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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Abstract

Extracellular vesicles (EV) are emergent therapeutic effectors that have reached clinical trial investigation. To translate EV-based therapeutic to clinic, the challenge is to demonstrate quality, safety, and efficacy, as required for any medicinal product. EV research translation into medicinal products is an exciting and challenging perspective. Recent papers, provide important guidance on regulatory aspects of pharmaceutical development, defining EVs for therapeutic applications and critical considerations for the development of potency tests. In addition, the ISEV Task Force on Regulatory Affairs and Clinical Use of EV-based Therapeutics as well as the Exosomes Committee from the ISCT are expected to contribute in an active way to the development of EV-based medicinal products by providing update on the scientific progress in EVs field, information to patients and expert resource network for regulatory bodies. The contribution of our work group "Extracellular Vesicle translatiOn to clinicaL perspectiVEs – EVOLVE France", created in 2020, can be positioned in complement to all these important initiatives. Based on complementary scientific, technical, and medical expertise, we provide EV-specific recommendations for manufacturing, quality control, analytics, non-clinical development, and clinical trials, according to current European legislation. We especially focus on early phase clinical trials concerning immediate needs in the field. The main contents of the investigational medicinal product dossier, marketing authorization applications, and critical guideline information are outlined for the transition from research to clinical development and ultimate market authorization.

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1. Introduction

Extracellular vesicles (EVs) are part of the cell "secretome" that also comprises soluble factors, participating in intercellular communication while being devoid of replicative capabilities [1]. EVs are subcellular entities delineated by a lipid bilayer similar to the plasma membrane, containing biomolecules from producer (parent) cells, released either spontaneously or after induction [1, 2]. The generic name "EV" covers numerous types of particle populations secreted by cells, such as exosomes, microvesicles (MVs)/ectosomes, microparticles, apoptotic bodies (ABs), or small/medium/large vesicles (**Figure 1**).

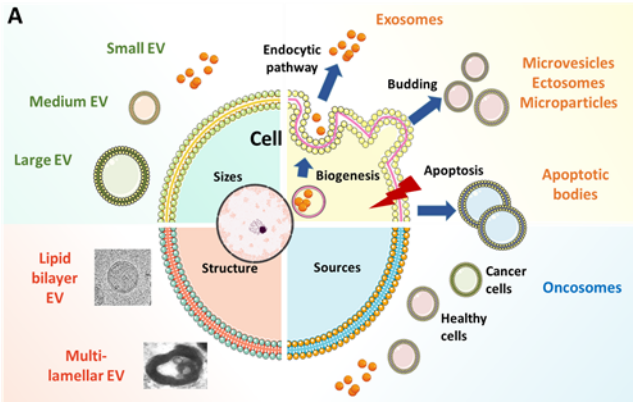


***Figure 1**: Example of sub-types of cell-released vesicles designated as Extracellular Vesicles (EVs). Sub-type diversity relates to size, structure, biogenesis, or source criteria.*

Exosomes (small EVs, 50-150 nm in diameter) generally follow the endocytic pathway and correspond to intraluminal vesicles secreted into the extracellular environment. They accumulate in multivesicular bodies (MVBs) that fuse with the plasma membrane before secretion occurs [3]. MVs (medium or large EVs, 100-1000 nm in diameter) are released into the extracellular environment after outward budding of the plasma membrane. ABs (large EVs, 1-5 µm in diameter) materialize from the subcellular fragments of apoptotic cells after their disassembly [4, 5]. The name of a vesicle population is often derived from the parent cell. The International Society for Extracellular Vesicles (ISEV) has suggested minimal information for studies of extracellular vesicle (MISEV) guidelines to help researchers interested in this delicate topic [6]. The current ISEV guidelines settle on the fact that "EV" remains a collective term describing a complex continuum of vesicles of different sizes and compositions, resulting from various mechanisms of formation and release.

There is growing research interest in EVs and their multifaceted physiological properties. Numerous biological effects of cells rely on their secretome and, more specifically EVs. Because they can recapitulate a substantial part of the parent cell's biological effects, EVs are considered potential therapeutic agents. Pre-clinical studies evidenced the beneficial effects of EVs/secretome from various cell sources to treat heart, kidney, liver, brain, and skin injuries, to name a few [7-11]. In addition to these cell-free regenerative approaches, EVs can be engineered in a pre-production or post-production step to convey natural or chemical molecules that improve their specific targeting or therapeutic properties. They can be used to encapsulate therapeutic products, protecting them from degradation and minimizing their toxicity [12, 13]. EVs also show the promising capacity to deliver transgene proteins, or RNA [14-16]. Therefore, there is today a wide range of clinical use proposed for EVs [17] and several clinical trials are ongoing in Europe and abroad (**Table 1**). Emerging companies and big pharma have recently become involved in the EV field [18] with the ultimate goal of translating EV research into clinics.

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***Table 1**: Overview of ongoing clinical trials with EVs.*

Rank	Title	Status	Conditions	Interventions	Phases	Country	URL
1	Expanded Access Protocol on Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicle Infusion Treatment for Patients With COVID-19 Associated ARDS	Available	Covid19 | ARDS | Hypoxia | Cytokine Storm	Biological: Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment	Not Applicable		https://ClinicalTrials.gov/show/NCT04657458
2	Autologous Serum-derived EV for Venous Trophic Lesions Not Responsive to Conventional Treatments	Recruiting	Ulcer Venous	Other: Autologous extracellular vesicles from serum	Not Applicable	Italy	https://ClinicalTrials.gov/show/NCT04652531
3	Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Pneumonia	Enrolling by invitation	Covid19 | SARS-CoV-2 PNEUMONIA | COVID-19	Drug: EXO 1 inhalation | Drug: EXO 2 inhalation | Drug: Placebo inhalation	Phase 2	Russian Federation	https://ClinicalTrials.gov/show/NCT04602442
4	A Clinical Study of Mesenchymal Stem Cell Exosomes Nebulizer for the Treatment of ARDS	Not yet recruiting	Acute Respiratory Distress Syndrome	Biological: low dose hMSC-Exos | Biological: medium dose hMSC-Exos | Biological: high dose hMSC-Exos | Biological: Dosage 1of hMSC-Exos | Biological: Dosage 2 of hMSC-Exos | Biological: No hMSC-derived exosomes	Phase 1 | Phase 2	China	https://ClinicalTrials.gov/show/NCT04602104
5	A Clinical Study of Mesenchymal Progenitor Cell Exosomes Nebulizer for the Treatment of Pulmonary Infection	Recruiting	Drug-resistant	Biological: Dosage 1 of MPCs-derived exosomes | Biological: Dosage 2 of MPCs-derived exosomes | Biological: No MPCs-derived exosomes	Phase 1 | Phase 2	China	https://ClinicalTrials.gov/show/NCT04544215
6	Extracellular Vesicle Infusion Therapy for Severe COVID-19	Not yet recruiting	Covid19 | ARDS | Pneumonia, Viral	Biological: DB-001	Phase 2		https://ClinicalTrials.gov/show/NCT04493242
7	Evaluation of Safety and Efficiency of Method of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia.	Completed	Covid19 | SARS-CoV-2 PNEUMONIA | COVID-19	Drug: EXO 1 inhalation | Drug: EXO 2 inhalation | Drug: Placebo inhalation	Phase 1 | Phase 2	Russian Federation	https://ClinicalTrials.gov/show/NCT04491240
8	COVID-19 Specific T Cell Derived Exosomes (CSTC-Exo)	Active, not recruiting	Corona Virus Infection | Pneumonia	Biological: COVID-19 Specific T Cell derived exosomes (CSTC-Exo)	Phase 1	Turkey	https://ClinicalTrials.gov/show/NCT04389385
9	the Safety and the Efficacy Evaluation of Allogenic Adipose MSC-Exos in Patients With Alzheimer's Disease	Recruiting	Alzheimer Disease	Biological: low dosage MSCs-Exos administrated for nasal drip | Biological: mild dosage MSCs-Exos administrated for nasal drip | Biological: high dosage MSCs-Exos administrated for nasal drip	Phase 1 | Phase 2	China	https://ClinicalTrials.gov/show/NCT04388982
10	Exosome of Mesenchymal Stem Cells for Multiple Organ Dysfuntion Syndrome After Surgical Repaire of Acute Type A Aortic Dissection	Not yet recruiting	Multiple Organ Failure	Biological: Exosome of Mesenchymal stromal cells	Not Applicable	China	https://ClinicalTrials.gov/show/NCT04356300
11	Safety Evaluation of Intracoronary Infusion of Extracellular Vesicles in Patients With AMI	Not yet recruiting	Heart Attack	Drug: PEP(extracellular vesicles) in Acute Myocardial Infarction	Phase 1	United States	https://ClinicalTrials.gov/show/NCT04327635
12	A Tolerance Clinical Study on Aerosol Inhalation of Mesenchymal Stem Cells Exosomes in Healthy Volunteers	Recruiting	Healthy	Biological: 1X level of MSCs-Exo | Biological: 2X level of MSCs-Exo | Biological: 4X level of MSCs-Exo | Biological: 6X level of MSCs-Exo | Biological: 8X level of MSCs-Exo | Biological: 10X level of MSCs-Exo	Phase 1	China	https://ClinicalTrials.gov/show/NCT04313647
13	Efficacy of Platelet- and Extracellular Vesicle-rich Plasma in Chronic Postsurgical Temporal Bone Inflammations	Completed	Otitis Media Chronic | Temporal Bone	Drug: Platelet- and extracellular vesicle-rich plasma | Drug: Standard conservative treatment	Not Applicable	Slovenia	https://ClinicalTrials.gov/show/NCT04281901
14	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes Treating Severe Novel Coronavirus Pneumonia	Completed	Coronavirus	Biological: Mesenchymal stromal cells-derived exosomes	Phase 1	China	https://ClinicalTrials.gov/show/NCT04276987
15	Evaluation of Adipose Derived Stem Cells Exo.in Treatment of Periodontitis	Recruiting	Periodontitis	Biological: adipose derived stem cells exosomes	Early Phase 1	Egypt	https://ClinicalTrials.gov/show/NCT04270006
16	Effect of UMSCs Derived Exosomes on Dry Eye in Patients With cGVHD	Recruiting	Dry Eye	Drug: Umbilical Mesenchymal Stem Cells derived Exosomes	Phase 1 | Phase 2	China	https://ClinicalTrials.gov/show/NCT04213248
17	The Use of Exosomes in Craniofacial Neuralgia	Enrolling by invitation	Neuralgia	Other: Neonatal stem cells Exosomes	Not Applicable	United States	https://ClinicalTrials.gov/show/NCT04202783
18	Focused Ultrasound and Exosomes to Treat Depression, Anxiety, and Dementias	Enrolling by invitation	Refractory Depression | Anxiety Disorders | Neurodegenerative Diseases	Other: Stem cells xosomes	Not Applicable	United States	https://ClinicalTrials.gov/show/NCT04202770
19	MSC EVs in Dystrophic Epidermolysis Bullosa	Not yet recruiting	Dystrophic Epidermolysis Bullosa	Drug: AGLE 102 (Mesenchymal Stromal Cells-derived extracellular vesicles)	Phase 1 | Phase 2		https://ClinicalTrials.gov/show/NCT04173650
20	iExosomes in Treating Participants With Metastatic Pancreas Cancer With KrasG12D Mutation	Not yet recruiting	KRAS NP_004976.2: p.G12D | Metastatic Pancreatic Adenocarcinoma | Pancreatic Ductal Adenocarcinoma | Stage IV Pancreatic Cancer AJCC v8	Drug: Mesenchymal Stromal Cells-derived Exosomes with KRAS G12D siRNA	Phase 1	United States	https://ClinicalTrials.gov/show/NCT03608631
21	Plant Exosomes and Patients Diagnosed With Polycystic Ovary Syndrome (PCOS) 17	Recruiting	Polycystic Ovary Syndrome	Other: Ginger exosomes | Other: Aloe exosomes | Other: Placebo	Not Applicable	United States	https://ClinicalTrials.gov/show/NCT03493984
22	MSC-Exos Promote Healing of MHs	Recruiting	Macular Holes	Biological: exosomes derived from mesenchymal stem cells (MSC-Exo)	Early Phase 1	China	https://ClinicalTrials.gov/show/NCT03437759
23	Allogenic Mesenchymal Stem Cell Derived Exosome in Patients With Acute Ischemic Stroke	Recruiting	Cerebrovascular Disorders	Allogenic mesenchymal stem cells derived exosome enriched by miR-124	Phase 1 | Phase 2	Iran	https://ClinicalTrials.gov/show/NCT03384433
24	Effect of Plasma Derived Exosomes on Cutaneous Wound Healing	Enrolling by invitation	Ulcer	Other: plasma-derived exosomes	Early Phase 1	Japan	https://ClinicalTrials.gov/show/NCT02565264
25	Effect of Microvesicles and Exosomes Therapy on β-cell Mass in Type I Diabetes Mellitus (T1DM)	Unknown status	Diabetes Mellitus Type 1	Biological: mesenchymal stem cells exosomes.	Phase 2 | Phase 3	Egypt	https://ClinicalTrials.gov/show/NCT02138331
26	Edible Plant Exosome Ability to Prevent Oral Mucositis Associated With Chemoradiation Treatment of Head and Neck Cancer	Active, not recruiting	Head and Neck Cancer | Oral Mucositis	Dietary Supplement: Grape extract | Drug: Lortab, Fentanyl patch, mouthwash	Phase 1	United States	https://ClinicalTrials.gov/show/NCT01668849
27	Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue	Active, not recruiting	Colon Cancer	Dietary Supplement: curcumin | Dietary Supplement: Curcumin conjugated with plant exosomes | Other: No intervention	Phase 1	United States	https://ClinicalTrials.gov/show/NCT01294072
28	Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Completed	Non Small Cell Lung Cancer	Biological: Dex2 Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Phase 2	France	https://ClinicalTrials.gov/show/NCT01159288

