman GR, Anderson TA, McBean J, Osteen KG.** Steroid and cytokine regulation of matrix metalloproteinase expression in endometriosis and the establishment of experimental endometriosis in nude mice. *Journal of Clinical Endocrinology & Metabolism* 2002;87(10):4782–4791. doi:10.1210/jc.2002-020418.

321. **Braundmeier AG, Dayger CA, Mehrotra P, Belton RJ, Nowak RA.** EMMPRIN is secreted by human uterine epithelial cells in microvesicles and stimulates metalloproteinase production by human uterine fibroblast cells. *Reprod Sci* 2012;19(12):1292–1301. doi:10.1177/1933719112450332.

322. **Harp D, Driss A, Mehrabi S, Chowdhury I, Xu W, Liu D, Garcia-Barrio M, Taylor RN, Gold B, Jefferson S, Sidell N, Thompson W.** Exosomes derived from endometriotic stromal cells have enhanced angiogenic effects in vitro. *Cell Tissue Res* 2016;365(1):187–196. doi:10.1007/s00441-016-2358-1.

323. **Texidó L, Romero C, Vidal A, García-Valero J, Fernández Montoli ME, Baixeras N, Condom E, Ponce J, García-Tejedor A, Martín-Satué M.** Ecto-nucleotidases activities in the contents of ovarian endometriomas: potential biomarkers of endometriosis. *Mediators Inflamm.* 2014;2014:120673. doi:10.1155/2014/120673.

324. **Ehrmann DA.** Polycystic Ovary Syndrome. *N. Engl. J. Med.* 2005;352(12):1223–1236. doi:10.1056/NEJMra041536.

325. **Stein IF, Leventhal ML.** Amenorrhea associated with bilateral polycystic ovaries - ScienceDirect. *Am. J. Obstet. Gynecol.* 1935.

326. **Koiou E, Tziomalos K, Katsikis I, Papadakis E, Kandaraki EA, Panidis D.** Platelet-derived microparticles in overweight/obese women with the polycystic ovary syndrome. *Gynecol. Endocrinol.* 2013;29(3):250–253.

2915          doi:10.3109/09513590.2012.743005.

2916    327.    **Willis GR, Connolly K, Ladell K, Davies TS, Guschina IA, Ramji D, Miners K,**
2917         **Price DA, Clayton A, James PE, Rees DA.** Young women with polycystic ovary
2918         syndrome have raised levels of circulating annexin V-positive platelet microparticles.
2919         *Human Reproduction* 2014;29(12):2756–2763. doi:10.1093/humrep/deu281.

2920    328.    **Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, Xing Q, Jin L, He L, Wu L,**
2921         **Wang L.** Identification of microRNAs in human follicular fluid: characterization of
2922         microRNAs that govern steroidogenesis in vitro and are associated with polycystic
2923         ovary syndrome in vivo. *J. Clin. Endocrinol. Metab.* 2013;98(7):3068–3079.
2924         doi:10.1210/jc.2013-1715.

2925    329.    **Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, Zhang B, Liang X, Li T, Chen J,**
2926         **Shen J, Zhao J, You L, Gao X, Zhu D, Zhao X, Yan Y, Qin Y, Li W, Yan J,**
2927         **Wang Q, Zhao J, Geng L, Ma J, Zhao Y, He G, Zhang A, Zou S, Yang A, Liu J,**
2928         **Li W, Li B, Wan C, Qin Y, Shi J, Yang J, Jiang H, Xu J-E, Qi X, Sun Y, Zhang**
2929         **Y, Hao C, Ju X, Zhao D, Ren C-E, Li X, Zhang W, Zhang Y, Zhang J, Wu D,**
2930         **Zhang C, He L, Chen Z-J.** Genome-wide association study identifies eight new risk
2931         loci for polycystic ovary syndrome. *Nat. Genet.* 2012;44(9):1020–1025.
2932         doi:10.1038/ng.2384.

2933    330.    **Chen Z-J, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, Liang**
2934         **X, Zhao X, Zhao J, Sun Y, Zhang B, Jiang H, Zhao D, Bian Y, Gao X, Geng L,**
2935         **Li Y, Zhu D, Sun X, Xu J-E, Hao C, Ren C-E, Zhang Y, Chen S, Zhang W,**
2936         **Yang A, Yan J, Li Y, Ma J, Zhao Y.** Genome-wide association study identifies
2937         susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and
2938         9q33.3. *Nat. Genet.* 2011;43(1):55–59. doi:10.1038/ng.732.

2939    331.    **McAllister JM, Modi B, Miller BA, Biegler J, Bruggeman R, Legro RS, Strauss**
2940         **JF.** Overexpression of a DENND1A isoform produces a polycystic ovary syndrome
2941         theca phenotype. *Proceedings of the National Academy of Sciences*
2942         2014;111(15):E1519–27. doi:10.1073/pnas.1400574111.

2943    332.    **Wang W, Fan J, Huang G, Zhu X, Tian Y, Tan H, Su L.** Meta-Analysis of
2944         Prevalence of Erectile Dysfunction in Mainland China: Evidence Based on

2945    Epidemiological Surveys. *Sex Med* 2017;5(1):e19–e30.
2946    doi:10.1016/j.esxm.2016.10.001.

2947  333.  **Esposito K, Ciotola M, Giugliano F, Schisano B, Improta L, Improta MR,**
2948        **Beneduce F, Rispoli M, De Sio M, Giugliano D.** Endothelial microparticles
2949        correlate with erectile dysfunction in diabetic men. *Int. J. Impot. Res.*
2950        2007;19(2):161–166. doi:10.1038/sj.ijir.3901500.

2951  334.  **Esposito K, Ciotola M, Giugliano F, Sardelli L, Maiorino MI, Beneduce F, De**
2952        **Sio M, Giugliano D.** Phenotypic assessment of endothelial microparticles in diabetic
2953        and nondiabetic men with erectile dysfunction. *J Sex Med* 2008;5(6):1436–1442.
2954        doi:10.1111/j.1743-6109.2008.00823.x.

2955  335.  **La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE.** Arterial erectile
2956        dysfunction: reliability of new markers of endothelial dysfunction. *J. Endocrinol.*
2957        *Invest.* 2011;34(10):e314–20. doi:10.1007/BF03346728.

2958  336.  **La Vignera S, Vicari E, Condorelli RA, Di Pino L, Calogero AE.** Arterial erectile
2959        dysfunction: reliability of penile Doppler evaluation integrated with serum
2960        concentrations of late endothelial progenitor cells and endothelial microparticles. *J.*
2961        *Androl.* 2012;33(3):412–419. doi:10.2164/jandrol.111.014712.

2962  337.  **Condorelli RA, Calogero AE, Vicari E, Di Pino L, Giacone F, Mongioì L, La**
2963        **Vignera S.** Arterial erectile dysfunction and peripheral arterial disease: reliability of
2964        a new phenotype of endothelial progenitor cells and endothelial microparticles. *J.*
2965        *Androl.* 2012;33(6):1268–1275. doi:10.2164/jandrol.111.015933.

2966  338.  **Park K, Ryu KS, Li WJ, Kim SW, Paick J-S.** Chronic treatment with a type 5
2967        phosphodiesterase inhibitor suppresses apoptosis of corporal smooth muscle by
2968        potentiating Akt signalling in a rat model of diabetic erectile dysfunction. *European*
2969        *Urology* 2008;53(6):1282–1288. doi:10.1016/j.eururo.2008.01.032.

2970  339.  **Porst H, Glina S, Ralph D, Zeigler H, Wong DG, Woodward B.** Durability of
2971        response following cessation of tadalafil taken once daily as treatment for erectile
2972        dysfunction. *J Sex Med* 2010;7(10):3487–3494. doi:10.1111/j.1743-
2973        6109.2010.01908.x.

2974    340.    **La Vignera S, Condorelli RA, Vicari E, D'Agata R, Calogero AE.** Endothelial
2975    apoptosis decrease following tadalafil administration in patients with arterial ED
2976    does not last after its discontinuation. *Int. J. Impot. Res.* 2011;23(5):200–205.
2977    doi:10.1038/ijir.2011.28.

