

## **Development of extracellular vesicle-based medicinal products: a position paper of the group “Extracellular Vesicle translatiOn to clinical perspectiVEs – EVOLVE France”**

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### **Supplementary Methods**

The Delphi method has been widely used, being regarded now as a valuable technique for reaching consensus in academic research [1]. The Delphi method was used for reaching recommendation consensus. All authors were survey respondents. We considered that consensus was achieved on a recommendation if at least 70% of the respondents were in agreement with it in two survey rounds. Agreement was assessed via a score using a 0 to 10 scale. A score of 0 represented total disagreement while a score of 10 represented total agreement. We considered that a score  $\geq 7$  indicated respondent agreement to the recommendation.

### **Supplementary Reference**

[1] M. Barrios, G. Guilera, L. Nuño, J. Gómez-Benito, Consensus in the delphi method: What makes a decision change?, *Technological Forecasting and Social Change*, 163 (2021) 120484.

## Supplementary Tables and Boxes

**Supplementary Table 1:** Advantages and limits of EVs originating from autologous or allogeneic cells in terms of their production and use.

	Autologous production		Allogenic production	
	Advantages	Limits	Advantages	Limits
Quantity of EV products		Depends on the amount of starting cell sources, the maximum number of cell passages from which EVs are produced	Possibility to produce large ammounts of EVs when using cells from a pool of donors	When only one donor, same limitations as for autologous
Availability		Long production time	Ready-to-use : frozen, lyophilized, etc [1]	
Quality controls		Number of tests limited due to a lack of time and ammount of EVs	Possibility of an extensive characterization [2]	
Heterogeneity		Variability in manufacturing quality: proliferation capacity / efficiency, depending on age	Possibility of choosing the most suitable donor	Donor cell source variability (sex, age, ethnic origin)
Reproducibility			Possibility to use immortalized cell line, or pool of donor	
Immunogenicity	No immunogenicity		Low immunogenicity reported [3, 4]	Unknown effect of MHC molecules transfer by DC-EVs [5]
Pathologic environment	Autologous tumor-derived EV can be packaged with chemotherapeutic drugs [6, 7]	Limited EV efficiency in particular condition due to the pathologic environment	EV production independent of the context of the pathology to treat. Possibility to adapt efficiency of EV by cell preconditioning [8]	

**Supplementary Box 1:** Our selection of relevant general information on raw material, reference standards or materials, container closure system/ storage conditions, stability and final product.

Raw material
<p>All raw materials employed in the manufacturing of active substances and final products should be listed to identify the step at which each material enters the process. Raw material suitability should be reported individually in terms of identity, safety, and functionality regarding its intended use in the manufacturing process. Safety in terms of microbial contaminants is of the utmost importance considering that terminal sterilization is not systematically possible (large EV sub-population loss and adsorption issues). Therefore, a reference to quality standards is required. Monographs available in European pharmacopeia apply to compendial excipients. In-house specifications should be set and justified considering non-compendial excipients. Information on the quality and control of non-compendial materials should be provided (EMA/CHMP/BWP/534898/2008 [9] and EMA/CAT/852602/2018 [10]). Particular attention should be paid to animal origin's raw materials because of the risk of undesirable immunological responses and adventitious agents. When applicable, they should be replaced by reagents of defined composition (EMA/CHMP/BWP/534898/2008) [9]. Information regarding the risk of potential contamination with adventitious agents concerning raw materials of human or animal origin should be documented (EMA/CHMP/BWP/534898/2008) [9]. Specific guidelines are dedicated to the use of porcine trypsin in the manufacture of human biological medicinal products (EMA/CHMP/BWP/814397/2011) [11] and another for albumin or any other plasma-derived medicinal product used as an excipient (EMA/CHMP/BWP/706271/2010) [12]. Detailed information concerning adventitious agents, such as fungi, mycoplasma, and bacteria, should be addressed in dedicated sections within the core dossier (EMA/CHMP/BWP/534898/2008) [9]. The guidance provided by the European pharmacopeia on microbiological [13], mycoplasma [14], and endotoxin [15] testing should be followed. A special appendix section of the IMPD is dedicated to viral safety and transmissible spongiform encephalopathy (TSE) agents (Appendix A2, [16]). In this section, according to the guidelines EMA/CHMP/BWP/534898/2008 [9] detailed information should be documented on the avoidance and control of TSE agents with specific guidance provided by EMEA/410/01 [17]. Information related to viral safety should also be provided in the appendix. A dedicated virus safety evaluation guideline specific for biotechnological investigational medicinal products should be followed (EMA/CHMP/BWP/398498/05 [18]).</p>
Reference standards or materials
<p>Currently, no international or European pharmacopeia standards are available for EV-enriched secretome products. Therefore, a batch developed in-house, shown to be stable and suitable for clinical trials, or a batch representative, should be used as a reference for identification, tests, and assays. The in-house reference preparation should be finely characterized (see the Characterization subsection).</p>
Container closure system/ storage conditions
<p>Storage conditions, such as state (dry or suspended), volume, weight, concentration, and single-or multi-dose samples, should be identified. The container closure material should be tested to ensure that the active substance is not partially or wholly adsorbed on the vessel walls, according to EMA/CHMP/BWP/534898/2008 [9]. Different storage conditions should be evaluated: vertical, horizontal, up, and down to ensure that no interactions occur with closure, according to ICH Q5C [19]. Whereas storage at -80°C is an interesting option if an appropriate buffer is used, the opportunity to freeze-dry EVs could be more convenient for shipment and long-term storage. As for any biological material, it makes sense to minimize freeze/thaw cycles. If possible, prevent the formation of ice crystals and reduce cryoprecipitation, it may be of interest to snap-freeze aliquots in liquid nitrogen, store aliquots at or below -80°C, and thaw at 37°C. It is necessary to validate freezing and thawing procedures.</p>
Stability

According to the EMA/CHMP/BWP/534898/2008 guidelines [9], for investigational products, stability data should be provided for at least one batch produced under the final manufacturing conditions and stored in the final container closure.