From a regulatory point of view, EV-derived products are medicinal products (discussed in section 2). Developer's final goal will be to apply for marketing authorization, which will allow both the wide availability and valuation of the product. To do so, developers will have to demonstrate that their EV-based product fulfills all requirements of quality, safety, and efficacy. This may be challenging because of the

variety and complexity of EV products. We believe that industrial developers need more information to understand how they can address the regulatory agencies' concerns.
Essential questions emerge: 1) How should the development of EV-based products be conducted to comply with existing regulatory frameworks? 2) How could the regulatory framework provide developers with further guidance adapted to the particular characteristics of EV-based products?

We attempted to address these issues collectively and prompted creating the group "Extracellular Vesicle translatiOn to clinicaL perspectiVEs – EVOLVE France" in 2020. Based on complementary scientific, technical, and medical expertise, we provide EV-specific recommendations for manufacturing, quality control, analytics, non-clinical development, and clinical trials. These recommendations are provided on an indicative basis exclusively reflecting our point of view as researchers/clinicians. Of note, these recommendations are neither a checklist nor a regulation and should be considered on a case-by-case basis. The requirements for early phase clinical trials are especially emphasized as it meets the immediate needs in the field. The main contents of the investigational medicinal product dossier (IMPD), marketing authorization applications, and critical guideline information are discussed to understand the transition from research to clinical development and ultimate market authorization.

This position paper may help developers and regulatory experts interested in the EV field to understand EV-based products' characteristics and the scientific and technological challenges associated with them and how they can impact the demonstration of product quality, safety, and efficacy. Finally, we believe that specific guidelines are required for EV-based products. Together with previously published reports, this paper may provide valuable information and advice for designing such documents.

2. Outlining the diversity of EV products and current regulatory classification

EV therapeutic products are complex and they depend highly on the type of cells used for production, the existence or not of modification of their content and their formulation. This chapter summarizes the main technical options currently proposed and their consequences in terms of regulatory classification.

2.1. Defining the cell source

EVs from various origins, such as humans, animals (tissues/body fluids), plants, or microorganisms, are currently being studied. However, we will focus here on EV sources for which future clinical uses in humans are more advanced: EVs derived from human cells.
The EV source affects many steps of the development, manufacturing, and control strategy of the process and the final product, impacting complexity. The critical strategic choices to make are: (i) primary cells or cell lines? (ii) Autologous or allogeneic cells? (iii) Native or modified (primed or genetically manipulated) cells?

2.1.1 Primary cells or cell lines?

EVs can be derived from cell lines or primary cells. GMP-compliant cell lines are commercially available; some are routinely used in Europe to produce biological medicinal products, such as recombinant proteins and vaccines. Mammalian cells (CHO, NS0, or Sp2/0), have been extensively used, however, human cells are often used in newly developed products. Marketing authorizations have been obtained for recombinant proteins produced in HEK293, HT-1080, or PER.C1 cell lines [19]. These cell lines may be of interest to produce a recombinant molecule for EV-mediated delivery. However, unmodified primary human cells are currently the main sources of EVs in clinical applications, particularly mesenchymal stromal cells (MSCs) and dendritic cells (DCs). This trend might change soon considering many different cell types are currently under investigation.

2.1.2 Allogeneic or autologous cells?

Both autologous and allogeneic cells can be used to produce EVs. This is a critical choice in somatic cell therapy, associated with different risks (immunogenicity, tumorigenicity, etc.) and constraints. The consequences are substantially different when it comes to EVs since they cannot replicate. Advantages

and limits of allogeneic and autologous cells are summarized in the Supplementary Table 1. Today, allogeneic cell sources appear to be the best option in most cases. On one hand, the use of allogeneic cells enables a large-scale manufacture of reproducible EV product, and on the other hand, it unlocks the untapped potential of making an off-the-shelf product.. Conversely, the autologous cell option is interesting for personalized medicine approaches.

The allogeneic strategy requires the manufacturing of large cell stocks or banks. The term "cell stock" is used for primary cells, whereas the term "cell bank" refers to cell lines (EMA/CAT/852602/2018 [20]). Master and working cell banks or stocks are designed for further EV production. This amplification in culture involves cell proliferation and passage steps (see Section 3.1) that could hasten replicative senescence [21] and reduce EV functionality [22]. Another aspect to be managed is the immunogenic potential of allogeneic products. Antigen-presenting cells and others can transfer major histocompatibility complex (MHC) molecules to recipient cells, in part via the secretion of EVs [23, 24]. Fortunately, low immunogenicity has been observed in studies investigating iterative injections of human EVs in immunocompetent mice [25, 26]. However, further clinical studies are necessary to validate the absence of an immune response to EVs derived from most if not all cell types, including immune cells.

2.1.3 Native or modified cells?

EVs from native unmodified cells

Many studies, including clinical trials, have focused on EVs derived from native, unmodified cells. Applications for tissue repair are under intense scrutiny, with substantial improvements in cardiac, cutaneous, lung, bone, and joint injuries. MSC-derived EVs remain at the forefront of these studies [7, 8, 27], but other cells seem promising [28, 29]. From a product development and regulatory perspective, using native EVs originating from unmodified cells may be the most manageable situation, as developed in Section 3.

However, there are cases where substantial modifications of the cells are desired to ensure the cell source's stability or to induce a selected molecule's expression at the surface or inside the EV.

Transient modification of EV-producing cell behavior (cell priming)

In somatic cell therapy, cell "priming" or "licensing" is commonly used. Classical priming methods consist of stimulation with inflammatory cytokines, growing the cells under hypoxia, or applying mechanical stress. Such stimuli can modify the contents of released EVs and their *in vitro* or *in vivo* functions [30, 31]. This is a transient modification of naturally occurring EV rather than EV engineering.

Stable modification of EV-producing cells

Cell modifications often aim to stabilize the cell source by immortalizing primary cells, introducing viral genes or oncogenes that regulate the cell cycle or manipulating human telomerase reverse transcriptase (hTERT). EVs can also be produced by cells differentiated from induced pluripotent stem cells (iPSC) [32-34] or by iPSC themselves [35, 36]. The first benefit of such approaches is that the cells can be amplified for many passages without major replicative senescence, thereby stabilizing the cell source. This could significantly improve batch consistency, which is a critical element of biological medicinal products. However, the risks and challenges of cell immortalization should be identified based on the type of transformation used and gene transfer type (see section 3.1). Specific controls of immortalized cell banks should address these risks.

2.2. Pre-production and post-production modifications of EVs content

EVs can be engineered in a pre-production or post-production step to convey natural or chemical molecules that improve their specific targeting or therapeutic properties. Moreover, EVs can encapsulate therapeutic molecules to protect them from degradation and potentiate their effects while minimizing their toxicity [12, 13].

Cells can be engineered to overexpress a naturally occurring molecule in EVs. Specific constructs designed by the fusion of a cargo (protein or peptide) to an EV-enriched protein [16] can be used to

address the cargo to the EVs. Other approaches aim to express membrane proteins, such as receptors, at the EV's surface to specifically target a cell type [37]. This implies a stable transfection of the cells, and modified EVs that contains the product of the transgene. The consequences in terms of product development are that specific risks due to the vector used for cell preparation (viruses) and/or to the presence of the transgene must be identified and relevant control strategy for the cell banks/stocks and the product should be implemented. Non-genetic modifications of the cells have also been proposed, such as loading the EV using the cell's natural capacity to take up exogenous material and drugs [38]. This strategy is performed after cell banking, in contrast to genetic modifications. Numerous methods and approaches have been described to modify EVs post-production by loading specific proteins, RNA, mi/pre-miRNA, or drugs as recently reviewed [39]. Here, the active substance should be clearly defined.