2978    341.    **La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE.** Endothelial
2979    antioxidant compound prolonged the endothelial antiapoptotic effects registered after
2980    tadalafil treatment in patients with arterial erectile dysfunction. *J. Androl.*
2981    2012;33(2):170–175. doi:10.2164/jandrol.111.013342.

2982    342.    **Schulz E, Gori T, Münzel T.** Oxidative stress and endothelial dysfunction in
2983    hypertension. *Hypertension Research* 2011.

2984    343.    **Higashi Y, Noma K, Yoshizumi M, Kihara Y.** Endothelial Function and Oxidative
2985    Stress in Cardiovascular Diseases. *Circulation Journal* 2009;73(3):411–418.
2986    doi:10.1253/circj.CJ-08-1102.

2987    344.    **Francomano D, Bruzziches R, Natali M.** Cardiovascular effect of testosterone
2988    replacement therapy in aging male. *Acta Bio Medica ...* 2010.

2989    345.    **La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero A.** Original
2990    immunophenotype of blood endothelial progenitor cells and microparticles in
2991    patients with isolated arterial erectile dysfunction and late onset hypogonadism:
2992    effects of androgen replacement therapy. *The Aging Male* 2011;14(3):183–189.
2993    doi:10.1016/j.ehj.2004.03.026.

2994    346.    **La Vignera S, Condorelli RA, Vicari E, D'Agata R, Calogero AE.** New
2995    Immunophenotype of Blood Endothelial Progenitor Cells and Endothelial
2996    Microparticles in Patients With Arterial Erectile Dysfunction and Late-Onset
2997    Hypogonadism. *J. Androl.* 2011;32(5):509–517. doi:10.2164/jandrol.110.011643.

2998    347.    **Sarker S, Scholz-Romero K.** Placenta-derived exosomes continuously increase in
2999    maternal circulation over the first trimester of pregnancy | Journal of Translational
3000    Medicine | Full Text. *Journal of ...* 2014.

3001    348.    **Salomon C, Torres MJ, Kobayashi M.** A Gestational Profile of Placental
3002    Exosomes in Maternal Plasma and Their Effects on Endothelial Cell Migration.

3003        *PLoS ...* 2014.

3004 349.   **Salomon C, Kobayashi M, Ashman K, Sobrevia L.** Hypoxia-Induced Changes in
3005        the Bioactivity of Cytotrophoblast-Derived Exosomes. *PLoS ...* 2013.

3006 350.   **Rice GE, Scholz-Romero K, Sweeney E, Peiris H, Kobayashi M, Duncombe G,**
3007        **Mitchell MD, Salomon C.** The Effect of Glucose on the Release and Bioactivity of
3008        Exosomes From First Trimester Trophoblast Cells. *J. Clin. Endocrinol. Metab.*
3009        2015;100(10):E1280–E1288. doi:10.1210/jc.2015-2270.

3010 351.   **Mitchell MD, Peiris HN, Kobayashi M, Koh YQ.** Placental exosomes in normal
3011        and complicated pregnancy - ScienceDirect. *American Journal of ...* 2015.

3012 352.   **Salomon C, Sobrevia L, Ashman K, E S, D M, E G.** The Role of Placental
3013        Exosomes in Gestational Diabetes Mellitus. In: *Gestational Diabetes- ...*. InTech;
3014        2013. doi:10.5772/55298.

3015 353.   **Redman C, Tannetta DS, Dragovic RA, Gardiner C.** Review: Does size matter?
3016        Placental debris and the pathophysiology of pre-eclampsia - ScienceDirect. *Placenta*
3017        2012.

3018 354.   **Ford HB, Schust DJ.** Recurrent pregnancy loss: etiology, diagnosis, and therapy.
3019        *Reviews in obstetrics and gynecology* 2009.

3020 355.   **Alijotas-Reig J, Palacio-Garcia C, Farran-Codina I, Zarzoso C, Cabero-Roura**
3021        **L, Vilardell-Tarres M.** Circulating Cell-Derived Microparticles in Women with
3022        Pregnancy Loss. *American Journal of Reproductive Immunology* 2011;66(3):199–
3023        208. doi:10.2147/VHRM.S955.

3024 356.   **Katz D, Beilin Y.** Disorders of coagulation in pregnancy. *Br J Anaesth*
3025        2015;115(suppl_2):ii75–ii88. doi:10.1093/bja/aev374.

3026 357.   **Rai R.** Is miscarriage a coagulopathy? *Current Opinion in Obstetrics and*
3027        *Gynecology* 2003;15(3):265.

3028 358.   **Patil R, Ghosh K, Satoskar P, Shetty S.** Elevated Procoagulant Endothelial and
3029        Tissue Factor Expressing Microparticles in Women with Recurrent Pregnancy Loss.
3030        *PLoS ONE* 2013.

3031  359.  **Laude I, Rongières-Bertrand C, Boyer-Neumann C, Wolf M, Mairovitz V,**
3032  **Hugel B, Freyssinet JM, Frydman R, Meyer D, Eschwège V.** Circulating
3033  Procoagulant Microparticles in Women with Unexplained Pregnancy Loss: a New
3034  Insight. *Thromb. Haemost.* 2001;85(1):18–21.

3035  360.  **Pasquier E, De Saint Martin L, Bohec C, Collet M.** Unexplained pregnancy loss: a
3036  marker of basal endothelial dysfunction? - ScienceDirect. *Fertil. Steril.* 2013.

3037  361.  **Katzenell S, Shomer E, Zipori Y, Zylberfisz A, Brenner B, Aharon A.**
3038  Characterization of negatively charged phospholipids and cell origin of
3039  microparticles in women with gestational vascular complications. *Thrombosis*
3040  *Research* 2012;130(3):479–484. doi:10.1016/j.thromres.2012.03.026.

3041  362.  **Karthikeyan VJ, Lip GYH.** Endothelial damage/dysfunction and hypertension in
3042  pregnancy. *Front Biosci (Elite Ed)* 2011;3:1100–1108.

3043  363.  **Jennifer Uzan MCOPRAJ-MA.** Pre-eclampsia: pathophysiology, diagnosis, and
3044  management. *Vascular Health and Risk Management* 2011;7:467.
3045  doi:10.2147/VHRM.S20181.

3046  364.  **Aharon A.** The role of extracellular vesicles in placental vascular complications.
3047  *Thrombosis Research* 2015;135 Suppl 1:S23–5. doi:10.1016/S0049-3848(15)50435-
3048  0.

3049  365.  **Jeyabalan A.** Epidemiology of preeclampsia: impact of obesity. *Nutr. Rev.*
3050  2013;71:S18–S25. doi:10.1111/nure.12055.

3051  366.  **Roberts JM, Escudero C.** The placenta in preeclampsia - ScienceDirect. *Pregnancy*
3052  *Hypertension: An International …* 2012.

3053  367.  **Germain SJ, Sacks GP, Sooranna SR, Soorana SR, Sargent IL, Redman CW.**
3054  Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of
3055  circulating syncytiotrophoblast microparticles. *J. Immunol.* 2007;178(9):5949–5956.

3056  368.  **Chen Y, Huang Y, Jiang R, Teng Y.** Syncytiotrophoblast-derived microparticle
3057  shedding in early-onset and late-onset severe pre-eclampsia. *Int J Gynaecol Obstet*
3058  2012;119(3):234–238. doi:10.1016/j.ijgo.2012.07.010.

3059   369.   **Goswami D, Tannetta DS, Magee LA, Fuchisawa A, Redman CWG, Sargent IL,**
3060       **Dadelszen von P.** Excess syncytiotrophoblast microparticle shedding is a feature of
3061       early-onset pre-eclampsia, but not normotensive intrauterine growth restriction.
3062       *Placenta* 2006;27(1):56–61. doi:10.1016/j.placenta.2004.11.007.