According to ICH Q5C [19] guidelines, a minimum of 6 months stability study in real conditions (temperature, buffer, container, etc.) is required if storage periods are greater than six months. In the case of storage periods of less than six months, the stability study extent's decision will be on a case-by-case basis. For the test frequency, if the storage duration is supposed to be one year or less, stability should be assessed every month during the first 3 months and every 3 months thereafter. If storage duration is superior to one year, stability studies could be performed every 3 months during the 1st year, every 6 months during the 2nd year, and every year thereafter.

Stability studies under accelerated and stress conditions are recommended even for investigational biologics according to the EMA/CHMP/BWP/534898/2008 guidelines [9]. These tests are of interest for establishing degradation patterns and developing and validating suitable analytical procedures. Accelerated tests generally imply an increase in temperature and humidity at shorter storage times than the requested ones. Stress tests should include the effect of temperatures above those of accelerated testing. They may also consider changes in humidity (e.g., 75% relative humidity or greater) and pH according to ICH Q1A (R2) [20].

The quality of products evaluated in the stability studies should be similar to that of products used in preclinical and clinical studies, as defined in the specifications. If storage at an intermediate step of the purification process is intended, stability should be evaluated according to ICH Q5C [19].

#### Drug substance and final product

According to the guidelines EMA/CHMP/BWP/534898/2008 [9], the analytical methods used for the control of the drug substance and final product should be listed. Methods complying with a monograph of a European country's pharmacopeia, the European Pharmacopeia, the United States Pharmacopoeia (USP), or the Japanese Pharmacopoeia are considered validated. Other methods, which are non-compendial analytical procedures, should be described. Importantly, validation is not required for these methods for phase I and II clinical trials; however, their suitability should be confirmed, and parameters such as linearity, specificity, accuracy, range, precision, quantification, and detection limit should be presented. By the end of phase III, robust method validation is required. Guidance on analytical method validation was provided by ICH Q2A [21] and Q2B [22].

In the transition from the drug substance to the final product, the formulation development should be described, and justification should be provided for all excipients, according to the EMA/CHMP/BWP/534898/2008 guidelines [9]. the pharmacopeia of a European country, the European Pharmacopeia, the United States Pharmacopoeia (USP), or the Japanese Pharmacopoeia may be used to set specifications for the excipients. For non-compendial excipients, in-house specifications should be set. Therefore, dedicated analytical procedures used to assess excipient quality should be indicated.

For investigational medicinal products (as well as for non-investigational ones), the batch release is performed by a Qualified Person taking into consideration the elements such as reports about in-process tests and release reports attesting compliance with the product specification file, among others, according to GMP guidelines annex 13 [23].

The finished product may be sterile filtered. However, adsorption issues and product losses should be considered. The finished product is then distributed in aseptic conditions into sterile containers (vials, syringes, ampoules). According to the GMP guidelines annex 13 [23], validation of sterilization processes is required. Due to the small batch size for investigational finished products, the filling and sealing steps are often manual or semi-automated operations. Particular attention should be paid to operator training and validation of the aseptic technique of individual operators according to the GMP guidelines annex 13 [23].

**Supplementary Table 2:** List of all the aspects concerning cellular banking (human) which could impact the production of EVs, inspired from guideline ICH Q5D [24]. ■ Blue part concerns “Origin, Source, and History of Cells”. For human cells, it is relevant to describe the following characteristics of the original donor: tissue or organ of origin, ethnic and geographical origin, age, sex and general physiological condition (medical history of the donor and any tests of the donor for pathogenic agents). The cultivation history of the cells should be documented. ■ Gray part concerns tests of identity, purity, stability and tumorigenicity. 1- Tests performed to determine if banked cells are what should be. 2- Assessment that the MCB and WCB are axenic biological products, i.e., are free from adventitious microbial agents and adventitious cellular contaminants. 3- Consistent production of the intended product and maintenance of stability during storage under defined conditions. At least, two time points should be examined in WCB: “one using cells which have received a minimal number of subcultures, and another using cells at/or above the limit of in vitro cell age for production use described in the marketing application”. 4- Evaluating the safety of a diploid cell line (ICH Topic Q5D) [24].

	Origin, Source, and History of Cells			Testing of Cell Banks			
Type of biological cell substrates	Description of cell origin/certificate of analysis for commercial cells	Cultivation history/certificate of analysis for commercial cells	Genotypic and phenotypic characterisation	Tests of Identity on MCB an primary cells	Tests of Purity on MCB and WCB on primary cells	Cell Substrate Stability : , at least two time points should be examined in WCB	Tests for Karyotype and Tumorigenicity
Human autologous primary cells	For each production	For each production	No	Genotype and phenotypic characterization on MCB and primary cells	Assessment of microbial/mycoplasma/ virus/ cross contamination by other cells	Morphological characteristics, growth characteristics, biochemical markers, immunological markers, productivity of the desired product, or other relevant genotypic or phenotypic markers	Generally not considered necessary for a no living cell final product
Human allogenic primary cells	One time (For each donor)	One time (For each donor)	No				
Human cell lines	One time (For each donor)	One time (For each donor)	No				
Transformed human cell	One time (For each donor)	One time (For each donor)	Yes			consistency of the coding sequence of the expression construct	