2.3. EV formulation

Depending on the administration route, one or more excipient(s) will be added during or after EV isolation to improve biodistribution. Excipients could be cryoprotectants, buffers, or synthetic matrices. For instance, EVs may be associated with hydrogels to facilitate delivery and retention at the site of interest while providing a combined mechanical effect [40]. The excipient is part of the final product and is considered in the development, with specific controls. Further, product classification depends on the role of the excipient/biomaterial in the therapeutic effect.

2.4. Administration and delivery of EVs

Many routes of administration can be used. This will affect both the choice of the formulation (injectable, integrated into biomaterials..) and isolation methods. Therefore, the choice of delivery route should be defined as early as possible and reassessed based on pre-clinical data.
Local administration may be advantageous for delivering EVs at the site of interest, limiting systemic circulation. Local treatments are currently studied, perhaps because much of the literature and trials focus on lesions well defined in space, such as in tissue repair or cancer. Many local administration routes can be proposed, ranging from topical administration to more complex radiologic, ultrasound, or endoscopy-guided routes. In case the site of action of EVs is not known, an administration strategy other than topic should be considered. Systemic administration has been widely used in pre-clinical models. It is a critical option if EVs target the immune system or are engineered to gain homing properties, allowing them to target specific tissues. Clinical trials using the intravenous administration route have been conducted in the field of cancer (NCT03608631), dementia (NCT04202770), or COVID-19 (NCT04493242).

2.5. Regulatory categorization depends of product complexity

An overview of EV product complexity is provided in **Figure 2**. The impact on product development is further discussed in Section 3.1. on producer cells; Section 3.7. on biomaterials in the finished product, and Section 3.8. on the particularities of engineered EVs containing a transgene product or drug.

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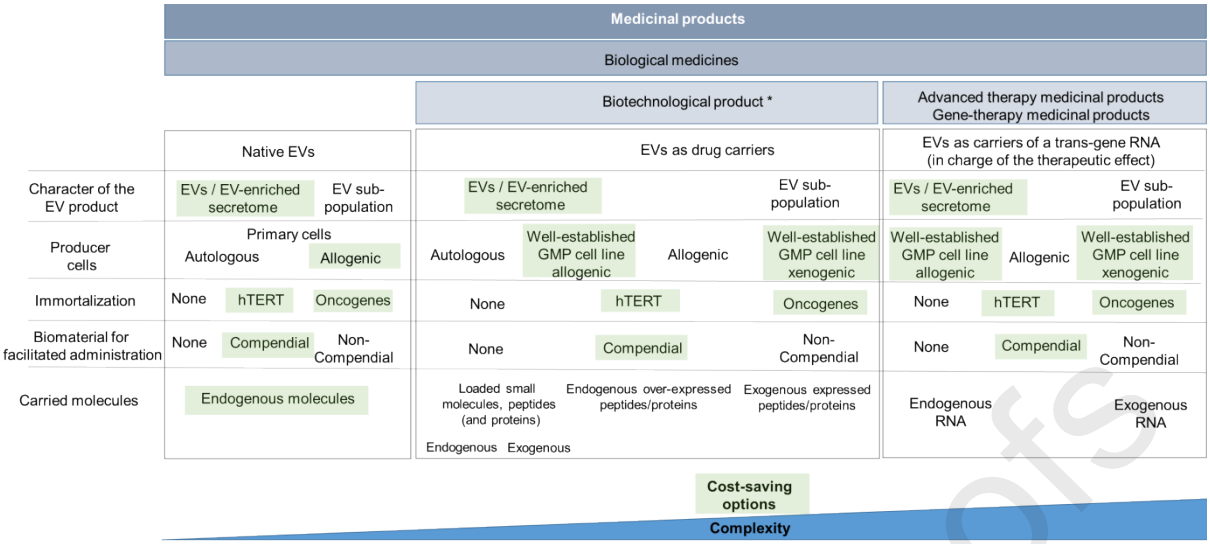


Figure 2: *Overview of EV-based medicinal products considering category, complexity, and cost-saving aspects. *The exception for biotechnological products in the scheme are the EVs loaded by methods other than genetic engineering approaches with peptides, proteins, and small molecules.*

2.6. EV-based medicinal products: where we are in the regulatory landscape

Some firms or clinics recently proposed "exosome" treatments with no clear regulatory framework, leading the US FDA to publish a public safety notification on exosome products in 2019 [41] and a Consumer alert on regenerative medicine products including stem cells and exosomes in 2020 [42]. The classification of these products is however clear: they should be considered medicinal products.

In Europe, the definition of a medicinal product is given in Directive 2001/83/EC [43]: "Any substance or combination of substances presented for treating or preventing disease in human beings. Any substance or combination of substances which may be administered to human beings with a view to making a medical diagnosis or to restoring, correcting or modifying physiological functions in human beings is likewise considered a medicinal product." The European Medicine Agency (EMA) glossary further states that such products act "by exerting a pharmacological, immunological or metabolic action." The EV-based therapeutic products, under development or to be developed, correspond to this definition. After extractive proteins, recombinant proteins, and cell- or tissue-based therapies, EVs emerge as the next generation of cell-derived therapeutics. Within the medicinal products framework, EV-based products are categorized as "biological medicinal products" and defined as following: "A biological medicinal product is a product, the active substance of which is a biological substance. A biological substance is a substance that is produced by or extracted from a biological source and that needs for its characterization and the determination of its quality a combination of physicochemical-biological testing, together with the production process and its control" (Directive 2003/63/EC)[44].

The subcategorization of EV-derived products will take into account their complexity and active substance, as proposed in an ISEV position paper [45]. EVs originating from unmodified primary cells are simpler products that belong to the biological medicinal product category, without having any further subcategory. The same could apply to EVs from genetically modified cells that do not contain a transgene product. Contrastingly, the ISEV position paper anticipated that EVs originating from genetically modified cells that contain a transgene product could be considered gene therapy products (GTP), a subclass of advanced therapy medicinal products (ATMP) [45]. Two recent recommendations of the EMA on the classification of ATMP support this view: in 2018 and 2021, the committee for advanced therapy (CAT) recommended that EVs containing recombinant RNA (mRNA and miRNA) should be considered gene therapy products [46, 47]. The CAT considers that the products fall within the definition of GTP (Directive 2001/83/EC, Annex I) [43], because they contain recombinant nucleic acids and that the effects of the products directly relates those molecules. It is currently unclear whether EVs containing

recombinant peptides or proteins would be considered GTP, we consider that it is not the case and that they should be considered biotechnological products (as recombinant proteins).

In any case, the active substance and mode of action will be decisive for the regulatory classification and therefore defining them should be a central concern during the product development, even if it may be very challenging and perhaps not fully elucidated for the first clinical testing [45, 48].

A particular case should be mentioned. If EVs are formulated with biomaterials that are responsible for the main therapeutic effect (EVs showing only an ancillary effect), the classification could shift from a biological medicine to a Class III medical device as described in the recent Regulation (EU) 2017/745 [49]. The regulation states that a medical device "does not achieve its principal intended action by pharmacological, immunological, or metabolic means, but which may be assisted in its function by such means." It should be noted that, from a development point of view, EV-related requirements are the same as those of any biological medicinal product.

In Europe, the marketing authorization of biological medicinal products can be under the EMA's responsibility through a centralized procedure (for biotechnological products and ATMP, for example), or sometimes under the responsibility of member states' agencies (for naturally derived biologicals).

3. Manufacturing process and control: Quality

The manufacturing process and control issues that constitute the "Quality" part of the application dossier for European marketing authorization, or the IMPD for clinical trials, should comply with the Common Technical Document (CTD) Module 3, as described in the ICH Topic M4Q guideline [50]. We display the CTD module 3 contents in **Table 2.** This table also shows our selection of the main guidelines we considered relevant for EVs taking into account that some guidance related to ATMPs may be appropriate for EVs. According to CTD Module 3 [50], a flow chart indicating sequential process steps, entry points for materials, relevant process parameters, and in-process testing should be provided, together with a detailed narrative description. In this document, the manufacturing process and related controls are divided into two parts: drug substance (active substance) and drug product (finished product).

Table 2: *Presentation of CTD module 3 content (ICH Topic M4Q) [50] for the manufacturing and control part named "quality." Our selection of guidelines relevant for EV-based products for clinical trials is indicated in the right column.*

CTD Module 3 content	Our selection of general relevant guidelines for EV-based products
"DRUG SUBSTANCE	
General information - Nomenclature - Structure - General Properties	EMA/CAT/852602/2018* [20] EMA/CHMP/BWP/534898/2008 [52] ICH Topic M4Q [50]
Manufacture - Manufacturer (name, address, and responsibilities) - Description of Manufacturing Process and Process Controls (flow diagram) - Control of Materials - Controls of Critical Steps and Intermediates - Process Validation and/or Evaluation - Manufacturing Process Development	EMA/CAT/852602/2018* [20] EMA/CHMP/BWP/534898/2008 [52] ICH Q5D [53] CPMP/BWP/3088/99 [54] EMA/CHMP/BWP/814397/2011 [55] EMEA/CHMP/BWP/398498/05 [56] EMEA/410/01 [57] EMA/CHMP/BWP/706271/2010 [58] GMP guidelines annex 13 [59] ICH Q9 [51] EMEA/CHMP/SWP/28367/07 [60] ICH Q5E [61]
Characterization - Elucidation of Structure and other Characteristics	ICH Topic Q6B [62]