3063   370.   **Li H, Han L, Yang Z, Huang W, Zhang X, Gu Y, Li Y, Liu X, Zhou L, Hu J, Yu**
3064       **M, Yang J, Li Y, Zheng Y, Guo J, Han J, Li L.** Differential Proteomic Analysis of
3065       Syncytiotrophoblast Extracellular Vesicles from Early-Onset Severe Preeclampsia,
3066       using 8-Plex iTRAQ Labeling Coupled with 2D Nano LC-MS/MS. *Cell. Physiol.*
3067       *Biochem.* 2015;36(3):1116–1130. doi:10.1159/000430283.

3068   371.   **Baig S, Kothandaraman N, Manikandan J, Rong L, Ee KH, Hill J, Lai CW, Tan**
3069       **WY, Yeoh F, Kale A, Su LL, Biswas A, Vasoo S, Choolani M.** Proteomic analysis
3070       of human placental syncytiotrophoblast microvesicles in preeclampsia. *Clin*
3071       *Proteomics* 2014;11(1):40. doi:10.1186/1559-0275-11-40.

3072   372.   **Baig S, Lim JY, Fernandis AZ, Wenk MR, Kale A, Su LL, Biswas A, Vasoo S,**
3073       **Shui G, Choolani M.** Lipidomic analysis of human placental Syncytiotrophoblast
3074       microvesicles in adverse pregnancy outcomes. *Placenta* 2013;34(5):436–442.
3075       doi:10.1016/j.placenta.2013.02.004.

3076   373.   **Truong G, Guanzon D, Kinhal V, Elfeky O, Lai A, Longo S, Nuzhat Z, Palma**
3077       **C, Scholz-Romero K, Menon R, Mol BW, Rice GE, Salomon C.** Oxygen tension
3078       regulates the miRNA profile and bioactivity of exosomes released from extravillous
3079       trophoblast cells – Liquid biopsies for monitoring complications of pregnancy.
3080       Bouma GJ, ed. *PLoS ONE* 2017;12(3):e0174514–27.
3081       doi:10.1371/journal.pone.0174514.

3082   374.   **Vargas A, Zhou S, Éthier-Chiasson M, Flipo D, Lafond J, Gilbert C, Barbeau B.**
3083       Syncytin proteins incorporated in placenta exosomes are important for cell uptake
3084       and show variation in abundance in serum exosomes from patients with
3085       preeclampsia. *FASEB J.* 2014;28(8):3703–3719. doi:10.1096/fj.13-239053.

3086   375.   **Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein**
3087       **FH, Romero R, Thadhani R, Karumanchi SA, CPEP Study Group.** Soluble
3088       endoglin and other circulating antiangiogenic factors in preeclampsia. *N. Engl. J.*

3089    *Med.* 2006;355(10):992–1005. doi:10.1056/NEJMoa055352.

3090    376.    **Estelles A, Gilabert J, Keeton M, Eguchi Y, Aznar J.** Altered expression of
3091            plasminogen activator inhibitor type 1 in placentas from pregnant women with
3092            preeclampsia and/or intrauterine fetal growth retardation | Blood Journal. *Blood*
3093            1994.

3094    377.    **Guller S, Tang Z, Ma YY, Di Santo S, Sager R, Schneider H.** Protein composition
3095            of microparticles shed from human placenta during placental perfusion: Potential
3096            role in angiogenesis and fibrinolysis in preeclampsia. *Placenta* 2011;32(1):63–69.
3097            doi:10.1016/j.placenta.2010.10.011.

3098    378.    **Gardiner C, Tannetta DS, Simms CA, Harrison P, Redman CWG, Sargent IL.**
3099            Syncytiotrophoblast microvesicles released from pre-eclampsia placentae exhibit
3100            increased tissue factor activity. *PLoS ONE* 2011;6(10):e26313.
3101            doi:10.1371/journal.pone.0026313.

3102    379.    **Tannetta DS, Hunt K, Jones CI, Davidson N, Coxon CH, Ferguson D, Redman
3103            CW, Gibbins JM, Sargent IL, Tucker KL.** Syncytiotrophoblast Extracellular
3104            Vesicles from Pre-Eclampsia Placentas Differentially Affect Platelet Function. *PLoS
3105            ONE* 2015;10(11):e0142538. doi:10.1371/journal.pone.0142538.

3106    380.    **Centlow M, Carninci P, Nemeth K, Mezey E, Brownstein M, Hansson SR.**
3107            Placental expression profiling in preeclampsia: local overproduction of hemoglobin
3108            may drive pathological changes. *Fertil. Steril.* 2008;90(5):1834–1843.
3109            doi:10.1016/j.fertnstert.2007.09.030.

3110    381.    **May K, Rosenlöf L, Olsson MG, Centlow M, Mörgelin M, Larsson I, Cederlund
3111            M, Rutardottir S, Siegmund W, Schneider H, Akerström B, Hansson SR.**
3112            Perfusion of human placenta with hemoglobin introduces preeclampsia-like injuries
3113            that are prevented by α1-microglobulin. *Placenta* 2011;32(4):323–332.
3114            doi:10.1016/j.placenta.2011.01.017.

3115    382.    **Olsson MG, Centlow M, Rutardóttir S, Stenfors I, Larsson J, Hosseini-Maaf B,
3116            Olsson ML, Hansson SR, Akerström B.** Increased levels of cell-free hemoglobin,
3117            oxidation markers, and the antioxidative heme scavenger alpha(1)-microglobulin in

3118    preeclampsia. *Free Radic. Biol. Med.* 2010;48(2):284–291.
3119    doi:10.1016/j.freeradbiomed.2009.10.052.

3120    383.    **Cronqvist T, Saljé K, Familari M, Guller S, Schneider H, Gardiner C, Sargent**
3121            **IL, Redman CW, Mörgelin M, Akerström B, Gram M, Hansson SR.**
3122            Syncytiotrophoblast vesicles show altered micro-RNA and haemoglobin content
3123            after ex-vivo perfusion of placentas with haemoglobin to mimic preeclampsia. *PLoS*
3124            *ONE* 2014;9(2):e90020. doi:10.1371/journal.pone.0090020.

3125    384.    **Aly AS, Khandelwal M, Zhao J, Mehmet AH, Sammel MD, Parry S.** Neutrophils
3126            are stimulated by syncytiotrophoblast microvillous membranes to generate
3127            superoxide radicals in women with preeclampsia. *The American Journal of*
3128            *Obstetrics & Gynecology* 2004;190(1):252–258. doi:10.1016/j.ajog.2003.07.003.

3129    385.    **Gilani SI, Weissgerber TL, Garovic VD, Jayachandran M.** Preeclampsia and
3130            Extracellular Vesicles. *Curr. Hypertens. Rep.* 2016;18(9):68. doi:10.1007/s11906-
3131            016-0678-x.

3132    386.    **Kohli S, Ranjan S, Hoffmann J, Kashif M, Daniel EA, Al-Dabet MM, Bock F,**
3133            **Nazir S, Huebner H, Mertens PR, Fischer K-D, Zenclussen AC, Offermanns S,**
3134            **Aharon A, Brenner B, Shahzad K, Ruebner M, Isermann B.** Maternal
3135            extracellular vesicles and platelets promote preeclampsia via inflammasome
3136            activation in trophoblasts. *Blood* 2016;128(17):2153–2164. doi:10.1182/blood-2016-
3137            03-705434.

3138    387.    **Lok CAR, Jebbink J, Nieuwland R, Faas MM, Boer K, Sturk A, Van Der Post**
3139            **JAM.** Leukocyte activation and circulating leukocyte-derived microparticles in
3140            preeclampsia. *American Journal of Reproductive Immunology* 2009;61(5):346–359.
3141            doi:10.1111/j.1600-0897.2009.00701.x.

3142    388.    **Mikhailova VA, Ovchinnikova OM, Zainulina MS, Sokolov DI, Sel'kov SA.**
3143            Detection of microparticles of leukocytic origin in the peripheral blood in normal
3144            pregnancy and preeclampsia. *Bull. Exp. Biol. Med.* 2014;157(6):751–756.
3145            doi:10.1007/s10517-014-2659-x.