**Supplementary Table 3:** Overview of physico-chemical characterisation methods.

Analysis Methods	Investigated parameter	Strengths	Weaknesses	Ref
<b>Nanoparticle Tracking Analysis (NTA)</b>	Concentration, Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Tracking of individual particle</li> <li>- Reduced influence of the scattered light intensity from larger particles = more adapted for multimodal samples</li> <li>- possible to study molecular content via fluorescence analysis in addition to the particle size measurements</li> </ul>	<ul style="list-style-type: none"> <li>- Measurement times may exceed those of DLS, due to the need to make multiple measurements to generate sufficient statistics</li> <li>- Highly susceptible to user bias in setting up the imaging conditions</li> <li>- Molecular analysis via fluorescence detection limited</li> </ul>	[25]
<b>Tunable Resistive Pulse Sensor (TRPS)</b>	Concentration, Hydrodynamic diameter Zeta potential	<ul style="list-style-type: none"> <li>- Tunable pore diameter: improve measurement sensitivity and resolution</li> <li>- Ability to alter pore geometry in TRPS, these pores provide a greater dynamic range of analysis</li> </ul>	<ul style="list-style-type: none"> <li>- Tunable pore diameter: increase possibilities of interlab variations</li> <li>- Pore blockages</li> <li>- Time-consuming disassembly and cleaning of the apparatus</li> <li>- Importance of the present electrolytes: conductive enough to allow resistivity, but not too high in salt concentration to avoid aggregation</li> </ul>	[25]
<b>Dynamic Light Scattering (DLS)</b>	Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Well established / statistically relevant/ easy to use</li> <li>- Easy to use for homogeneously dispersed samples.</li> <li>- High dynamic range (from 10 nm to 10µm)</li> </ul>	<ul style="list-style-type: none"> <li>- Non adapted to heterogeneous sample : large particles such as aggregates or agglomerates dominate the intensity of the scattered light signal minimizing the presence smaller particles.</li> </ul>	[25]
<b>Differential centrifugal sedimentation (DCS)</b>	Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Identification of multiple populations of particles (according to their sedimentation rate)</li> <li>- Applicable to agglomerated samples and/or complex media</li> </ul>	<ul style="list-style-type: none"> <li>- Less common than DLS</li> <li>- Not suited to measure the size of agglomerates accurately, unless the effective density of the agglomerates can be reliably determined.</li> <li>- Multiple parameter to know to obtain robust results</li> </ul>	[25]
<b>Field-Flow Fractionation (FFF)</b>	Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Absolute hydrodynamic diameter quantification is properly calibrated with standard and if the applied force is known.</li> <li>- Can separate sample population to generate monodisperse suspension</li> <li>- Different forces can be used to separate (magnetic forces, dielectric properties, thermal gradient, sedimentation forces)</li> </ul>	<ul style="list-style-type: none"> <li>- Not a commonly used method for EV analysis rendering difficult inter-laboratory comparison</li> </ul>	[25]
<b>Transmission electron microscopy (TEM)</b>	Size, Shape (2D projection), Structure	<ul style="list-style-type: none"> <li>- Access to particles morphology</li> <li>- High-resolution images (0.1 to 0.05nm)</li> </ul>	<ul style="list-style-type: none"> <li>- Labor intensive to obtain a statistically significant particle size measurement.</li> <li>- Work in high vacuum environment + high energy electron beam: demands chemical fixing for biological samples</li> </ul>	[25]
<b>CryoTEM</b>	Size, Shape (2D projection), Structure	<ul style="list-style-type: none"> <li>- Allows direct imaging of EVs that cannot be dried with limited damage.</li> <li>- EV visualization</li> <li>- Identification of multilamellar EVs</li> </ul>	<ul style="list-style-type: none"> <li>- Labor intensive to obtain a statistically significant particle size measurement.</li> <li>- Low signal-to-noise ratio</li> <li>- Cost, access</li> </ul>	[26] [27] [28]
<b>Scanning electron microscopy (SEM)</b>	Size, shape (2D projection),	<ul style="list-style-type: none"> <li>- Single-particle resolution,</li> <li>- Lower energy beams than TEM (less expensive)</li> <li>- Possible analysis of thick samples (&gt;100nm)</li> </ul>	<ul style="list-style-type: none"> <li>- Lower resolution compared to TEM : 2–3 nm to &gt;10 µm</li> </ul>	[25]
<b>Atomic force microscopy (AFM)</b>	Size Size distribution (number-based), Shape (3D imaging) , Young modulus	<ul style="list-style-type: none"> <li>- High compatibility with different samples and measurement Environments</li> <li>- No extensive preparation of samples required</li> </ul>	<ul style="list-style-type: none"> <li>- Samples need to be deposited on hard surface, limited throughput</li> <li>- Resolution: 10–20 nm lateral resolution., &lt;1 nm vertical resolution</li> </ul>	[25]