- Impurities	EMA/CHMP/BWP/534898/2008 [52] EMEA/CHMP/BWP/398498/05 [56] ICH Topic Q5A (R1) [63]
Control of Drug Substance - Specification - Analytical Procedures - Validation of Analytical Procedures - Batch Analyses - Justification of Specification	ICH Topic Q6B [62] EMA/CHMP/BWP/534898/2008 [52] ICH Q2A [64] ICH Q2B [65] EMA/CAT/852602/2018 [20]
Reference Standards or Materials	EMA/CHMP/BWP/534898/2008 [52]
Container Closure System	EMA/CHMP/BWP/534898/2008 [52]
Stability - Stability Summary and Conclusions - Post-approval Stability Protocol and Stability Commitment - Stability Data	EMA/CHMP/BWP/534898/2008 [52] ICH Q5C [66]
DRUG PRODUCT	
Description and Composition of the Drug Product	EMA/CAT/852602/2018* [20] EMA/CHMP/BWP/534898/2008 [52] ICH Topic M4Q [50]
Pharmaceutical Development (manufacturing process, container closure system, microbiological attributes and usage instructions) - Components of the Drug Product - Drug Product (formulation development; overage justification if any; physicochemical and biological properties; manufacturing process development; container closure system; microbiological attributes; compatibility)	EMA/CAT/852602/2018* [20] EMA/CHMP/BWP/534898/2008 [52] ICH Topic M4Q [50]
Manufacture - manufacturer; - batch formula, - description of manufacturing process and process controls; - controls of critical steps and intermediates); - process validation and/or evaluation	EMA/CHMP/BWP/534898/2008 [52] GMP guidelines annex 13 [59] EMA/CAT/852602/2018* [20] ICH Topic Q6B [62]
Control of Excipients - Specifications - Analytical Procedures - Validation of Analytical Procedures - Justification of Specifications - Excipients of Human or Animal Origin - Novel Excipients	EMA/CHMP/BWP/534898/2008 [52] EMEA/CHMP/BWP/398498/05 [56] EMEA/410/01 [57] EMA/CHMP/BWP/706271/2010 [58] EMA/CAT/852602/2018* [20]
Control of Drug Product - Specification(s) - Analytical Procedures - Validation of Analytical Procedures - Batch Analyses - Characterization of Impurities - Justification of Specification(s)	EMA/CHMP/BWP/534898/2008 [52] EMA/CAT/852602/2018* [20] ICH Topic Q6B [62] ICH Q2A [64] ICH Q2B [65]
Reference Standards or Materials	EMA/CHMP/BWP/534898/2008 [52]
Container Closure System	EMA/CHMP/BWP/534898/2008 [52]
Stability - Stability Summary and Conclusion - Post-approval Stability Protocol and Stability Commitment - Stability Data	EMA/CHMP/BWP/534898/2008 [52] ICH Q5C [66]
APPENDICES	

Journal Pre-proofs

A.1 Facilities and Equipment	Considered "Not applicable" for biological investigational medicinal products in clinical trials according to EMA/CHMP/BWP/534898/2008 [52]
A.2 Adventitious Agents Safety Evaluation	ICH Topic Q 5 A (R1) [63] EMEA/410/01 [57] EMEA/CHMP/BWP/398498/05) [56]
A.3 Excipients (novel excipients)	EMA/CHMP/BWP/534898/2008 [52]
A.4 Solvents for reconstitution and diluents"	This appendice is not in the ICH Topic M4 Q. However, it is recommended by EMA/CHMP/BWP/534898/2008 [52]

** While EVs do not necessarily fulfill the definition of ATMPs, the underlying scientific principles outlined in this guideline may be applicable.*

We present an overview of the typical sequential steps for EV-based product manufacturing in **Figure 3**: cell culture (typically starting with one or more vials of the cell bank), cell priming (if any), an EV-secretion step, EV harvest, EV purification, formulation, filling and finishing, storage, and shipping. In this simplified workflow overview, the "Drug substance" steps start with the cell substrate, and the "Drug product" steps begin with the formulation. Key aspects related to the cells, raw material, manufacturing process, and control will be discussed below according to the CTD Module 3 [50] data requirements.

For all the manufacturing steps (and beyond), a risk-based approach (evaluation of probability and severity) is an essential issue in EMA/CAT/852602/2018 [20] and ICH Q9 [51]. The risk-based approach starts by identifying the risk related to the unfavorable effects that the product may elicit in patients (*that is,* immunogenicity, toxicity, and treatment failure) and identifying the risk factors to be addressed (origin of cells, level of cell manipulation, aspects of the manufacturing process, etc.). The next step consists in mapping the related data/knowledge available to identify risk factors and risk relationships. The overall risk management strategy, including risk control, is detailed in ICH Q9 [51]. Quality risk management can be applied to evaluate suppliers, manufacturers, starting materials, critical process parameters, and other aspects.

The next subsections will focus on the manufacturing process from the starting material to the final product. EV-related specificities will be outlined and some recommendations will be provided. Some general information of interest (but not specific to EV-based products) on raw material, reference standards or materials, container closure system/ storage conditions, stability and final product is provided in the **Supplementary Box 1**.

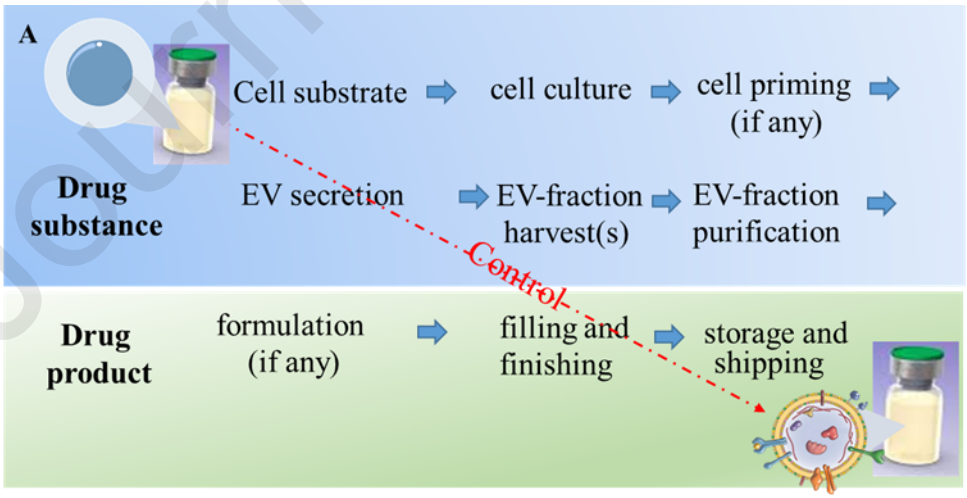


***Figure 3**: Simplified workflow of the main steps related to EV-based product manufacturing.*

3.1. Starting material (producer cell source)

EV-based products may be produced from autologous or allogeneic donated cellular materials (cells or tissues) from single or multiple donors. According to investigational ATMP guidelines (EMA/CAT/852602/2018) [20], three different strategies can be identified: (i) primary cells used directly, (ii) primary cells cultured for a few passages (cell stocks), and (iii) cells based on a well-defined cell bank system consisting of a master cell bank (MCB) and a working cell bank (WCB). An equivalent banking approach can be considered for cell stocks from primary cells. Other possibilities include genetically modified cells. Immortalized cells are attractive candidates, and as previously pointed out, their use may significantly improve batch consistency by reducing replicative senescence. Oncogenes (c-myc, SV40 or EBV T antigen) or hTERT could be used. The risks and challenges of EVs produced by immortalized cells should be identified based on the type of cell transformation used and gene transfer type. Recommendation R 3.1 relates to this issue.

Recommendation R 3.1:
Considering the choice of the immortalization strategy for EV-producing cells, safety remains the main issue. When possible, we recommend considering non-tumorigenic immortalization approaches.

Additional documentation and testing levels must be considered based on the different possibilities (allogeneic/autologous options and the choice of primary cells, cell lines, or genetically modified cells). These data are listed in **Supplementary Table 2,** based on the ICH Q5D data [53]. For instance, the use of genetically modified (transformed) cells requires additional analysis of the coding sequence's consistency for the expression construct to determine the subculturing limit. When applicable, gene integrity, replication-competent virus screening, expression and stability, residual vector, or nucleic acids should be analyzed according to the note for guidance CPMP/BWP/3088/99 [54]. If the product's therapeutic effect is ascribed to the transgene product, guidance for the starting material is provided at EMA/CAT/852602/2018 [20] in the investigational gene therapy product sections.

A two-tiered cell bank approach for a cell line, in which the MCB is used to generate the WCBs, is considered the best strategy to enable a consistent and continued product manufacture. The same two-tiered approach can be used for cell stocks (primary cells) with the exact same requirements in characterization and qualification. The advantage is to start from a well-characterized common source to prepare each production batch (ICH Q5D) [53].

Manufacturers should expose their strategy to enable a continued supply of cells from their cell bank(s), including the expected intervals between the generation of new cell bank(s) and the criteria to be considered to qualify cell bank(s) (ICH Q5D) [53]. It is essential to define a limit in terms of the number of population doublings. According to the ICH Q5D [53], the population doubling level at which senescence occurs should be determined for diploid cells. Recommendation R 3.2 attempts to address this issue considering EV-producer cells.

Recommendation R 3.2:
We recommend examining the effect of the cell amplification process on the quantity and quality of the EVs produced (independent of senescence). This corresponds to the cell stability evaluation, which implies "appropriateness for intended use in production," as indicated in ICH Q5D [53]*. In our opinion, cell stability evaluation should be the main point that defines the cell sub-cultivation (amplification) limit.*

EV pooling or cell pooling strategies may be considered when the size of the bank is limited. According to the ATMP guidelines EMA/CAT/852602/2018 [20], cell pooling should be avoided. A donor cell could become more dominant, with a risk for product consistency. The EMA/CHMP/BWP/187338/2014 [67] states that suitable pooling strategies can ensure product consistency in multiple harvests originating from one cell culture. A similar approach could be proposed for EV harvests originating from different cell cultures including for cells from different donors, as addressed in Recommendation R 3.3.

Recommendation R 3.3:
Our recommendation is to optionally pool equivalent EV-based products from different cell stocks (primary cell from different donors) after the manufacturing step while maintaining traceability, rather than pooling cells from different cell stocks.

3.2. Manufacturing steps and in-process control

In biological products, the process largely defines a product. EV-based products are very new and complex. The mode of action, and precise nature of EV's active substances are poorly understood. This is especially true in the case of native, but not engineered EVs. The influence of process robustness on their quality attributes is not known. Therefore, it is imperative to build quality into the process and not only rely on product quality in the final release testing specification, which is only an element of the control strategy. According to ICH Topic M4Q [50] in-process control and acceptance criteria should be set throughout the manufacturing process. Each step related to EV-based product manufacturing is briefly discussed below. Besides, we present a set of recommendations for in-process control and testing (**Table 2**) for each manufacturing step.

Cell culture step

The first step in the manufacturing process (**Figure 3**) is the culture of the producer cells for amplification in order to obtain enough cells for EV production. Currently, different cGMP culture platforms [68] may be used for cell culture (and for EV secretion), such as flasks, hyperflasks, and bioreactors (hollow fiber, fixed bed, or stirred tank). They are overviewed in another paper of this theme issue [69]. To some extent, the control strategy will depend on the culture platform used. Of note, our recommendation for control during the cell culture step R 3.4 and **Table 3** considers this point and also takes into account that extensive testing was performed on the cell-substrate in terms of identity, stability, and safety from a qualified MCB (the most widely used approach).