3146    389.    **Holder B, Jones T, Sancho Shimizu V, Rice TF, Donaldson B, Bouqueau M,**

3147   **Forbes K, Kampmann B.** Macrophage Exosomes Induce Placental Inflammatory

3148   Cytokines: A Novel Mode of Maternal-Placental Messaging. *Traffic*

3149   2016;17(2):168–178. doi:10.1111/tra.12352.

3150 390. **Holder BS, Tower CL, Jones CJP, Aplin JD, Abrahams VM.** Heightened pro-

3151   inflammatory effect of preeclamptic placental microvesicles on peripheral blood

3152   immune cells in humans. *Biol. Reprod.* 2012;86(4):103.

3153   doi:10.1095/biolreprod.111.097014.

3154 391. **Joerger-Messerli MS, Hoesli IM, Rusterholz C, Lapaire O.** Stimulation of

3155   monocytes by placental microparticles involves toll-like receptors and nuclear factor

3156   kappa-light-chain-enhancer of activated B cells. *Front Immunol* 2014;5:173.

3157   doi:10.3389/fimmu.2014.00173.

3158 392. **Ling L, Huang H, Zhu L, Mao T, Shen Q, Zhang H.** Evaluation of plasma

3159   endothelial microparticles in pre-eclampsia. *J. Int. Med. Res.* 2014;42(1):42–51.

3160   doi:10.1177/0300060513504362.

3161 393. **Elfeky O, Longo S, Lai A, Rice GE, Salomon C.** Influence of maternal BMI on the

3162   exosomal profile during gestation and their role on maternal systemic inflammation.

3163   *Placenta* 2017;50:60–69. doi:10.1016/j.placenta.2016.12.020.

3164 394. **Shomer E, Katzenell S, Zipori Y, Sammour RN, Isermann B, Brenner B,**

3165   **Aharon A.** Microvesicles of women with gestational hypertension and preeclampsia

3166   affect human trophoblast fate and endothelial function. *Hypertension*

3167   2013;62(5):893–898. doi:10.1161/HYPERTENSIONAHA.113.01494.

3168 395. **Chen L, Mayo R, Chatry A, Hu G.** Gestational Diabetes Mellitus: Its

3169   Epidemiology and Implication beyond Pregnancy. *Current Epidemiology Reports*

3170   2016;3(1):1–11.

3171 396. **Erem C, Kuzu UB, Deger O, Can G.** Prevalence of gestational diabetes mellitus

3172   and associated risk factors in Turkish women: the Trabzon GDM Study. *Arch Med*

3173   *Sci* 2015;11(4):724–735. doi:10.5114/aoms.2015.53291.

3174 397. **Salomon C, Scholz-Romero K, Sarker S, Sweeney E, Kobayashi M, Correa P,**

3175   **Longo S, Duncombe G, Mitchell MD, Rice GE, Illanes SE.** Gestational Diabetes

3176    Mellitus Is Associated With Changes in the Concentration and Bioactivity of
3177    Placenta-Derived Exosomes in Maternal Circulation Across Gestation. *Diabetes*
3178    2016;65(3):598–609. doi:10.2337/db15-0966.

3179  398.  **Moro L, Bardají A, Macete E, Barrios D, Morales-Prieto DM, España C,**
3180        **Mandomando I, Sigaúque B, Dobaño C, Markert UR, Benitez-Ribas D, Alonso**
3181        **PL, Menéndez C, Mayor A.** Placental Microparticles and MicroRNAs in Pregnant
3182        Women with Plasmodium falciparum or HIV Infection. *PLoS ONE*
3183        2016;11(1):e0146361. doi:10.1371/journal.pone.0146361.

3184  399.  **Tan KH, Tan SS, Ng MJ, Tey WS, Sim WK, Allen JC, Lim SK.** Extracellular
3185        vesicles yield predictive pre-eclampsia biomarkers. *Journal of Extracellular Vesicles*
3186        2017;6(1):1408390. doi:10.1080/20013078.2017.1408390.

3187  400.  **Tan KH, Tan SS, Sze SK, Lee WKR, Ng MJ, Lim SK.** Plasma biomarker
3188        discovery in preeclampsia using a novel differential isolation technology for
3189        circulating extracellular vesicles. *Am. J. Obstet. Gynecol.* 2014;211(4):380.e1–13.
3190        doi:10.1016/j.ajog.2014.03.038.

3191  401.  **Campello E, Spiezia L, Radu CM, Dhima S, Visentin S, Valle FD, Tormene D,**
3192        **Woodhams B, Cosmi E, Simioni P.** Circulating microparticles in umbilical cord
3193        blood in normal pregnancy and pregnancy with preeclampsia. *Thrombosis Research*
3194        2015;136(2):427–431. doi:10.1016/j.thromres.2015.05.029.

3195  402.  **Salomon C, Guanzon D, Scholz-Romero K, Longo S, Correa P, Illanes SE, Rice**
3196        **GE.** Placental Exosomes as Early Biomarker of Preeclampsia: Potential Role of
3197        Exosomal MicroRNAs Across Gestation. *J. Clin. Endocrinol. Metab.*
3198        2017;102(9):3182–3194. doi:10.1210/jc.2017-00672.

3199  403.  **Chaparro A, Gaedechens D, Ramírez V, Zuñiga E, Kusanovic JP, Inostroza C,**
3200        **Varas-Godoy M, Silva K, Salomon C, Rice G, Illanes SE.** Placental biomarkers
3201        and angiogenic factors in oral fluids of patients with preeclampsia. *Prenat. Diagn.*
3202        2016;36(5):476–482. doi:10.1002/pd.4811.

3203  404.  **Motta-Mejia C, Kandzija N, Zhang W, Mhlomi V, Cerdeira AS, Burdujan A,**
3204        **Tannetta D, Dragovic R, Sargent IL, Redman CW, Kishore U, Vatish M.**

3205     Placental Vesicles Carry Active Endothelial Nitric Oxide Synthase and Their

3206     Activity is Reduced in Preeclampsia. *Hypertension* 2017;70(2):372–381.

3207     doi:10.1161/HYPERTENSIONAHA.117.09321.

3208  405.  **Hilfiker-Kleiner D, Sliwa K.** Pathophysiology and epidemiology of peripartum

3209     cardiomyopathy. *Nat Rev Cardiol* 2014;11(6):364–370.

3210     doi:10.1038/nrcardio.2014.37.

3211  406.  **Walenta K, Schwarz V, Schirmer SH, Kindermann I, Friedrich EB, Solomayer**

3212     **EF, Sliwa K, Labidi S, Hilfiker-Kleiner D, Böhm M.** Circulating microparticles as

3213     indicators of peripartum cardiomyopathy. *Eur. Heart J.* 2012;33(12):1469–1479.

3214     doi:10.1093/eurheartj/ehr485.

3215  407.  **Halkein J, Tabruyn SP, Ricke-Hoch M, Haghikia A, Nguyen N-Q-N, Scherr M,**

3216     **Castermans K, Malvaux L, Lambert V, Thiry M, Sliwa K, Noel A, Martial JA,**

3217     **Hilfiker-Kleiner D, Struman I.** MicroRNA-146a is a therapeutic target and

3218     biomarker for peripartum cardiomyopathy. *Journal of Clinical Investigation*

3219     2013;123(5):2143–2154. doi:10.1172/JCI64365.

3220  408.  **Reiner AT, Witwer KW, van Balkom BWM, de Beer J, Brodie C, Corteling RL,**

3221     **Gabrielsson S, Gimona M, Ibrahim AG, de Kleijn D, Lai CP, Lötvall J, del**

3222     **Portillo HA, Reischl IG, Riazifar M, Salomon C, Tahara H, Toh WS, Wauben**

3223     **MHM, Yang VK, Yang Y, Yeo RWY, Yin H, Giebel B, Rohde E, Lim SK.**

3224     Concise Review: Developing Best-Practice Models for the Therapeutic Use of

3225     Extracellular Vesicles. *Stem Cells Transl Med* 2017;6(8):1730–1739.

3226     doi:10.1002/sctm.17-0055.