<b>Electrophoretic Light Scattering</b>	Zeta potential, Surface charge	<ul style="list-style-type: none"> <li>- Statistical values</li> <li>- Already used to control Red Blood Cell stability (Quality standard available)</li> </ul>	-Depends on the particle environment, and the pH and ionic strength of the solution or the particle concentration strongly affect its value	[25]
<b>Analytical Ultracentrifugation (AUC)</b>	Hydrodynamic diameter Density	<ul style="list-style-type: none"> <li>- High sensibility : allow study of nanoparticle (NP) surface coverage (surface modification, or plasma protein adsorption,...)</li> <li>- Possible integration of multiwavelength optical characterisation during sedimentation : allow NP-protein interaction, particle shape, identification of polydisperse NP</li> </ul>	<ul style="list-style-type: none"> <li>- Sedimentation parameter strongly depend on sample concentration or sample interactions.</li> <li>- Possible variation in data interpretation</li> <li>- High cost of instrumentation</li> <li>- High analysis time</li> </ul>	[25]
<b>Size exclusion chromatography (SEC)</b>	Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Also used for isolation -obtention of subpopulations in monodisperse sample</li> <li>- Highly compatible with industrial production</li> <li>- Possibility of in-line analysis of proteins and amino-acid content</li> </ul>	<ul style="list-style-type: none"> <li>- Not quantitative even if NP of known dimension are used to correlate elution time to NP size.</li> <li>- Unpredictable interactions with stationary phase (due to electrostatic, VDW, hydrophobic interactions depending on the sample)</li> </ul>	[25]
<b>Asymmetric flow field-flow fractionation coupled to DLS</b>	Hydrodynamic diameter	<ul style="list-style-type: none"> <li>- Also used for isolation -obtention of subpopulations in monodisperse sample</li> <li>- Highly compatible with industrial production</li> <li>- Possibility of in-line analysis of proteins and amino-acid content</li> </ul>	<ul style="list-style-type: none"> <li>- Costly</li> <li>- Advanced expertise required</li> </ul>	[29]
<b>SAXS: small-angle X-ray scattering</b>	Size, Size distribution Shape Agglomeration	<ul style="list-style-type: none"> <li>- High sensitivity</li> <li>- Compatible for dried and suspended particles</li> <li>- Low sample time preparation</li> <li>- Non destructive</li> </ul>	<ul style="list-style-type: none"> <li>- Previous knowledge of particle morphology is required for fitting the data, especially for polydisperse and heterogeneous samples</li> </ul>	[25]
<b>Fluorescence Correlation Spectroscopy (FCS)</b>	Encapsulation efficiency	<ul style="list-style-type: none"> <li>- No separation required</li> </ul>	<ul style="list-style-type: none"> <li>- Low concentration required</li> <li>- Dedicated to fluorescent molecule encapsulation</li> </ul>	[25]
<b>Super resolution microscopy</b>	Possible interaction of fluorescently labelled proteins with materials	<ul style="list-style-type: none"> <li>- Possible detection of single emitting fluorophore Resolution comparable to electronic microscopy</li> </ul>	<ul style="list-style-type: none"> <li>- Not a widely used method for EV analysis rendering difficult inter-laboratory comparison</li> </ul>	[30]
<b>Single Molecules Fluorescence Microscopy</b>	Host-guest interactions of NP	<ul style="list-style-type: none"> <li>- Study of diffusion coefficient of encapsulated molecules</li> <li>- -</li> </ul>	<ul style="list-style-type: none"> <li>- Demandst dye or fluorescent labelling</li> </ul>	[25]
<b>Video drop analysis</b>	Hydrodynamic diameter Concentration	<ul style="list-style-type: none"> <li>- Low volumes required</li> <li>- More straightforward than NTA for operators</li> </ul>	<ul style="list-style-type: none"> <li>-Requires samples at a concentration range superior to NTA</li> <li>- Limited detection of EVs &lt; 80 nm</li> </ul>	

**Supplementary Table 4:** Overview of immuno-chemical properties characterisation methods.

Analysis Methods	Investigated parameter	Strengths	Weaknesses	Ref
<b>Western blot</b>	Protein qualification/identification	Easy to performs in bio lab Information on protein size	Semi quantitative Detection limit: $10^5$ – $10^6$ EV per biomarker to analyze Extensive processing time, large sample volume required : poorly adapted to high throughput analysis	[31] [32]
<b>ELISA</b>	Protein qualification/identification	Quantification of specific molecules	Detection limit : $10^5$ – $10^6$ EV per biomarker to measure protein Difficult to develop simultaneous biomarker analysis	[31] [32] [33]
<b>Mass spectrometry</b>	Protein identification proteomic	High throughput quantitative and comparative analysis of full protein content	- Variation regarding peptide fractionation method (SDS-Page, 2D liquid chromatography, isoelectric focusing based fractionation) could strongly affect EV proteomic profiling - Proper identification, quantitation, validation demands expertise	
<b>“Conventional” flow cytometry</b>	Protein qualification/identification	- Characterize size and allow biomarker detection - Possible multiplex detection (39 biomarker till now using a bead-based method) - small sample volume required	- For EVs larger than 300-500 nm in diameter - High optical background (due to smaller particle, as well as suspension media quality) - Not adapted to quantification - Single vesicle analysis impossible: use of latex microparticles to bind multiple vesicles thereafter labelled with biomarker	[28] [34] [35]
<b>Small particle flow cytometry (including image-tream)</b>	Size Quantification and biomarker detection	Quantification, size measurement of > 100nm EV and simultaneous biomarker detection	- High cost, specialized cytometers - Limited detection of EV < 100 nm.	[36] [37] [38]
<b>Nanoflow cytometer</b>	Size Quantification and biomarker detection	Dedicated to small particle detection with and without biomarker	Moderate cost Limited detection of large EVs	[39]
<b>Micro nuclear magnetic resonance (<math>\mu</math>NMR)</b>	Biomarker analysis	- EV detection in complex environment (even optically turbid media) - Sensitivity : $10^2$ – $10^3$ EV / biomarker to analyze	- Requirement of magnetic nanoparticles association to EV - Relatively high costs of analytical instruments (e.g., NMR detector) - Low throughput	[40]
<b>Surface plasmon resonance (SPR) - Nano-Plasmonic Exosome (nPLEX)</b>	Biomarker quantification	Label free High sensitivity/ Detection limit : $10^3$ EV per biomarker to analyze High throughput Assay time < 30min	- Technical complexities involved in device fabrication and operation - High costs of analytical instruments	[41] [42]
<b>Integrated magnetic electrochemical exosomes sensor -</b>		- Isolation and detection in a single hand-portable device for electrochemical detection - measurement in 10 $\mu$ l of complex media	- Simultaneous measurement of 4 biomarker - Medium throughput	[31] [43]