Recommendation R 3.4:
Regarding the culture step for cells from a qualified MCB or cell stock, we recommend, for instance, performing a metabolic activity test on a monitoring basis, rather than using acceptance criteria during cell culture. In particular, an in-line analysis of glucose and lactate levels should be performed when technically applicable. We recommend cell counting to be conducted if technically feasible (depending on the cell culture platform, this may not be feasible for instance with hyperflasks and hollow fiber bioreactors). We also recommend performing a risk analysis to identify critical process parameters and set manufacturing controls as well as acceptance criteria in accordance with quality-by-design approach ICH Q8 R2 [70], EMA/CAT/852602/2018 2018 [20, 71] *and ICH Q9* [51]*, especially for phase III clinical trials, using data from pharmaceutical development studies (before clinical batch production).*

Cell priming step

Priming strategies have been investigated to modulate the potency of cells and EVs [72, 73]. Cell priming may be conducted at the end of the cell culture step, for instance, via cytokine incubation and removal before the EV secretion step. Particular attention should be paid to the undesirable co-purification of cytokine contaminants with EVs. The elimination of the priming agent is addressed in the Recommendation R 3.5.

Recommendation R 3.5:
We recommend the in-process determination of the concentration of priming molecules that remain in the conditioned medium, at the end of the priming procedure after washing off the substance, only in case the clearance capacity efficiency of the purification step is not satisfactory or not sufficiently validated. Otherwise, acceptance ranges for the residual concentration of priming molecules should

be set only for the drug substance and finished product.
Additionally, we recommend the same control and testing strategy for the culture step (R 3.4).

EV secretion step

EV secretion may be spontaneous or induced. These strategies are overviewed in another paper of this theme issue [69].

- Spontaneous EV secretion

Spontaneous EV production may be chosen to preserve the basal cell characteristics. In this case, the complete cell culture medium is replaced by an EV-deprived medium. Although this may affect cell viability, it enables to minimize the co-purification of EVs present in the serum or platelet lysate [6]. Additionally, other particles like protein aggregates are also present in the serum or platelet lysates. Recommendation R 3.6 relates to this issue. The term "particle" is used herein in a non-specific way for protein aggregate or EVs when we cannot discriminate them.

Recommendation R 3.6:
When the EV secretion step is performed in the presence of sera or platelet lysates, we recommend, as a prior step, maximal removal of EVs/particles contributed by sera or platelet lysates and the quantitative documentation of the concentration of residual particles. Thereby, the residual particle content should be a quality attribute of this raw material.

***Table 3**: Our recommendations, on an indicative basis, about in-process control and in-process testing strategy for each manufacturing process step of EV-based products. This should be combined with a risk analysis to detect any critical process parameters according to ICH Q9* [51].

Step	In-process control	In-process testing (quality attributes)
Upstream process		
Cell culture	Metabolic activity test on a monitoring basis rather than as acceptance criteria: at-line monitoring analysis of glucose and lactate levels, when applicable (In-line pH, temperature, pO2, glucose and lactate levels, cell culture duration with acceptance criteria as a function of risk analysis, especially for phase III clinical trials)	We recommend cell counting to be carried out if technically feasible
Cell priming		Residual priming molecule concentration (if any) in the conditioned medium after washing We recommend cell counting to be carried out if technically feasible
EV secretion		Control the initial particle baseline concentration as well as particle concentration and size distribution at the end of the EV secretion step as acceptance criteria We recommend cell counting to be carried out if technically feasible. It is of interest to document EV yield per cell considering, if possible, cell count at the beginning of the EV secretion step or, otherwise, cell counting during seeding in the cell culture step In case a chemical substance or biomaterial agent is used for enhancing EV release, acceptance ranges for the residual agent concentration after the EV secretion step should be set

Downstream process		
EV harvest	If multiple harvests, the metabolic activity test on a monitoring basis, rather than as acceptance criteria: at-line monitoring analysis of glucose and lactate levels before each harvest. (In-line pH, temperature, pO_2, glucose and lactate levels, cell culture duration with acceptance criteria as a risk analysis function, especially for phase III clinical trials).	If multiple harvests, our recommendation is to pool them (pooling can be performed after isolation and storage) and analyze particle concentration and size distribution. We recommend determining particle concentration increment indicating the baseline particle concentration as well as particle concentration and size distribution at harvest time for each harvest as acceptance criteria. This can also make it possible to eliminate a harvested batch if the concentration is insufficient or if the size distribution is atypical.
Isolation step	(Protein load, concentration fold of the volume, load/wash conductivity, elution pH, operating pressure in case tangential flow filtration is used and as a function of risk analysis)	Analysis of the ratio particle concentration/micrograms of protein, as well as particle size distribution.

- Induced EV secretion

Cell culture under starvation [74] is the most straightforward strategy to collect EVs. Indeed, platelet lysate or serum removal during the EV secretion step reduces the presence of particle impurities. Of note, starvation may impact producer cell physiology and consequently EV content and quality, with a relative increase in apoptotic bodies. Moreover, limited cell survival duration, may decrease EV production.

EV secretion may be triggered by stimulating factors such as cytochalasin B [75]. These approaches are overviewed in another paper of this theme issue [69]. In all cases, these strategies will introduce additional complexity, which may impact the control strategy as addressed in the Recommendation R 3.7. Approaches for induced EV secretion will need to be proven safe and cGMP. The co-purification of stimulating agents with EV should be avoided.

Recommendation R 3.7:
After EV secretion is complete, we recommend setting acceptance ranges for residual concentrations of relevant stimulating agents used to induce EV release only in case the clearance capacity efficiency of the purification step is not satisfactory or not sufficiently validated.

As expected, the main in-process test for EV secretion relies on particle analysis as indicated in the Recommendation R 3.8.

Recommendation R 3.8:
We recommend controlling the baseline particle concentration and size distribution before and at the end of EV secretion as acceptance criteria.
The strategy described for cell culture is also recommended here (R 3.4.)
We recommend documenting EV production yields per cell, and when technically feasible, considering cell counts at the beginning of EV secretion or cell counts during seeding for cell culture.

EV harvest

Depending on the strategy used for EV secretion, producer cells may remain alive for several days and sometimes proliferate. Multiple harvests may thus be considered, raising the question of the variability between them. This risk can be managed by pooling multiple harvests and testing, as described in EMA/CHMP/BWP/187338/2014 [67]. We consider this may be applied to EV harvests as indicated in

the Recommendation R 3.9.

Recommendation R 3.9:
Our recommendation is to pool multiple harvests of EV-based product from the same producer cell batch (pooling can be performed after isolation and storage) and analyzing particle concentration and size distribution. We recommend determining the increment in particle concentration indicating the initial particle concentration baseline as well as particle concentration and size distribution at harvest time for each harvest as acceptance criteria. We also recommend combining this in-process test with the strategy described for the cell culture step (R 3.4.).

EV purification/enrichment
Different methods may be used to purify or rather enrich EVs [76]. A detailed description of these methods will be the focus of another paper in this theme issue [77]. The choice of a purification method should be based on the medium's volume to be processed and the analytic resolution targeted. For small volumes, ultracentrifugation using closed and sterile tubes is of interest. If a specific sub-population of EVs is the target, gradient ultracentrifugation would be more appropriate. However, in both cases, the main limitation is scalability. Tangential flow filtration (TFF) can be considered the method of choice for scalable EV concentration/purification; besides, adding a diafiltration step allows "washing" the EV fraction via buffer exchange. The diafiltration offers an additional advantage for formulation by introducing the excipient in the buffer or by using the final formulation buffer (cryoprotectants or others) [78]. A strategy of in-process control is suggested in the Recommendation 3.10.

Recommendation R 3.10:
Regarding in-process testing, we recommend analyzing the ratio of particles/micrograms of proteins as well as particle size distribution before and after purification. In the case of EV secretion under starvation or in protein-depleted medium in general, protein levels may eventually be very low and close to the quantification limits of current detection techniques because of the reduced presence of protein aggregate impurities. In these cases, we recommend the analysis of particle concentration and size distribution before and after purification.

Most isolation methods currently available only allow an enrichment of the secretome in EVs without eliminating all other soluble factors. In most cases, the therapeutic product will be composed of a continuum of different types of vesicles and a certain amount of soluble proteins that may participate in the final product's biological and therapeutic activity. EVs may also release their contents over time during product processing or storage, modifying EVs and soluble components' amounts. These aspects were taken into consideration in the Recommendation R 3.11.

Recommendation R 3.11:
The definition of the product should be precise and take into consideration the heterogeneity of the final preparation, including soluble factors. Often the term "EV-enriched secretome" could be proposed rather than "EVs."

3.3. Characterization

Characterization studies conducted throughout the development process (all steps before phase I clinical trial) will provide a comprehensive picture and knowledge of the EV-based product to allow appropriate choice of the control parameters in the manufacturing process. Characterization studies will be used to establish the product's specifications according to EMA/CHMP/BWP/534898/2008 [52]. Besides, according to the guidelines ICH Topic Q6B [62], based on characterization studies, it will be possible to select an appropriate subset of batch release methods by justifying selection choice.

According to the guidelines on investigational biologicals EMA/CHMP/BWP/534898/2008 [52],

characterization should include the determination of physicochemical and immunochemical properties, biological activity, purity, and impurities. Following this guideline, an ATMP guideline (EMA/CAT/852602/2018) [20], and MISEV guidance [6], we recommend using multiple approaches to characterize EV properties to increase the reliability of the results.

This position paper will not fully address all the existing methods used to characterize EVs. The reader may refer to recent reviews in the field, such as Shao *et al.* [79]. Nevertheless, several analytical methods, the related investigated parameters, method strengths, and weaknesses are provided in **Supplementary Table 3** for physicochemical properties and **Supplementary Table 4** for immunochemical properties. Our goal here is to discuss a specific set of characterization techniques allowing quantification and qualification during the development phase of EV-enriched secretome products in **Box 1**. These suggestions and the related Recommendations 3.12-14 are provided for the general guidance based on current available techniques. The suggested techniques, on an indicative basis, are neither exclusive nor exhaustive.

Recommendation R 3.12:
We recommend adapting the physicochemical characterization considering the particularities related to the EV-enriched secretome. We recommend considering the following tests (see Table 3 for the specific steps):
- Quantification: via particle quantification, for instance, using nanoparticle tracking analysis (NTA) combined with protein quantification;
- Physical properties: e.g., hydrodynamic diameter using NTA, for instance;
- Structure: e.g., via transmission electron microscopy (TEM) or cryo-TEM [80-82], for instance.

Concerning immunochemical characterization, ICH Topic Q6B [62] recommends investigating biological identity, homogeneity, purity, and quantity via immunochemical procedures (*e.g.*, ELISA, western blotting). Our recommendations regarding the evaluation of EV immuno-chemical properties and purity are indicated in the Recommendation 3.13 and 14, respectively. Biological activity will be discussed in Section 4.