3227  409.  **Jang SC, Kim OY, Yoon CM, Choi D-S, Roh T-Y, Park J, Nilsson J, Lötvall J,**

3228     **Kim Y-K, Gho YS.** Bioinspired exosome-mimetic nanovesicles for targeted delivery

3229     of chemotherapeutics to malignant tumors. *ACS Nano* 2013;7(9):7698–7710.

3230     doi:10.1021/nn402232g.

3231  410.  **Tan S, Li X, Guo Y, Zhang Z.** Lipid-enveloped hybrid nanoparticles for drug

3232     delivery. *Nanoscale* 2013;5(3):860–872. doi:10.1039/c2nr32880a.

3233  411.  **Haney MJ, Klyachko NL, Zhao Y, Gupta R, Plotnikova EG, He Z, Patel T,**

3234        **Piroyan A, Sokolsky M, Kabanov AV, Batrakova EV.** Exosomes as drug delivery

3235        vehicles for Parkinson's disease therapy. *J Control Release* 2015;207:18–30.

3236        doi:10.1016/j.jconrel.2015.03.033.

3237   412.   **Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, Wei J, Nie G.** A doxorubicin

3238        delivery platform using engineered natural membrane vesicle exosomes for targeted

3239        tumor therapy. *Biomaterials* 2014;35(7):2383–2390.

3240        doi:10.1016/j.biomaterials.2013.11.083.

3241   413.   **Ohno S-I, Takanashi M, Sudo K, Ueda S, Ishikawa A, Matsuyama N, Fujita K,**

3242        **Mizutani T, Ohgi T, Ochiya T, Gotoh N, Kuroda M.** Systemically injected

3243        exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol.*

3244        *Ther.* 2013;21(1):185–191. doi:10.1038/mt.2012.180.

3245   414.   **Kooijmans SAA, Aleza CG, Roffler SR, van Solinge WW, Vader P, Schiffelers**

3246        **RM.** Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles

3247        promotes tumour cell targeting. *Journal of Extracellular Vesicles* 2016;5:31053.

3248   415.   **Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM.** Active loading into

3249        extracellular vesicles significantly improves the cellular uptake and photodynamic

3250        effect of porphyrins. *J Control Release* 2015;205:35–44.

3251        doi:10.1016/j.jconrel.2014.11.029.

3252   416.   **Ohno S-I, Drummen GPC, Kuroda M.** Focus on Extracellular Vesicles:

3253        Development of Extracellular Vesicle-Based Therapeutic Systems. *IJMS*

3254        2016;17(2):172. doi:10.3390/ijms17020172.

3255   417.   **Syn NL, Wang L, Chow EK-H, Lim CT, Goh B-C.** Exosomes in Cancer

3256        Nanomedicine and Immunotherapy: Prospects and Challenges. *Trends Biotechnol.*

3257        2017;35(7):665–676. doi:10.1016/j.tibtech.2017.03.004.

3258   418.   **Katz-Jaffe MG, Schoolcraft WB, Gardner DK.** Analysis of protein expression

3259        (secretome) by human and mouse preimplantation embryos. *Fertil. Steril.*

3260        2006;86(3):678–685. doi:10.1016/j.fertnstert.2006.05.022.

3261   419.   **Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ.**

3262        Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors:

3263    evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*
3264    2006;20(5):847–856. doi:10.1038/sj.leu.2404132.

3265    420.    **Saadeldin IM, Kim SJ, Choi YB, Lee BC.** Improvement of cloned embryos
3266    development by co-culturing with parthenotes: a possible role of
3267    exosomes/microvesicles for embryos paracrine communication. *Cell Reprogram*
3268    2014;16(3):223–234. doi:10.1089/cell.2014.0003.

3269    421.    **Nguyen HPT, Simpson RJ, Salamonsen LA, Greening DW.** Extracellular Vesicles
3270    in the Intrauterine Environment: Challenges and Potential Functions. *Biol. Reprod.*
3271    2016;95(5):109. doi:10.1095/biolreprod.116.143503.

3272    422.    **Greening DW, Nguyen HPT, Evans J, Simpson RJ, Salamonsen LA.** Modulating
3273    the endometrial epithelial proteome and secretome in preparation for pregnancy: The
3274    role of ovarian steroid and pregnancy hormones. *J Proteomics* 2016;144:99–112.
3275    doi:10.1016/j.jprot.2016.05.026.

3276    423.    **Kim TS, Lee SH, Gang GT, Lee YS, Kim SU, Koo DB, Shin MY, Park CK, Lee
3277    DS.** Exogenous DNA uptake of boar spermatozoa by a magnetic nanoparticle vector
3278    system. *Reprod. Domest. Anim.* 2010;45(5):e201–6. doi:10.1111/j.1439-
3279    0531.2009.01516.x.

3280    424.    **Campos VF, de Leon PMM, Komninou ER, Dellagostin OA, Deschamps JC,
3281    Seixas FK, Collares T.** NanoSMGT: transgene transmission into bovine embryos
3282    using halloysite clay nanotubes or nanopolymer to improve transfection efficiency.
3283    *Theriogenology* 2011;76(8):1552–1560. doi:10.1016/j.theriogenology.2011.06.027.

3284    425.    **Liang X, Zhang L, Wang S, Han Q, Zhao RC.** Exosomes secreted by
3285    mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-
3286    125a. *J. Cell. Sci.* 2016;129(11):2182–2189. doi:10.1242/jcs.170373.

3287    426.    **Griffiths GS, Galileo DS, Reese K, Martin-DeLeon PA.** Investigating the role of
3288    murine epididymosomes and uterosomes in GPI-linked protein transfer to sperm
3289    using SPAM1 as a model. *Mol. Reprod. Dev.* 2008;75(11):1627–1636.
3290    doi:10.1002/mrd.20907.

3291    427.    **Lösche W, Scholz T, Temmler U, Oberle V, Claus RA.** Platelet-derived

3292   microvesicles transfer tissue factor to monocytes but not to neutrophils. *Platelets*
3293   2009;15(2):109–115. doi:10.1080/09537100310001649885.

3294   428.   **Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, Becker A,**
3295          **Hoshino A, Mark MT, Molina H, Xiang J, Zhang T, Theilen T-M, García-**
3296          **Santos G, Williams C, Ararso Y, Huang Y, Rodrigues G, Shen T-L, Labori KJ,**
3297          **Lothe IMB, Kure EH, Hernandez J, Doussot A, Ebbesen SH, Grandgenett PM,**
3298          **Hollingsworth MA, Jain M, Mallya K, Batra SK, Jarnagin WR, Schwartz RE,**
3299          **Matei I, Peinado H, Stanger BZ, Bromberg J, Lyden D.** Pancreatic cancer
3300          exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.*
3301          2015;17(6):816–826. doi:10.1038/ncb3169.

3302   429.   **Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Mark MT,**
3303          **Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop**
3304          **N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T,**
3305          **Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH,**
3306          **Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH,**
3307          **Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K,**
3308          **Batra SK, Jarnagin WR, Brady MS, Fodstad O, Müller V, Pantel K, Minn AJ,**
3309          **Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado**
3310          **H, Bromberg J, Lyden D.** Tumour exosome integrins determine organotropic
3311          metastasis. *Nature* 2015:1–19. doi:10.1038/nature15756.

3312   430.   **Fong MY, Zhou W, Liu L, Alontaga AY, Chandra M, Ashby J, Chow A,**
3313          **O'Connor STF, Li S, Chin AR, Somlo G, Palomares M, Li Z, Tremblay JR,**
3314          **Tsuyada A, Sun G, Reid MA, Wu X, Swiderski P, Ren X, Shi Y, Kong M, Zhong**
3315          **W, Chen Y, Wang SE.** Breast-cancer-secreted miR-122 reprograms
3316          glucose metabolism in premetastatic niche to promote metastasis. *Nat. Cell Biol.*
3317          2015;17(2):183–194. doi:10.1038/ncb3094.