<b>iMEX</b>		- limit of detection : $10^4$ EV per biomarker to analyze/ possible analyse till $10^8$ EV - Assay time : 1h - Affordable device		
<b>Exoscreen (luminescence proximity assay)</b>	Biomarker detection	- No EV purification step necessary - Possible multiwell plate use -	- Dedicated to EV <200nm	[31] [44]
<b>Micro BCA/BCA ; Bradford Nanodrop</b>	Total protein quantification	- High throughput - Assay time : 2h - Easy to perform - Read-out using classical spectrophotometer	- Possible EV overestimation if protein contamination - Interference of compounds in the medium must be considered (BCA/microBCA)	[45]
<b>Sulfophosphovanilin assay</b>	Total lipid quantification	- Easy to perform	- Low sensitivity (requirement of high EV amount) - Low reproducibility	[46] [47]
<b>Fourier-transform infrared spectroscopy (FTIR)</b>	Total lipid quantification	- Fast and simple for labs in which the technique is already implemented	- Requires equipment usually not available in biomedical laboratories.	[48]
<b>Capillary electrophoresis instruments</b>	Total RNA quantification	- Important parameter to report	- Interference of contaminants such as co-isolated free RNA	[49]
<b>Raman (micro)spectroscopy</b>	Molecular signature Biochemical composition	- Global biomolecular information (lipids, proteins, carbohydrates, nucleic acids) in one acquisition - Superposition of the single EV signal for an overall biochemical characterization of the sample - Label-free (therefore unspecific to limited biomarkers)	- Complex sample preparation (except for dried samples) - Identification of specific molecule difficult even impossible	[50] [51]
<b>Exoview</b>	Size Concentration (not total) Biomarker detection	- Kit-based: easier standardization and inter-lab comparability - possibility to check membrane and cytosolic markers	- Capture biomarker choice limited to available kits but customization possibility via the supplier	[52]
<b>Super-resolution microscopy</b>	Size Shape Structure Biomarker detection	- Less time consuming than electron microscopy - Detection of membrane and cytosolic biomarkers	- Not high-throughput	[30]

**Supplementary Box 2:** Our selection of relevant literature information and examples on pharmacology investigation based on *in vitro*, *ex vivo* and *in vivo* studies for EV-enriched secretome products.

#### ***In vitro* assays**

**Target Binding assays:** Assays can be established using non-human/human cell lines expressing the relevant target or stable cell line expressing the target. Antibody-based approaches such as Flow cytometer, EV array, Surface Plasmon Resonance Imaging (SPRi) combined with antibody microarray, nanoplasmonic exosome assay (nPLEX), Micro-NMR and bead-based microfluidic assays could be exploited for EV phenotyping and/or target binding experiments [53]. If required, ligand blocking/activation assays can be executed.

**Specificity assays:** They are a variant of target binding essays performed to evaluate the specificity of EVs to their therapeutic target rather than closely homology related targets. (e.g. in case of G protein-coupled receptors (GPCRs) based targets). Potency in terms of fold based on specificity to unrelated targets may predict the potential adverse effects in the later stage of development of EVs.

**Cross reactivity assays:** They are also a variant of target binding essays. They can be planned to find out the species with selective binding to human EVs. Generally used species are mouse, rat, rabbit, dog or monkey. The binding potential obtained from these cross-reactivity assays can be utilized for dose selection and relevant animal model selection for efficacy, toxicology and safety pharmacology studies.

**Efficacy proof-of-concept (potency, functional assays or biological activity assays):** The final readout of efficacy assays should have direct or indirect link to patho-physiological state of the disease in the TPP. For example [54], an *in vitro* cell-based functional assay using the murine macrophage-like cell line RAW 264.7 cells has been developed to compare different preparations of EVs based on their anti-inflammatory properties. In this assay, LPS-induced IL-6 secretion was reduced upon treatment with appropriate concentration of EV, which suppressed the polarization of M0 macrophages to M1 phenotype due to LPS stimulation. In addition, correlation of *in vitro* functional assay results with the mouse model of LPS-Induced inflammation were also observed. In another example [55], the potency of EVs for chronic wound healing was evaluated using *in vitro* fibroblasts proliferation, migration assays and human umbilical vein endothelial cells tube formation assays. Fibroblasts from both healthy donors and diabetic wound patients were used for more clinical relevance [55].

**Recipient cell Internalization assays:** It is of interest to check the internalization capacity of EVs before proceeding to assays to delineate their cellular and molecular mechanism of action. A widely used method includes labeling EVs with lipidic dyes such as PKH26 followed by live fluorescence imaging [55]. However, dye aggregation can mislead the tracking [56]. In a more elegant strategy, the uptake of EVs was followed via GFP-Hsp70 signal based on genetic modification [57]. However, this is a more complex strategy to implement.

**Intercellular target modulation assays (mode of action, mechanism of action - MoA studies):** To propose an acceptable MoA for an EV-based therapy product, it is important to delineate the intracellular downstream pathway modulation (i.e. target phosphorylation) using various appropriate methodologies such as Western blot, PCR, Fluorescence resonance energy transfer assay or Elisa-based assays. These assays will provide useful information regarding possible pharmacological action of EVs and also to derive pharmacodynamic (PD) markers which could be used in the preclinical animal efficacy and clinical development stage of EVs. For example [55], the wound healing and angiogenic potential of EVs was investigated by performing immunoblotting and qRT-PCR to reveal their ability to modulate pivotal signaling pathways such as Akt, ERK, STAT3 and induced expression of plethora of growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1) involved in wound healing process. This type of *in vitro* studies may further reveal the ability of EVs to impact disease-modifying pathways at molecular levels [55].