Recommendation R 3.13:
We recommend investigating the immuno-chemical properties of EV-enriched secretome products (identity attributes) via an antibody-based technique such as western blotting, ELISA, flow cytometry (for instance using the MACSPlex Exosome Kit [83] or small particle flow cytometry or nano-flow cytometry [84]) or with ExoView [85]*, for instance. We also recommend the investigation of RNA and DNA content using capillary electrophoresis [86] or other methods.*

In 2019, members of four international societies (including the ISEV) concentrated efforts on defining MSC-EVs for therapeutic applications [87]. They proposed minimal information required for MSCs as EV-producer cells such as the ratio of MSC to non-MSC surface antigens, ratio of specific membrane lipids to proteins, ratio of two specific lipids, concentration of membrane lipid vesicles (detection of lipid particles after lipid labeling via fluorescence-augmented NTA) and the assessment of biological integrity of a MSC-EV preparation via the enzyme activity of surrogate proteins [87]. Rohde *et al* published their multimodal matrix for release testing of umbilical cord MSC-EVs [88]. This included the assessed parameters for producer cell characterization, as well as EV identity, purity and impurity, indicating the related ranges, marker profiles and test methods that were selected [88]. Although these two works focus on MSC-EVs, the general principles of their approaches can be proposed/adapted to EVs from other cell types. We consider that these initiatives are quite valuable. Our recommendations and the proposed control strategies in Table 2 and 3 are complementary to these previous papers.

Recommendation R 3.14:
We recommend investigating purity indirectly via the ratio of particle concentration/micrograms of proteins [89] and reporting the enrichment factor at the end of the purification process. When expressing relative purity in terms of specific activity (units of biological activity per mg of product), we recommend taking into consideration the fact that it can be method-dependent and that a single biological activity test will not cover the multiple biological effects of EV-enriched secretomes.

Box 1*: Our selection, technical considerations, and recommendations in the choice of characterization methods for the development phase, in-process testing, drug substance, finished product analysis, and stability studies. The steps at which these methods are applied are listed in Table 3.*

Particle quantification and hydrodynamic diameter analysis
Nanoparticle tracking analysis (NTA) allows single-particle measurement analysis. It is consequently assumed to be less prone to interference caused by aggregates or larger particles than the dynamic light scattering (DLS) technique. Moreover, this method was validated for the size determination of synthetic nano-particles following quality criteria if particles are superior to 50 nm [90]. NTA is widely used, facilitating inter-laboratory comparison. We recommend, for instance, the NTA method for particle quantification for the development phase, in-process control, drug substance analysis, stability studies, and finished product analysis.
The used NTA analysis parameters must be specified. They can significantly impact results: concerning the sample (concentration, media, etc.), method-specific settings, and data processing (*i.e.,* laser wavelength, camera level, concentration range, threshold value, temperature, number, and duration of videos). A standard operating procedure should be used to facilitate inter-users, inter-lab, inter-apparatus comparison. Validation following the guidance provided by ICH Q2A [64] and Q2B [65] is essential for NTA and the other analytical methods indicated herein in Box 1.
NTA, DLS, or tunable resistive pulse sensor (TRPS) cannot differentiate EV from non-EV particles (*i.e.,* lipoproteins, protein aggregates). Even if fluorescence detection associated with NTA could theoretically allow biomarker-specific detections, sensitivity is limited, especially for low-sized particles. Therefore, our suggestion is, for instance, to combine NTA with characterization methods involving EV marker detection (see immune-chemical characterization) for the development phase, drug substance analysis, stability studies, and finished product analysis.
Size and structure
To evaluate the structure and differentiate EVs from non-vesicle particles, transmission electron microscopy (Cryo)TEM is currently the most reliable method. (Cryo)TEM is particularly useful to characterize the content of EV samples with the advantage of being label-free. However, labeling may be performed via antibody-functionalized nanoparticles for biomarker detection [80-82]. (Cryo)TEM-based methods allow physical diameter analysis compared to hydrodynamic diameter (size + surrounding solvent molecules) for NTA [80-82]. Our suggestion is to perform (Cryo)TEM-based methods for size and structure characterization complemented to NTA during the development phase, for instance.
Surface charge
The zeta potential (ZP) is determined by the net electrical charge of molecules exposed at EVs' surface. Zeta potential of particles is one of the fundamental parameters known to affect dispersion and particle suspension stability. Its measurement can therefore help to investigate EV aggregation. The ZP of EVs is generally slightly negative (ζ EV ≈ -15/-10mV) [81, 91]. As it is the case for monitoring red blood cells stability [91, 92], one possibility is to monitor ZP to assess EV stability in the development phase even if this method has limitations.

Total protein quantification and purity

Detailed protocols to allow total protein quantification can be found in European Pharmacopoeia [93] and EV scientific papers [94]. Proposed methods include measurement of sample absorbance at 280 nm, colorimetric assays (Lowry, Bradford, micro-bicinchoninic acid, Biuret), and fluorimetric-based assays. To favor online characterization processes, if size exclusion chromatography is used, we recommend associating it with a UV detection at 280 nm. We suggest investigating total protein quantification for composition analysis by colorimetric assays and analyzing purity indirectly via the ratio of particle concentration/micrograms of proteins [89], for instance, for the development phase, in-process testing (if EV secretion step in complete medium), drug substance analysis, stability studies, and finished product analysis.

Total lipid quantification

Currently, total lipid quantification is less investigated than protein quantification. Indeed, high amounts of EV-enriched secretome are generally required to achieve lipid quantification. Although attenuated total reflection Fourier-transform infrared spectroscopy [95] and sulfovanilin assay [96] provide interesting results, no method is currently compatible with a reproducible analytic procedure with high sensitivity. Therefore, we suggest performing total lipid quantification only optionally for the development phase.

Immunochemical characterization

Based on MISEV, we recommend investigating the presence of some EV positive markers (Categories 1 and 2) [6], but we consider that the other categories are only optional. Western blot (WB) is a long-standing immunochemical analysis method. However, it requires high EV quantities and extensive processing. Even if WB is helpful at the lab scale, it is poorly adapted for in-process testing. High-performance tandem mass spectrometry could identify a panel of proteins (ranging from 700 to nearly 2000 proteins depending on equipment). This method and transcriptomic ones can be very informative for the development phase. However, they are costly, labor, and time-consuming for in-process testing and active substance and drug product analysis. In general, we suggest using techniques enabling multiplexing and requiring low EV quantities. This is the case of, for instance, MACSPlex Exosome Kit [83], which is based on bead immune-capture to enable 37 biomarker detection in conventional flow cytometers. Elisa kits are also of interest. However, both Elisa and MACSPlex Exosome Kit do not allow single EV analysis. In doing so, flow cytometers enabling the detection of small particles in the EV range should be used, which is quite complex. Guidance on this is provided by MIFlowCyt-EV [97]. Another possibility for single EV analysis is to use nanoflow cytometry [84] with appropriate detection limit for EVs (except large ones). Exoview method [85] is of interest for multiplex biomarker detection for single EVs with additional imaging capabilities. Although WB is considered an appropriate method, our suggestion is to use Elisa, MACSPlex Exosome Kit, Exoview, small particle flow cytometers, or nanoflow cytometry for the development phase, drug substance analysis, stability studies, and finished product analysis.

DNA content

Although DNA presence in EV cargo is currently discussed and probably in small quantity compared to co isolated or membrane adsorbed DNA molecules [98-100], the presence of various DNA species such as single-stranded (ss)DNA, double-stranded (ds)DNA, and mitochondrial (mt)DNA in the final product should therefore be analyzed. Following DNA isolation, size and concentration could be determined using automated electrophoresis tools such as the capillary electrophoresis system [88]. If present, DNA may also be an impurity when non-encapsulated into EVs. Therefore, our suggestion to discriminate the DNA content present in EVs from impurities is to perform DNA analysis with and without DNase treatment [101, 102]. This is our recommendation for the development phase, drug substance analysis, and finished product analysis.

RNA content

Except for nuclear RNA, the presence of cytoplasmic RNA of multiple types such as tRNA, miRNA, Y-RNA, mRNA, SRP-RNA, rRNA, lncRNA, piRNA, snRNA, snoRNA, and scaRNA has been reported for EVs [6, 86, 103, 104]. To date, multiple widely used methods (microarrays and RT-qPCR techniques), as well as the latest advanced methods such as small RNA seq, are available to analyze and characterize the presence of RNA in EVs. The identification of a specific miRNA may be performed depending on the involved mechanism of action. We suggest performing a capillary electrophoresis test to investigate RNA content in the development phase, drug substance analysis, and finished product analysis. RNA analysis can be performed with and without RNase treatment to discriminate the RNA content present in EVs from impurities (*i.e.,* extracellular protein-RNA complexes) [102, 105].

As mentioned above, according to the ICH Topic Q6B guidelines [62], based on characterization studies, it will be possible to select an appropriate subset of methods for batch release. Recommendation R 3.15 deals with this issue.

Recommendation R 3.15:
Our recommendation is that characterization studies should be as exhaustive as possible during the development phase; however, a pragmatic approach should be considered in order to select the most technically relevant parameters for in-process control and testing, drug substance analysis, stability studies, and finished product analysis, considering feasibility in terms of related cost, labor, and time efforts. This is in agreement with existing guidelines, such as ICH Topic Q6B [62] *and ICH Q9* [51].

3.4. Impurities

Impurities may be process-related and/or product-related. According to ICH Topic Q6B [62], process-related impurities are derived from the manufacturing steps, originating from cell substrates, cell culture (*i.e.,* antibiotics, serum, and other media components), or downstream processing (*i.e.,* column leachables). Product-related impurities (*i.e.,* degradation products) may appear during manufacture and/or storage, displaying properties that are not comparable to those of the desired product concerning activity, efficacy, and safety.

Our Recommendation R 3.16 concerns process-related impurities.

Recommendation R 3.16:
Residual fibrinogen levels may be detected in the final product, especially when the EV secretion step is performed in media containing platelet lysate or serum. We recommend analyzing this impurity, for instance, with an immunoassay, and setting upper limits in case the clearance capacity efficiency of the purification step is not satisfactory or not sufficiently validated.

According to EMA/CHMP/BWP/534898/2008 [52] and ICH Topic Q6B [62] concerning biological medicines or biologics, host cell proteins and DNA are considered process-related impurities. We consider that, to some extent, this does not apply to EV-based products, as indicated in Recommendation R 3.17.

Recommendation R 3.17:
We recommend considering host cell proteins as EV-based product attributes.
We recommend discriminating the encapsulated DNA content of EVs, which is a product attribute, from that of non-encapsulated DNA content, which reflects process-related impurities, by performing adequate DNA analyses, with and without DNase treatment [101, 102].