3318   431.   **Wood MJ, O'Loughlin AJ, Lakhal S.** Exosomes and the blood–brain barrier:
3319          implications for neurological diseases. *Therapeutic Delivery* 2011;2(9):1095–1099.
3320          doi:10.4155/tde.11.83.

3321   432.   **Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, Gil CD,**
3322          **Nerviani A, Dell'Accio F, Pitzalis C, Oliani SM, Jan LY, Perretti M.** Neutrophil-

3323       derived microvesicles enter cartilage and protect the joint in inflammatory arthritis.
3324       *Sci Transl Med* 2015;7(315):315ra190–315ra190. doi:10.1126/scitranslmed.aac5608.

3325   433.   **Suetsugu A, Honma K, Saji S, Moriwaki H, Ochiya T, Hoffman RM.** Imaging
3326       exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic
3327       nude-mouse models. *Advanced Drug Delivery Reviews* 2012:1–8.
3328       doi:10.1016/j.addr.2012.08.007.

3329   434.   **Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA.** Delivery of
3330       siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat.*
3331       *Biotechnol.* 2011:1–7. doi:10.1038/nbt.1807.

3332   435.   **Wahlgren J, Karlson TDL, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen**
3333       **P, Valadi H.** Plasma exosomes can deliver exogenous short interfering RNA to
3334       monocytes and lymphocytes. *Nucleic Acids Research* 2012;40(17):e130–e130.
3335       doi:10.1093/nar/gks463.

3336   436.   **Ju Z, Ma J, Wang C, Yu J, Qiao Y, Hei F.** Exosomes from iPSCs Delivering
3337       siRNA Attenuate Intracellular Adhesion Molecule-1 Expression and Neutrophils
3338       Adhesion in Pulmonary Microvascular Endothelial Cells. *Inflammation* 2016:1–11.
3339       doi:10.1007/s10753-016-0494-0.

3340   437.   **Gopal SK, Greening DW, Rai A, Chen M, Xu R, Shafiq A, Mathias RA, Zhu H-**
3341       **J, Simpson RJ.** Extracellular vesicles: their role in cancer biology and epithelial-
3342       mesenchymal transition. *Biochem. J.* 2017;474(1):21–45. doi:10.1042/BCJ20160006.

3343   438.   **Lener T, Gimona M, Aigner L, Börger V, Buzas E, Camussi G, Chaput N,**
3344       **Chatterjee D, Court FA, del Portillo HA, O'Driscoll L, Fais S, Falcon-Perez JM,**
3345       **Felderhoff-Mueser U, Fraile L, Gho YS, Görgens A, Gupta RC, Hendrix A,**
3346       **Hermann DM, Hill AF, Hochberg F, Horn PA, de Kleijn D, Kordelas L,**
3347       **Kramer BW, Krämer-Albers E-M, Laner-Plamberger S, Laitinen S, Leonardi**
3348       **T, Lorenowicz MJ, Lim SK, Lötvall J, Maguire CA, Marcilla A, Nazarenko I,**
3349       **Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl**
3350       **IG, Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D,**
3351       **Tonn T, Vader P, van Balkom BWM, Wauben M, Andaloussi SE, Théry C,**
3352       **Rohde E, Giebel B.** Applying extracellular vesicles based therapeutics in clinical

3353              trials - an ISEV position paper. *Journal of Extracellular Vesicles* 2015;4:30087.

3354   439.   **Barkalina N, Jones C, Wood MJA, Coward K.** Extracellular vesicle-mediated

3355              delivery of molecular compounds into gametes and embryos: learning from nature.

3356              *Hum. Reprod. Update* 2015;21(5):627–639. doi:10.1093/humupd/dmv027.

3357   440.   **Crescitelli R, Lässer C, Szabó TG, Kittel Á, Eldh M, Dianzani I, Buzás EI,**

3358              **Lötvall J.** Distinct RNA profiles in subpopulations of extracellular vesicles:

3359              apoptotic bodies, microvesicles and exosomes. *Journal of Extracellular Vesicles*

3360              2013;2. doi:10.3402/jev.v2i0.20677.

3361   441.   **Ji H, Greening DW, Barnes TW, Lim JW, Tauro BJ, Rai A, Xu R, Adda C,**

3362              **Mathivanan S, Zhao W, Xue Y, Xu T, Zhu H-J, Simpson RJ.** Proteome profiling

3363              of exosomes derived from human primary and metastatic colorectal cancer cells

3364              reveal differential expression of key metastatic factors and signal transduction

3365              components. *Proteomics* 2013;13(10-11):1672–1686. doi:10.1002/pmic.201200562.

3366   442.   **Xu R, Simpson RJ, Greening DW.** A Protocol for Isolation and Proteomic

3367              Characterization of Distinct Extracellular Vesicle Subtypes by Sequential Centrifugal

3368              Ultrafiltration. *Methods Mol. Biol.* 2017;1545:91–116. doi:10.1007/978-1-4939-

3369              6728-5_7.

3370   443.   **Chen X, Gao C, Li H, Huang L, Sun Q, Dong Y, Tian C, Gao S, Dong H, Guan**

3371              **D, Hu X, Zhao S, Li L, Zhu L, Yan Q, Zhang J, Zen K, Zhang C-Y.**

3372              Identification and characterization of microRNAs in raw milk during different

3373              periods of lactation, commercial fluid, and powdered milk products. *Nature*

3374              *Publishing Group* 2010;20(10):1128–1137. doi:10.1038/cr.2010.80.

3375   444.   **Carrasco-Ramírez P, Greening DW, Andrés G, Gopal SK, Martín-Villar E,**

3376              **Renart J, Simpson RJ, Quintanilla M.** Podoplanin is a component of extracellular

3377              vesicles that reprograms cell-derived exosomal proteins and modulates lymphatic

3378              vessel formation. *Oncotarget* 2016;7(13):16070–16089.

3379              doi:10.18632/oncotarget.7445.

3380   445.   **Gopal SK, Greening DW, Hanssen EG, Zhu H-J, Simpson RJ, Mathias RA.**

3381              Oncogenic epithelial cell-derived exosomes containing Rac1 and PAK2 induce

3382      angiogenesis in recipient endothelial cells. *Oncotarget* 2016;7(15):19709–19722.

3383      doi:10.18632/oncotarget.7573.

3384  446.  **Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJP, Hole P,**

3385      **Carr B, Redman CWG, Harris AL, Dobson PJ, Harrison P, Sargent IL.** Sizing

3386      and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis.

3387      *Nanomedicine* 2011;7(6):780–788. doi:10.1016/j.nano.2011.04.003.

3388  447.  **Gardiner C, Shaw M, Hole P, Smith J, Tannetta D, Redman CW, Sargent IL.**

3389      Measurement of refractive index by nanoparticle tracking analysis reveals

3390      heterogeneity in extracellular vesicles. *Journal of Extracellular Vesicles*

3391      2014;3:25361.

3392  448.  **Palmieri V, Lucchetti D, Gatto I, Maiorana A, Marcantoni M, Maulucci G, Papi**

3393      **M, Pola R, De Spirito M, Sgambato A.** Dynamic light scattering for the

3394      characterization and counting of extracellular vesicles: a powerful noninvasive tool. *J*

3395      *Nanopart Res* 2014;16(9):2583. doi:10.1007/s11051-014-2583-z.

3396  449.  **Franchi A, Cubilla M, Guidobaldi HA, Bravo AA, Giojalas LC.** Uterosome-like

3397      vesicles prompt human sperm fertilizing capability. *Mol. Hum. Reprod.*

3398      2016;22(12):833–841. doi:10.1093/molehr/gaw066.

3399  450.  **Al-Dossary AA, Martin-DeLeon PA.** Role of exosomes in the reproductive tract

3400      Oviductosomes mediate interactions of oviductal secretion with gametes/early

3401      embryo. *Front Biosci (Landmark Ed)* 2016;21:1278–1285.