#### ***Ex vivo* assays**

*Ex vivo* assays can be performed using blood, immune cells or biopsy tissue from animals or human volunteers. These approaches could be applied in the absence of established cell lines for *in vitro* cell-based assays. In addition, *ex vivo* based assays may provide more useful information regarding the bioactivity of EVs with a better translational value. For instance, various immune cell-based assays can be performed using peripheral blood mononuclear cell (PBMCs) from healthy volunteers or from patients with the targeted disease to unravel the immunomodulatory properties of EVs [58, 59]. For example, immunosuppressive activity of exosomes evaluated using following assays: Apoptosis assays in CD8+ cells, CFSE-based CD4+ proliferation assay, CD39 induction in CD4+ T cells, Adenosine production by CD4+CD39+ Treg, downregulation of NKG2D expression on NK cells and cytotoxicity activity of NK cells. In another example [59], immune-stimulant activity of EVs from RBC was assessed by measuring cytokines using supernatants of PBMC. Moreover, some mechanisms of action studies may be more feasible in *ex vivo* settings than *in vivo*. In the case of topically-administered EV therapy products targeting dermatological conditions, testing of EVs using

artificial human skin transplants, skin biopsy and skin substitutes is highly recommended as they have high predictive and clinical translational value. Likewise, various *ex vivo* assays can be selected and performed so that *in vivo* efficacy testing can be minimized through the use of *ex vivo* assays.

#### ***In vivo* assays**

**Chemically induced animal models:** These models represent a widely used and straightforward approach to induce disease in animals. For instance, a mouse model of dextran sulfate sodium inflammatory bowel disease (IBD) was induced in order to investigate the therapeutic effect of EVs from human umbilical cord mesenchymal stem cells (MSC) injected intravenously. These EVs inhibited the expression of IL-7 in macrophages, reducing the inflammatory responses and attenuating colitis [60].

**Surgically induced animal models:** These models require more technical expertise than chemically induced ones, especially considering the precision in surgery requested for small animals. For instance, colo-cutaneous fistulas were induced by a surgical connection between the cecum and the skin in order to test the therapeutic effect of mouse MSCs administered locally into a hydrogel. A pro-healing, anti-fibrotic and anti-inflammatory effect was reported for this therapeutic approach [61].

**Genetically modified animal models:** These complex models are often used when other possibilities are not available. For instance, there is no inducible animal model for Duchenne Muscular Dystrophy. In a recent study, the potency of EVs from cardiosphere-derived cells delivered intramyocardially was carried out in mdx mice, a widely used genetically modified model for studying Duchenne muscular dystrophy (DMD). A transiently restored partial expression of full-length dystrophin in mdx mice was reported as therapeutic effect [62].

**Supplementary Box 3:** Our selection of relevant literature information and examples on toxicological studies for EV-enriched secretome products.

#### Single dose and repeated dose studies

**Acute toxicity:** Acute systemic toxicity can be assessed by administering a range of single doses of EVs to rodents and recording maximum non-lethal dose, visual, behavioral signs of toxicity and mortality for 24h. In addition, acute toxicological testing methods will guide the selection of dose, duration of dosing and dosing frequency for subacute and chronic toxicology study. Route of administration must correspond to the one intended for the clinic and the dose-response relationship must be established. Considering the inherent nature of EVs, it is unlikely they exhibit acute toxic effects in a single-dose regime. However, contaminants which may be obtained during production, isolation process, cell derived apoptotic bodies and unknown debris may cause off target and non-specific toxicity could be detected.

**Subacute toxicity:** Subacute toxicity can be determined on the animals that remain alive in each dose group with observation for a total of 14 days after the acute toxicity tests. Subacute toxicity should be performed to evaluate the toxic potential of EV on body weight, plasma and blood chemistry profiles [4]. Route of administration must be the clinical intended route. Dosing scheme and dose need to be selected based on results obtained from acute toxicological studies if performed. Animal species selection is based on the source of EVs and its cross-reactivity properties. However, rodents can be selected for initial toxicological screening of EVs obtained from mouse or non-human cell lines and EVs derived from human primary cells. In order to evaluate EVs derived from genetically-engineered human primary cells intended to target known disease-linked pathway, the selection of the appropriate species (non-human species or humanized rodent model) should be essential to elucidate the on- or off-target mediated toxicities. Given the complex nature of EVs with its surface receptors expression and intracellular components, precise and relevant *in vitro* or *ex vivo* based target-mediated pathway modulation experiments should be established.

**Chronic toxicity:** Chronic toxicity can be assessed on animals receiving the highest non-lethal dose for a total of 90 days. Visual signs of toxicity, loss of body weight and gross/histopathological changes in vital organs have to be recorded and compared to those of control mice [63]. As with small molecules and biologics, chronic toxic evaluation is recommended for EV-based products intended to treat chronic diseases like cancer, autoimmune disorders and metabolic disorders. Selection and consideration of the most relevant species are crucial before conducting a chronic toxicology study. Non-human primates may be suitable species for long-term studies for EVs based therapies derived from human primary cells intended to use as allogeneic injections in patients. Rodents, rabbits, pigs and dogs could also be considered in case of cross-reactivity of EVs with other species.

To this day, few toxicological studies have been reported for EV-based therapies. The administration of 10 total doses of EVs isolated from human HEK-293T cells in immunocompetent mice, did not reveal any abnormalities in visual signs of toxicity, body weight and gross/histopathological changes indicating absence of toxicity [4]. A study using mesenchymal stem cell (MSC)-derived EVs, shown that oral administration of  $4 \times 10^{10}$  particles/kg (equivalent to the injection of  $0.4\text{--}0.9 \times 10^6$  MSC/kg body weight) did not induce mortalities, clinical signs or abnormal findings on gross examination of organs. Similarly, intravenous injection of bovine milk-derived EVs ( $2 \times 10^{12}$  particles/200 $\mu$ L/mouse) did not elicit any acute or subacute toxicity in immunocompetent mice as revealed by histopathological analysis [64]. By contrast, injection of an equivalent dose in zebrafish embryos resulted in increased mortality and organ malformations, suggesting the possibility to use an alternative *in vivo* model for refining the maximum non-lethal dose inducing systemic toxicity before testing on rodents or larger animals.