Degradation products are main product-related impurities according to ICH Topic Q6B [62]. Degradation products can notably come from vesicle disruption during storage, releasing the inner content of EVs. As soluble proteins are always present in EV-enriched secretome products, it is difficult to discriminate them from those due to vesicle disruption. Strategies are suggested in Recommendation R.18 and R.19 for degradation investigation in complement to the MISEV 2018 recommendation to determine the topology of EV-associated components by mild digestions and permeabilization studies.

Recommendation R 3.18:
We recommend adopting an indirect monitoring strategy for analyzing EV degradation for the drug substance and the final product based on particle concentration (using NTA, for instance) and the concentration of biomarker-positive EVs (for instance using ExoView, flow cytometry enabling small particle detection or nano-flow cytometry). This implies monitoring "intact" EVs rather than the occurrence of degradation products from EV disruption.

Recommendation R 3.19:
We recommend performing biophysical destabilization tests (for instance, hypo-osmotic stress sonication or freeze-thaw cycles that are known to destabilize membranes [2]) followed by quantification and hydrodynamic particle size analysis (using NTA, for instance). Despite the limitations of this approach, it may be informative to investigate degradation products.

According to ICH Topic Q6B [62], product aggregates are considered product-related impurities. We consider that this does not apply to EV-based products, as indicated in the Recommendation R.3.20. In the case of EVs, it is challenging to discriminate single large EVs from small EV aggregates, considering (i) the wide EV size range, (ii) current technical limitations, and (iii) the fact that aggregation is a dynamic phenomenon.

Recommendation R 3.20:
We recommend considering EV aggregates as product-related substances up to a size range that should be defined on a case-by-case basis. Therefore, we consider that there is no need to discriminate aggregates from single EVs that are in this size range. We recommend documenting the EV size range using two different methods: single particle characterization (NTA, for instance) and a biomarker-based analysis (for instance, ExoView, flow cytometry, or nano-flow cytometry). This recommendation applies to the analysis of the drug substance, stability studies, and analysis of the final product.

As purification increases, there may be a decrease in potency, as recently published [106]. Thus, it is reasonable to question as to what extent should impurities be reduced. An important issue is the potential beneficial role of impurities such as albumin, which may improve stability. In this regard, a relevant parameter to consider is batch consistency via molecular fingerprinting analysis. This is the issue of the Recommendation R 3.21.

Recommendation R 3.21:
We recommend not minimizing process-related impurities at all costs, but to characterize the most predominant impurities and set acceptable upper limits to ensure batch consistency.

The issue of contaminants is a stand-alone topic. Semantically, contaminants are unintentionally present chemical, biochemical, and/or microbial species that are not part of the manufacturing process in contrast with impurities, according to ICH Topic Q6B [62]. According to the European Pharmacopeia, standard tests for microbial contaminants include determining endotoxin levels [107], sterility [108], and the absence of mycoplasma [109]. Certified laboratories should perform these tests.

Concerning viral safety, ICH Topic Q6B [62] refers to the ICH guidelines Topic Q5A [63] concerning biologicals. Although the basic principles of ICH Q5A [63] apply to biologicals in clinical development, a guideline on virus safety issues dedicated to investigational biologics is available (EMEA/CHMP/BWP/398498/05 [56]. According to this guideline, which is most the relevant to EVs products, in addition to a risk assessment, viral safety evaluation should include (i) the testing of the cell banks or cell stock; (ii) the evaluation of biological raw materials (appropriate documentation should be provided to support their viral safety); (iii) testing for viruses in unprocessed bulk (with tests repeated if there is a significant change in production manufacturing such as a scale change); and (iv) via the

validation of virus reduction (by characterizing and evaluating processes for inactivating/removing viruses and also quantitatively estimating the reduction level of viral particles). Given the similarities between EVs and viral particles in terms of size and composition, we consider classical clearance steps traditionally performed for biopharmaceutical products (chemical inactivation step, size exclusion chromatography, or filtration) are inappropriate for EV-enriched secretome. Therefore, as for cell-based medicinal products, the viral safety relies on stringent sourcing and acceptance criteria of all biological products (EMEA/CHMP/410869/2006 [110]), as further commented in the Recommendation R 3.22.

Recommendation R 3.22:
We recommend adopting a viral security strategy based on the classic 3 pillars of ICH Q5A [63] *(selection and testing of the materials, virus elimination and product testing). When the implementation of a final virus elimination step is technically difficult/impossible to achieve, the control of the manufacturing process, the selection/testing of raw and starting materials, and the testing of the product at relevant manufacturing steps should be reinforced.*

3.5. Control of the active substance (specification, analytical procedures, and validation)

According to the EMA/CHMP/BWP/534898/2008 [52], the specifications required for the active substance are mandatory for the following quality attributes: (i) quantity, (ii) identity, (iii) purity, and (iv) microbiological quality. Upper limits for impurities should be defined. It is also mandatory to include a test for biological activity. This could be challenging for native EV for which the mode of action is probably complex and often not fully characterized. A recent publication has reviewed critical considerations for the development of potency tests [48]. Although this work focuses on MSC-derived small EVs, the general strategy proposed by the authors could apply to EVs originating from other cells.

As an indicative basis, the recommended selection of control tests for the drug substance is provided in the Recommendation R.3.23 and **Table 4**.

Recommendation R 3.23*:*
We recommend considering the following tests in order to set active substance specifications in general (additional tests will be required as product complexity increases; see R 3.26).
(i) Quantity tests by particle quantification and total protein quantification; (ii) identity tests by hydrodynamic diameter analysis of single particles, immunochemical characterization, DNA and RNA content; (iii) purity test by the ratio of particle counts/micrograms of protein, and (iv) contaminants (microbiological quality) according to the European Pharmacopoeia.
We recommend testing for impurities such as albumin or fibrinogen quantification (if the EV secretion step is performed in complete medium and in case the clearance capacity efficiency of the purification step is not satisfactory or not validated). DNA (and optionally RNA too) not encapsulated in EVs may be analyzed for impurity quantification by DNase (and RNase) treatment.
We recommend testing the biological activity on a monitoring basis using a potency test in vitro, if relevant, or else, in vivo.

Table 4*: Suggested selection of characterization analyses for the overall control strategy in general (additional tests will be required as product complexity increases—see R 3.26). Abbreviations: M, AC, and UL stand for: monitoring, acceptance criteria, and upper limits, respectively.*

Our suggested tests for	**Development**	**Clinical batch production**

the critical quality attributes and other required tests	phase	In-process control	Drug substance control	Stability test (drug substance and finished product)	Finished product control
Quantity attribute					
Particle quantification by NTA	M		AC	AC	AC
Total protein quantification by colorimetric assays	M	M	AC	AC	AC
Identity attribute					
Size and structure by TEM-based methods	M				
Hydrodynamic diameter analysis by NTA	M	M	AC	AC	AC
Immunochemical characterization by Elisa, MACSPlex Exosome Kit, Exoview, small particle cytometry or nanoflow cytometry	M		AC	AC	AC
DNA content (with/without DNase treatment)	M		AC		AC
RNA content (optionally with/without RNase treatment)	M		AC		AC
Purity attribute					
Ratio of particle counts/micrograms of proteins	M	M	AC	AC	AC
Impurity / contaminants					
Albumin or fibrinogen quantification (if EV secretion step in complete medium) DNA (optionally RNA) quantification with and without DNase (optionally RNase) treatment, as indicated above Priming molecule concentration (if relevant)	M		UL		UL

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Endotoxin, sterility and mycoplasma test according to the Eur. Pharm.) and virus testing (*in vitro* and/or *in vivo*)			AC		AC
Biological activity					
Potency tests *in vitro*	M		M	M	M
Potency tests *in vivo* (if any)	M				M
Others					
Appearance and description: physical state (eg., solid, liquid), color, etc.	M			AC	AC
General tests: pH and osmolarity	M			AC	AC

3.6. Stability

Following ICH Q5C [66] guidance, it is necessary to design a stability study to detect changes related to the product's identity, purity, and potency. Our selected methods for testing the stability of EV-based products are provided in Recommendation R3.24.

Recommendation R 3.24:
We recommend considering the following methods to investigate EV-based products stability: (i) particle hydrodynamic diameter and particle quantification; (ii) total protein quantification, as well as the ratio of particle counts/micrograms of proteins; (iii) immunochemical characterization; and (iv) an in vitro potency test.

3.7. The finished product (final product, medicinal product, or drug product)

The formulation of the EV-enriched secretome may play a decisive role in therapeutic outcomes. Preservation needs and administration strategies mainly drive the formulation choice.

Cryoprotectants may be added to the formulation such as sugars, diols, and amino acids, as reviewed elsewhere [111]. Depending on the administration route and therapeutic goals, the EV-enriched secretome may be associated or formulated with different excipients. For instance, they may be incorporated into a biomaterial for controlled spatiotemporal release with compendial and/or non-compendial excipients. The choice of a biomaterial/excipient approved for clinical use or listed in the pharmacopeia is of interest. It is necessary to provide data on EV release kinetics under the administration route's physiological conditions. Furthermore, when associating the EV-enriched secretome with a biomaterial/excipient before the storage, it is essential to provide data on EV stability during storage. EV-enriched secretome dilution with the biomaterial can be performed immediately before use (in case this can be done by the clinician) or some hours before use (in case the mixing should be performed by the hospital pharmacy, for instance). According to the EMA/CHMP/BWP/534898/2008 guidelines [52], stability-in-use data (requested for preparations used after reconstitution, mixing, or dilution) are not required if the preparation will be used immediately following reconstitution.

The finished product should be adequately characterized. Some principles described for the active

substance also apply to the finished product, such as in-process tests, the need for comparability exercises if formulation changes, the control of excipients, reference standards or materials, container closure system, stability, analytical procedures, validation, and the set-up of specifications (tests plus acceptance criteria). According to the EMA/CHM /BWP/534898/2008 [52], specifications are mandatory for quantity, identity, purity, and microbiological quality. It is also compulsory to include a test for biological activity and set the impurities' upper limits.

In an indicative basis, our suggested selection of control tests for the drug products is provided in the Recommendation R3.25 and **Table 4**.

Recommendation R 3.25:
We recommend considering the following tests in order to set drug product specifications in general (additional tests will be required as product complexity increases; see R 3.24).
(i) Quantity tests by particle quantification and total protein quantification; (ii) identity tests by particle hydrodynamic diameter analysis, immunochemical characterization, DNA and RNA content; (iii) purity test by the ratio of particle counts/micrograms of protein; and (iv) contaminants (microbiological quality) according to the European Pharmacopoeia.
We recommend testing for impurities such as albumin or fibrinogen quantification (if the EV secretion step is performed in complete medium and in case the clearance capacity efficiency of the purification step is not satisfactory or not validated). DNA (and optionally RNA too) not encapsulated in EVs may be analyzed for impurity quantification by DNase (and RNase) treatment.
We recommend testing on a monitoring basis the biological activity using a potency test in vitro, if relevant, or else in vivo. In addition, appearance and description (visual description of the appearance of the product) as well as general tests (pH and osmolarity) may be addressed.