3402

3403

3404

3405

3406

3407

Table 1: Classification of the methods of isolation of extracellular vesicles based on their principle.

| METHOD | TECHNIQUE | ISOLATION PRINCIPLE | GENERAL WORKFLOW | ADVANTAGES | LIMITATIONS | REFERENCES |
|---|---|---|---|---|---|---|
| Centrifugation | Serial differential centrifugation | Sedimentation velocity | Serial or differential centrifugation: (1) 300 x g, 10' remove cells → (2) 2.000 x g, 10' remove cell debris, apoptotic bodies → (3) 10.000/20.000 x g, 30' to isolate microvesicles → (4) 100.000/200.000 x g, 70' to isolate exosomes. | • Broad application<br>• Standardization.<br>• Ease of use<br>• Reproducibility<br>• Yield | • Sedimentation dependent on density, tube length, sample viscosity, concentration and vesicle aggregation apart from size. | (16,95,99,440) |
| | Density gradient | Buoyant density | Generally introduced to further purify distinct types of EVs (i.e., microvesicles or exosomes). Various different reagents including sucrose or ionidxanol. Crude EV populations loaded either on top (float down) or at bottom (float up) of gradient. Ultracentrifugation performed under pre-established conditions | • Purification - increases EVs populations purity from: protein aggregates, RNA-protein complexes, separation of EVs subpopulations within the same type. | • Yield<br>• Reproducibility<br>• Trained user<br>• Time-consuming. | (4,95-98,100-102,441) |

| | | | | • Soft isolation approach <br> • Clinically applicable medium (ionixodonal) <br> • EV homogeneisty | | |
|---|---|---|---|---|---|---|
| Size-exclusion | Filtration | Size/shape | Generally interspersed within centrifugation steps: prior to centrifugation, supernatants are challenged through syringe filters of determined pore size. | • Easy to use. <br> • Further stringency of the populations based on their canonical sized. <br> • Reproducibility | • Yield loss within filtering membrane. <br> • Risk of vesicles deformation or fragmentation. | (95,103,104) |

| | Ultrafiltration | Size | Centrifugal filtration units of prefixed molecular size range that selectively retain vesicles Previous studies shown to isolate distinct subtypes of EVs using this strategy | • Easy to use.<br>• Quick technique.<br>• Reproducible | • Yield loss within filtering membrane.<br>• Risk of vesicles deformation or fragmentation. | (64,105-108,442) |
|---|---|---|---|---|---|---|
| | Chromatography | Size/charge | Purification of EVs based on surface charge or size | • High resolving power – improved purification of EVs from proteins and lipid particles.<br>• Limits EVs and proteins aggregation based on buffer utilised.<br>• Less sensitive to the viscosity of the media. | • Usually coupled to centrifugation in order to remove cell debris and recoverEV contianingfractions.<br>• Often issues with volume or buffer associated with elution | (109-111,113) |

| | | | | • Respectful with EVs functionalities and biological properties.<br>• Shorter isolation times. | | |
|---|---|---|---|---|---|---|
| Immunoaffinity | | Presence of specific EV surface molecules | Microbeads coupled to antibodies are incubated with EVs for specific surface markers recognition (i.e., A33, EpCAM, CD63). Afterwards, beads are washed and recovered by precipitation or magnetism. | • Separation based on specific molecules further than by size.<br>• Selectivity<br>• Resolution<br>• Speed of isolation | • Sometimes coupled to centrifugation and/or filtration to initially remove larger cellular debris.<br>• Select surface markers of EVs are not always known/available.<br>• Cost<br>• Yield | (87,89,118-121) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Polymeric precipitation | | Weight increase to pellet at low centrifugal force | Incubation of polymerization kit reagents with EVs solution and recovery by low-speed centrifugation- | • High speed<br>• Simple procedure | • Possibility of co-precipitating impurities.<br>• Unable to separate EVs fractions.<br>• Ideal only for small (60 to 180 nm) EVs populations. | (94,122,123) |
| Microfluidics | | Different possible principles:<br><br>(1) Presence of specific molecules.<br><br>(2) Physical properties such as size.<br><br>(3) Microfluidic filtration. | (1) EVs are passed through microfluidic system and EVs specific markers are recognised by antibodies in a device surface.<br><br>(2) Still not applicable for EVs.<br><br>(3) Combination of microfluidics and polymer filter that allow passing EVs under a certain size. | • Reduced sample volume needed.<br>• Smaller processing times and costs, maintaining high sensitivity.<br>• Possibility to process, quantify and image the samples within the system itself. | • Habitually couple to centrifugation in order to remove undesired EVs populations.<br>• Unable to differentiate EVs populations.<br>• Still under development. | (125,126,138,443) |

Table2: Classification of the methods of characterization of extracellular vesicles based on their principle.

| METHOD | TECHNIQUE | PRINCIPLE | MAIN FEATURES | QUANTITATIVE / QUALITATIVE | REFERENCES |
|---|---|---|---|---|---|
| Microscopy | Transmission electron microscopy (TEM) | Negitive staining of EVs with electron-dense molecules (heavy metals). | • Direct imaging of EV size<br>• Size distribution<br>• Can be couples to immunogold labelling to stain specific structures. | • Semi-quantitative.<br>• Dehydrating (fixation)<br>• Possibility to take measures within the imaging field. | (129-131,441,444) |
| | Scanning electron microscopy (SEM) | Covering of molecules with microgold particles and electron reflexion scanning. | • Three-dimensional imaging of EVs structures. | • Semi-quantitative.<br>• Possibility to take measuresmentswithin the imaging field. | (133,134,445) |
| | Cryo-electron microscopy (Cryo-EM) | Plunge-frozen in liquid ethane/nitrogen. | • Avoids fixation and contrasting steps. | • Semi-quantitative.<br>• Possibility to take measures within the imaging field. | (64,132) |

| | | | • Allows to see structures closer to their native states.<br>• Size distribution | • Highly trained user | |
|---|---|---|---|---|---|
| | Atomic force microscopy (AFM) | Use of a cantilever with a free end that touches the surface to obtain topographical information. | • Resolution at the nanometric level.<br>• Possibility to analyse both dry and aqueous samples.<br>• Can be combined with microfluidic isolation devices.<br>• It does not provide direct imaging of EVs. | • Quantitative.<br>• Size-distribution profiles determination.<br>• Require homogeneous EV purification | (135-138) |
| Size distribution analysis techniques | Nanoparticle tracking analysis (NTA) | Particles are challenged with a laser beam and forward scattered light is real-time captured by a microscope to calculate sizes based in particles their Brownian motion. | • Size measures in the range of 50 to 1000 nm.<br>• Standardization is not needed but possible (interest for concentration assessments).<br>• Size distribution<br>• Low sample use | • Qualitative: not only size populations but also EVs markers can be analysed by fluorescent labelling.<br>• Quantitative: possibility to get precise size distributions and their associated concentrations in 1 nm intervals.<br>• Cost | (139,446,447) |

| | | | | | |
|---|---|---|---|---|---|
| | | | • Compatibility of fluorescence detectors | | |
| | Dynamic Light Scattering (DLS) | Particles are challenged with a laser beam and reflected light is captured by a detector in a certain variable angle. The detector converts time dependent fluctuations in the scattered light intensity into particle size data. | • Size measurements in the range of 1 to 6000 nm for EVs concentrations from $10^6$ to $10^9$ particles/mL.<br><br>• Samples can be recovered after the analysis.<br><br>• Limitations with polydisperse samples and those containing big EVs. | • Mainly qualitative.<br>• Semi-quantitative if standards are used. | (140,448) |
| | Tunable resistive pulse sensing (TRPS) | A transmembrane voltage is established in a porous membrane. The crossing of EVs through the pores alters the electrophoretic flow causing a resistance that can be translated into size data. | • Size measurements in the range of 70 nm to 10 µm for EVs concentrations from $10^5$ to $10^{12}$ particles/mL.<br><br>• Single EVs measures that allow multimodal EVs populations study. | • Qualitative.<br>• Quantitative. | (141-143) |