#### Immunogenicity and Immunotoxicology studies

***In vitro* tests:** These tests are commonly done for immunological assessments in two-tiered manner, a first tier assessing bone-marrow suppression (myelotoxicity) and a second tier focused on lymphotoxicity. Optimized colony-forming assays may be used to assess myelotoxicity, while some of the better known *in vitro* immunotoxicity assays include T-cell dependent antibody response, T-cell proliferation assays, *ex vivo* immunophenotyping and mixed lymphocyte reaction assays [65, 66]. In addition, *in vitro* based immunological assessments like *in vitro* transcriptomics assays using cell lines evaluating changes in the expression of genes related to the inflammatory response, immune cell activation and cytokine release upon treatment with EVs.

***In vivo* tests:** The immunotoxicity can be assessed in standard toxicity studies especially *in vivo* models by

evaluating the total leukocyte counts, immunoglobulin levels [67], and evaluating lymphoid organs such as the spleen for weight and histopathological changes. The selected test should have adequate sensitivity, specificity and evidence backing. It is furthermore highly advised to perform such studies in chronic dosing mode to properly assess the immunogenic potential. Furthermore, if the EVs are used as a drug-delivery system for an active drug, the immune properties of the EVs should be studied in this context, to determine potential synergic or antagonist effects of the EVs and the loaded drug on the immune system. If needed, comparative studies using EVs and their parental cells on immunogenicity and immunotoxicology may be performed during non-clinical evaluation.

Generally rodent species are widely used to assess the immunogenicity [4]. In vivo rodent studies include the evaluation of serum levels of inflammatory cytokines and changes in spleen cell phenotypes i.e. expression levels of B and T-cell markers [4, 68]. Another study was carried out with C57BL/6 mice administered with clinical grade GMP EVs [3]. Immunogenicity studies revealed no significant changes in lymphocytes (CD3+, CD4+, CD8+, and CD19+) and myeloid cells (CD11b+, F4/80+) in spleen, bone marrow and thymus. In the same study, modified EVs containing a siRNA did not cause any immunogenicity compared to unloaded exosomes suggesting the importance of immunogenicity studies for EV loaded with drugs, proteins or siRNA however this may not provide concrete prediction for immunogenicity in humans. Given the surface expressing protein and intracellular components of EVs, rodent-based immunotoxicology testing may not exactly predict the immune response due to genetic makeup of the system. Non-human primates and humanized animal models may serve as an important test system to address the immunogenicity problems associated with EVs.

**Supplementary Box 4:** Protocol content according to the guidance of the Annex I of Regulation 536/2014.

Protocol
<p>“The protocol shall describe the objective, design, methodology, statistical considerations, purpose and organisation of the clinical trial.”</p>
<p>“The protocol shall be identified by: (a) the title of the clinical trial; (b) the EU trial number; (c) the sponsor's protocol code number specific for all versions of it (if relevant); (d) the date and number of the version, to be updated when it is amended; (e) a short title or name assigned to the protocol; and (f) the name and address of the sponsor, as well as the name and function of the representative or representatives of the sponsor authorised to sign the protocol or any substantial modification to the protocol.”</p>
<p>“The protocol shall at least include: (a) a statement that the clinical trial is to be conducted in compliance with the protocol, with this Regulation and with the principles of good clinical practice; (b) a comprehensive list of all investigational medicinal products and of all auxiliary medicinal products; (c) a summary of findings from non-clinical studies that potentially have clinical significance and from other clinical trials that are relevant to the clinical trial; (d) a summary of the known and potential risks and benefits including an evaluation of the anticipated benefits and risks to allow assessment in accordance with Article 6; for subjects in a clinical trial in an emergency situation, the scientific grounds for expecting that the participation of the subjects has the potential to produce a direct clinically relevant benefit shall be documented; (e) where patients were involved in the design of the clinical trial, a description of their involvement; (f) a description of, and justification for, the dosage, the dosage regime, the route and mode of administration, and the treatment period for all investigational medicinal products and auxiliary medicinal products; (g) a statement of whether the investigational medicinal products and auxiliary medicinal products used in the clinical trial are authorised; if authorised, whether they are to be used in the clinical trial in accordance with the terms of their marketing authorisations, and, if not authorised, a justification for the use of non-authorised auxiliary medicinal products in the clinical trial; (h) a description of the groups and subgroups of the subjects participating in the clinical trial, including, where relevant, groups of subjects with specific needs, for example. age, gender, participation of healthy volunteers, subjects with rare and ultra rare diseases; (i) references to literature and data that are relevant to the clinical trial, and that provide background for the clinical trial; (j) a discussion of the relevance of the clinical trial in order to allow assessment in accordance with Article 6; (k) a description of the type of clinical trial to be conducted and a discussion of the trial design (including a schematic diagram of trial design, procedures and stages, if relevant); (l) a specification of the primary end-points and the secondary end-points, if any, to be measured during the clinical trial; (m) a description of the measures taken to minimise bias, including, if applicable, randomisation and blinding; (n) a description of the expected duration of subject participation and a description of the sequence and duration of all clinical trial periods, including follow-up, if relevant; (o) a clear and unambiguous definition of the end of the clinical trial in question and, if it is not the date of the last visit of the last subject, a specification of the estimated end date and a justification thereof; (p) a description of the criteria for discontinuing parts of the clinical trial or the entire clinical trial; (q) arrangements for the maintenance of clinical trial treatment randomisation codes and procedures for breaking codes, if relevant; (r) a description of procedures for the identification of data to be recorded directly on the Case Report Forms considered as source data; (s) a description of the arrangements to comply with the applicable rules for the collection, storage and future use of biological samples from clinical trial subjects, where applicable, unless contained in a separate document; (t) a description of the arrangements for tracing, storing, destroying and returning the investigational medicinal product and unauthorised auxiliary medicinal product in accordance with Article 51; (u) a description of the statistical methods to be employed, including, if relevant: — timing of any planned interim analysis and the number of subjects planned to be enrolled; — reasons for choice of sample size; — calculations of the power of the clinical trial and clinical relevance; — the level of significance to be used; — criteria for the termination of the clinical trial; — procedures for accounting for missing, unused, and spurious data and for reporting any deviation from the original statistical plan; and — the selection of subjects to be included in the analyses; (v) a description of the subject inclusion and exclusion criteria, including criteria for</p>



withdrawing individual subjects from treatment or from the clinical trial; (w) a description of procedures relating to the withdrawal of subjects from treatment or from the clinical trial including procedures for the collection of data regarding withdrawn subjects, procedures for replacement of subjects and the follow-up of subjects that have withdrawn from treatment or from the clinical trial; (x) a justification for including subjects who are incapable of giving informed consent or other special populations, such as minors; (y) a justification for the gender and age allocation of subjects and, if a specific gender or age group is excluded from or underrepresented in the clinical trials, an explanation of the reasons and justification for these exclusion criteria; (z) a detailed description of the recruitment and informed consent procedure, especially when subjects are incapable of giving informed consent; (aa) a description of the treatments, including medicinal products, which are permitted or not permitted, before or during the clinical trial; (ab) a description of the accountability procedures for the supply and administration of medicinal products to subjects including the maintenance of blinding, if applicable; (ac) a description of procedures for monitoring subject compliance, if applicable; (ad) a description of arrangements for monitoring the conduct of the clinical trial; (ae) a description of the arrangements for taking care of the subjects after their participation in the clinical trial has ended, where such additional care is necessary because of the subjects' participation in the clinical trial and where it differs from that normally expected for the medical condition in question; (af) a specification of the efficacy and safety parameters as well as the methods and timing for assessing, recording, and analysing these parameters; (ag) a description of ethical considerations relating to the clinical trial if those have not been described elsewhere; (ah) a statement from the sponsor (either in the protocol or in a separate document) confirming that the investigators and institutions involved in the clinical trial are to permit clinical trial-related monitoring, audits and regulatory inspections, including provision of direct access to source data and documents; (ai) a description of the publication policy; (aj) duly substantiated reasons for the submission of the summary of the results of the clinical trials after more than one year; (ak) a description of the arrangements to comply with the applicable rules on the protection of personal data; in particular organisational and technical arrangements that will be implemented to avoid unauthorised access, disclosure, dissemination, alteration or loss of information and personal data processed; (al) a description of measures that will be implemented to ensure confidentiality of records and personal data of subjects; (am) a description of measures that will be implemented in case of data security breach in order to mitigate the possible adverse effects.”

“ The protocol shall describe the procedures for: (a) eliciting and recording adverse events by the investigator, and the reporting of relevant adverse events by the investigator to the sponsor; (b) reporting by the investigator to the sponsor of those serious adverse events which have been identified in the protocol as not requiring immediate reporting; (c) reporting of suspected unexpected serious adverse reactions by the sponsor to the Eudravigilance database; and (d) follow-up of subjects after adverse reactions including the type and duration of follow-up.”

“Issues regarding labelling and the unblinding of investigational medicinal products shall be addressed in the protocol, where necessary.”

“The protocol shall be accompanied by the Charter of the Data Safety Monitoring Committee, if applicable.”

“The protocol shall be accompanied by a synopsis of the protocol.”

**Supplementary Box 5:** Contents of the CTD Module 5 according to ICH M4E (R1) [69].

<b>"5.1 Table of Contents OF MODULE 5</b>
5.2 Tabular Listing of All Clinical Studies
5.3 Clinical Study Reports
5.3.1. Reports of Biopharmaceutic Studies
5.3.1.1 Bioavailability (BA) Study Reports
5.3.1.2 Comparative BA and Bioequivalence (BE) Study Reports
5.3.1.3 In vitro-In vivo Correlation Study Reports
5.3.1.4 Reports of Bioanalytical and Analytical Methods for Human Studies
5.3.2. Reports of Studies Pertinent to Pharmacokinetics using Human Biomaterials
5.3.2.1 Plasma Protein Binding Study Reports
5.3.2.2 Reports of Hepatic Metabolism and Drug Interaction Studies
5.3.2.3 Reports of Studies Using Other Human Biomaterials
5.3.3. Reports of Human Pharmacokinetic (PK) Studies
5.3.3.1 Healthy Subject PK and Initial Tolerability Study Reports
5.3.3.2 Patient PK and Initial Tolerability Study Reports
5.3.3.3 Intrinsic Factor PK Study Reports
5.3.3.4 Extrinsic Factor PK Study Reports
5.3.3.5 Population PK Study Reports
5.3.4. Reports of Human Pharmacodynamic (PD) Studies
5.3.4.1 Healthy Subject PD and PK/PD Study Reports
5.3.4.2 Patient PD and PK/PD Study Reports
5.3.5. Reports of Efficacy and Safety Studies
5.3.5.1 Study Reports of Controlled Clinical Studies Pertinent to the Claimed Indication
5.3.5.2 Study Reports of Uncontrolled Clinical Studies
5.3.5.3 Reports of Analyses of Data from More Than One Study
5.3.5.4 Other Clinical Study Reports
5.3.6. Reports of Post-Marketing Experience
5.3.7. Case Report Forms and Individual Patient Listings
5.4 Literature References

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