| | | | • By modifying pores configuration the analysable EVs size and sample volume can be regulated. | | |
|---|---|---|---|---|---|
| | Flow cytometry | EVs are swept along by a liquid stream to align them in single file in the centre of the stream until the interrogation point, where they are excited by a laser beam. Laser scattered light is gathered by detectors situated 180º (size data) and 90º (morphology or fluorescently stained structures data) to the laser beam. | • Analysis of EVs with a lower size limit of 250-500 nm and ability to distinguish vesicles that differ 200 nm in size. <br> • New technological developments have reduced the limit of detection to ~100 nm and the discrimination power to 100-200 nm. <br> • Possibility to coupling to latex beads for easy marker analysis. <br> • No sorting capacity <br> • Dependent on EV surface markers or use of EV fluorescent labels | • Qualitative: not only size populations but also EVs markers can be analysed. <br> • Quantitative. | (144-149) |

| Molecular markers characterization techniques | Western blotting / ELISA | Both techniques share the same principle: proteins are attached to support (membranes or plates, respectively) and challenge with antibodies carrying a certain label. | • Easy to perform.<br>• Cheap and available.<br>• Relatively quick. | • Qualitative.<br>• Semi-quantitative in the case of Western blot and quantitative for ELISA. | (30,150,151) |
|---|---|---|---|---|---|
| | ExoScreen | ELISA sandwich-like system with modifications in the detection tandem. The method relies in that all the components of the system must stay closed (~200 nm, within the same vesicle) for a laser stimuli transfer and detection. | • Reduced time consumption.<br>• Increased sensitivity.<br>• EVs isolation is not mandatory.<br>• Little sample volumes are required. | • Qualitative.<br>• Quantitative. | (152) |
| | μNMR | Labelling of specific EVs surface molecular markers with antibodies coupled to magnetic nanoparticles and detection by microfluidic μNMR. | • Greatly higher sensitivity. | • Qualitative.<br>• Quantitative. | (153) |
| | Nano-plasmonic exosome assay (nPLEX) | A gold film with nanoholes coated with specific antibodies for the recognition of exosomal proteins is light-excited, generating surface plasmons. Joining of EVs to the antibodies cause plasmon intensity changes that are proportional to the amount of joined EVs. | • Label-free.<br>• Easy to miniaturize.<br>• Scalable for higher throughput detection.<br>• A magnitude order more detection sensitivity than μNMR. | • Qualitative.<br>• Quantitative. | (154,155) |

Table 3: Main functions of extracellular vesicles in reproductive physiology classified by their origin.

| EV TYPE | MAIN FEATURES | TARGET | FUNCTIONS | REFERENCES |
|---|---|---|---|---|
| Epididymosomes | - First described by Piko *et al* in 1967.<br>- Sizes: 50 to 8000 nm or even 2-10 µm.<br>- Two main classes: CD9-positive (affinity for live SPZ) and ELSPBP-1-enriched (affinity for dead SPZ) epididymosomes | SPZ | Transfer of molecules involved in sperm maturation (P25b, GliPr1L1, MIF, SPAM1, PMCA4) | (239-241,245,247) |
| | | | Protection from oxidative stress (BLVRA) | (244) |
| | | | Protection from lipid peroxidation (GPX5) | (250) |
| | | | Morphology and membrane composition regulation (ADAM7) | (248,249) |
| | | | Sperm motility (ADAM7, PMCA4) | (247-249) |
| | | | Small RNA regulation of gene expression | (256,257) |
| Prostasomes | - First described by Ronquist *et al* in 1978.<br>- Sizes: 30 to 500 nm. | SPZ | Enhancement of sperm motility (progesterone receptors, $Ca^{2+}$ cascade signalling components, aminopeptidase N. | (267,271,272, 274,275) |
| | | | Protection from acidic female reproductive tract environment. | (268) |
| | | | Protection from oxidative stress (PMCA4). | (272) |
| | | | Prevention of premature capacitation and acrosome reaction (cholesterol) | (265,279-281) |

| | | | | | |
|---|---|---|---|---|---|
| | - Unusual lipid composition that provide them with increased ordered structure, rigidity and viscosity. | | | Posterior induction of capacitation, SPZ hypermotility and acrosome reaction at the moment of fertilization (cAMP, progesterone receptors, hydrolases, lipoxygenases). | (282,285,286, 288,289) |
| | | | | Protection from the hostile female reproductive tract: immunity, oxidative stress, bacteria. | (259,265,282) |
| Uterine microenvironment EVs | - Wide variety of origins: serum transudates, residues from womb cell apoptosis, endometrial epithelial cells and conceptus.<br>- Variations throughout the menstrual cycle. | Endometrium | Endometrial origin | Promotion of embryo implantation (specific miRNA cargo) | (149) |
| | | | Embryo origin | Regulation of endometrial angiogenesis (specific miRNA and protein cargo) and uterine spiral arteries remodelling. | (314,348) |
| | | Embryo | Endometrial origin | Embryo development (enJSRV *env* gene RNA) and subsequent priming of the endometrium for embryo harbouring. | (311-313) |
| | | | | Promotion of embryo implantation (miR-30d, specific protein cargo, influenced by uterine hormones – functional with trophoderm). | (82,133) |
| | | | Embryo origin | Enhancing of trophoblast cells migratory ability and implantation efficiency (laminin, fibronectin). | (79) |
| | | SPZ | Sperm maturation (SPAM1) | | (426) |
| | | | Capacitation, acrosome reaction and motility promotion (PMCA4). | | (217,449) |
| Oviductal EVs | - First described for their implications in | SPZ | Regulation of SPZ storage and promotion of capacitation, acrosome reaction and hypermotility (PMCA4a). | | (217,304,305, 450) |
| | | | Regulation of molecule delivery into SPZ (Integrins α5β1 and αvβ3). | | (216) |

| | | | | |
|---|---|---|---|---|
| | SPZ final competence acquisition. | Embryo | Enhancement of embryo quality and early development. | (306) |
| Follicular EVs | - First described by da Silvera *et al*. in 2012.<br>- miRNA cargo variation with female age and reproductive aging. | Cumulus-oocyte complex | Follicle development and oocyte growth (specific miRNA cargo, ACVR1, ID2) | (300,302) |
| | | | Follicle maturation: proliferation of small follicles and inflammatory response of large developed follicles (specific miRNA signatures). | (301) |
| | | | Cumulus-oocyte complex expansion and related genes upregulation. | (303) |

Table 4: Involvement of extracellular vesicles in reproductive-related pathologies.

| DISEASE | EVs PATHOGENIC ROLE | | | REFERENCES |
|---|---|---|---|---|
| Endometriosis | Promotion of endometriotic lesions invasion and progression. | | | (319);(321); (323) |
| | Enhancement of angiogenic potential. | | | (319); (322) |
| Polycystic Ovaries Syndrome | miRNA expression regulation towards PCOS phenotype. | | | (328) |
| Erectile dysfunction | Promotion of endothelial dysfunction, vascular damage and atherogenesis. | | | (333); (335) |
| Early pregnancy loss | Induction of an excessive pro-coagulant activity. | | | (358); (359) |
| | Promotion of endothelial dysfunction. | | | (360) |
| Pre-eclampsia | Placental origin | Promotion of abnormal remodelling of uterine spiral arteries. | | (402) |
| | | Enhancement of angiogenic failure and subsequent endothelial dysfunction. | | (377) |
| | | Stimulation of pro-inflammatory and pro-coagulant activities. | | (367);(377);(378);(379); (390) |
| | | Generation of oxidative stress into the placenta and mother vasculature. | | (383); (384) |
| | Maternal origin | General | Transportation of PE risk factors. | (385) |
| | | | Failure to ensure appropriate vascular development. | (394) |
| | | Platelet EVs | Unleashing of thrombo-inflammatory placental response. | (386) |
| | | Leukocytes EVs | Promotion of pro-inflammatory cytokines release by the placenta | (390) |
| Gestational diabetes mellitus | Promotion of pro-inflammatory cytokines production by endothelial cells. | | | (397) |