3.8. Particularities: engineered EVs containing a transgene product or a drug in charge of the therapeutic effect

Loading EVs with a transgene product or a drug will modify the control strategy and product specification since the final product's mode of action will mainly rely on this molecule (which can be considered the product's primary active substance). Therefore, the control strategy will include, in addition to what has been described so far, a part focusing on this active substance that should demonstrate pharmaceutical quality and consistency.

In the case of a gene therapy product, the guideline EMA/CAT/852602/2018 [20] should be followed. For instance, it is indicated that control tests and acceptance criteria concerning the drug substance in terms of genetic identity, integrity, and quantity should be established. Besides, a test for biological activity based on infection/transduction assays and detection of expression/activity of the therapeutic sequence should be included. Additionally, for genetically modified cells transduced using retro/lentiviral vectors, each virus batch should be tested for the presence of replication competent virus with a validated method. The vector integrity, biological activity (including transduction capacity), and strength should be systematically included in the stability tests.

Regulatory guidance is less clear in the case of EV-enriched secretome products containing a drug. Therefore, we address this issue in the Recommendation R 3.26.

Recommendation R 3.26:
We recommend considering the following approaches for EV-enriched secretome products loaded with a drug molecule (in addition to Recommendations R 3.9 and R 3.21–23).
In-process control:
- Analysis of EV producer cell drug loading (in case of pre-production loading) on a monitoring basis.
For drug substance and finished product control, as well as stability studies:
- Analyses of the drug molecules in terms of quantity, identity, and purity may be established. In addition, a test for biological activity specifically related to the drug may be established.

4. Non-clinical (pre-clinical) development

The non-clinical development phase aims to provide *in vitro*, *ex vivo,* and *in vivo* data on the pharmacodynamics (PD), pharmacokinetics (PK), and toxicity profile of the EV-enriched secretome product at the proposed route of administration to support the feasibility of its investigation in a clinical trial in terms of efficacy and safety.

In this regard, necessary guidance on non-clinical studies is provided by the EMEA/CHMP/SWP/28367/07 guidelines (on how to mitigate risks for first-in-human and early clinical trials) [60]. However, its content is highly "target-oriented." Considering the multitude of biomolecules present in EV-enriched secretome products, the therapeutic effect involves multiple targets. In this regard, the ATMP guideline EMA/CAT/852602/2018 [20] is particularly relevant considering that the complexity of ATMP similarly involves a multi-target effect. Herein, we highlight some guideline issues that we deem appropriate for EV-enriched secretome products by selecting recommendations from both EMA/CAT/852602/2018 [20] and EMEA/CHMP/SWP/28367/07 [60] and others.

Significantly, according to both EMA/CAT/852602/2018 [20] and EMEA/CHMP/SWP/28367/07 [60], the product tested in non-clinical studies should be representative of the one administered in clinical studies. Indeed, toxicity tests are performed later in the development phase with a product equivalent to the one to be tested in clinical trials (sometimes the same product to be tested in clinical trials). However, PD and PK studies were performed earlier in the development phase, with a more preliminary version of the final product. In all cases, important EV data should be provided as indicated in the Recommendation R 4.1.

Recommendation R 4.1:
We recommend documenting the following data for the EV-enriched secretome investigated through in vitro, ex vivo, and in vivo studies: particle concentration, total protein content, size distribution, and concentration of marker-positive EVs. We also recommend providing information on producer cell equivalents (the amount of producer cells needed to obtain the tested dose of EV-enriched secretome).

As in the previous section, there is a formal format for the non-clinical data indicated in the CTD module 4 content (ICH Topic M4S) [112]. We present this in **Table 5**. Several entries are listed in CTD Module 4; however, that does not mean that all tests are required. The main entries are outlined and discussed in the context of EV-enriched secretome products.

4.1. Pharmacology

The first part, "Pharmacology," concerns the primary PD (associated with the proposed therapeutic indication) and secondary PD (unintentional effects distinct from the proposed therapeutic indication). Safety pharmacology is related to undesired pharmacodynamic effects. In general, safety pharmacology tests are the core battery for assessing adverse product effects on vital functions (respiratory, cardiovascular, and central nervous system, for instance). The last item of the pharmacology section concerns pharmacodynamic drug interactions with current drugs that will probably be administered for the same disease. Guidelines for pharmacology studies are provided in the guidelines ICH S7A (on safety pharmacology) [113] and ICHS7B (on ventricular repolarization effects more specifically) [114], although the latter is much less relevant for EVs.

Importantly, all these tests are not required before the beginning of early phase clinical trials. According to EMA/CAT/852602/2018 [20], the extent of the non-clinical data required for an ATMP before a clinical trial will depend on the risk associated with the product, which in turn will depend on certain risk factors (i.e., cell type, genetic modification, or not). Therefore, this guideline recommends selecting tests based on risk analysis, which is not the strategy recommended in the EMEA/CHMP/SWP/28367/07 guidelines [60]. Our position on that is stated in the Recommendation R 4.2.

Recommendation R 4.2:
We recommend adapting preclinical safety evaluation strategies used classically for biological medicinal products (ICH S6 (R1) [115]*). We recommend using a risk-based approach to identify the necessary non-clinical data for EV-enriched secretome products on a case-by-case basis as applied to ATMPs.*

Table 5: *Presentation of CTD module 4 content (ICH Topic M4S) [112] for the non-clinical (pre-clinical) part. Our selection of guidelines relevant for EV-enriched secretome products for clinical trials is indicated in the right column.*

CTD Module 4 content	**Our selection of general relevant guidelines for EV-enriched secretome products**
"Table of Contents of Module 4	
Study reports	
Pharmacology - Primary Pharmacodynamics - Secondary Pharmacodynamics - Safety Pharmacology - Pharmacodynamic Drug Interactions	EMA/CAT/852602/2018 [20]* EMEA/CHMP/SWP/28367/07 [60] ICH S7A [113] ICH S7B [114]
Pharmacokinetics** - Analytical Methods and Validation Reports - Absorption - Distribution - Metabolism - Excretion - Pharmacokinetic Drug Interactions - Other Pharmacokinetics studies	EMA/CAT/852602/2018 [20]* EMEA/CHMP/SWP/28367/07 [60] ICH S3A [116] ICH S6 (R1) [115] ICH M3 (R2) [117]
Toxicology - Single-Dose Toxicity (in order by species, by route) - Repeat-Dose Toxicity (in order by species, by route, by duration; including supportive toxicokinetics evaluations) - Genotoxicity In vitro In vivo (including supportive toxicokinetics evaluations) - Carcinogenicity (including supportive toxicokinetics evaluations) Long-term studies (in order by species; including range finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics) Short- or medium-term studies (including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics) Other studies - Reproductive and Developmental Toxicity (including range-finding studies and supportive toxicokinetics evaluations) (If modified study designs are used, the following sub-headings should be modified accordingly.) Fertility and early embryonic development Embryo-fetal development Prenatal and postnatal development, including maternal function Studies in which the offspring (juvenile animals) are dosed and/or further evaluated. - Local Tolerance - Other Toxicity Studies (if available) Antigenicity Immunotoxicity Mechanistic studies (if not included elsewhere) Dependence	EMA/CAT/852602/2018 [20]* EMEA/CHMP/SWP/28367/07 [60] ICH S6 (R1) [115] ICH M3 (R2) [117] ICH S9 [118] EMA/CHMP/SWP/169215/2005 [119] ICH S2 (R1) [120]

Metabolites Impurities Other	
Literature References"	

** While EVs do not necessarily fulfill the definition of ATMPs, the underlying scientific principles outlined in this guideline may be applicable.*

*** Technological challenges should be taken into account when considering ADME studies for EVs as it will be discussed in this section 4.*

In addition to this general approach, EMA/CAT/852602/2018 [20] guides the minimum non-clinical data required before a clinical trial. Concerning the pharmacology section, an efficacy proof-of-concept in a relevant *in vivo* model mimicking the disease and related *in vitro* or *ex vivo* studies are required. These tests should demonstrate the potency of the product and the transgene's expression in gene therapy medicinal products. We consider the exact requirements to be valuable for EV-enriched secretome products. For gene therapy medicinal products, the expression and therapeutic effect of the transgene should be demonstrated in a relevant *in vivo* model (and via *in vitro* or *ex vivo* studies if applicable), according to EMA/CAT/852602/2018 [20]. The pharmacology studies for drug-loaded EVs are not totally clear, therefore we addressed this issue in the Recommendation R 4.3.

Recommendation R 4.3

In the specific case of EV-enriched secretomes loaded with a drug molecule, we recommend testing the potency of the drug molecule in a relevant *in vivo* model (and through in vitro or ex vivo studies when applicable). MoA may only be supported by the literature for drugs with market approval, as their MoA is already known.

The tests described in **Supplementary Box 2** can be performed to investigate the primary or secondary PD of EV-enriched secretome products and to provide relevant information for future toxicity investigations. *In vitro* and *ex vivo* tests are expected to investigate the intended therapeutic action in addition to assisting researchers in planning, selecting, and executing an appropriate *in vivo* model to derive proof-of-concept efficacy information. Target binding, specificity, and cross-reactivity assays are of particular importance in the case of EVs derived from genetically manipulated cells with transgene expression on the EV surface. For these products, assays should be planned with unmodified EVs and EVs with transgenes using recipient cells expressing the intended target. The tests are listed in **Supplementary Box 2** on an informative basis. It is not compulsory to perform all of these tests. Test selection should be based on a case-by-case basis, and justification should be given based on the target product profile of the product being developed.

The replacement of animal testing by *in vitro* pharmacology studies should be explored whenever possible. However, animal testing (following ethics committee approval) is crucial before administration in humans, particularly for PD and biodistribution studies and toxicology assessment (as discussed in the following sub-sections). It is essential to use appropriate preclinical animal models relevant to human pathology to support primary pharmacodynamic (potency) studies. Various tools adapted to small animals are currently available to measure quantitative, relevant, and functional outputs. This is particularly important for qualitatively and quantitatively estimating the potency *in vivo*. Progress has been made in animal model development, especially in rodents, to be as close as possible to human diseases (i.e., via chemical or chemical induction.)

In some cases, large animal models could be relevant because they have physiological and pathological similarities with humans. In particular, surgery-induced models and local treatment applications can be more accessible in large animal models. Strategies that intend to use a novel clinical delivery device may need to include feasibility testing in a large animal model. In case no inducible relevant animal model is available, alternative genetically modified animal models should be considered for efficacy evaluation (**Supplementary Box 2**). The use of immunocompromised animal models (avoiding toxic effects and the possible immunogenicity related to the xenogenic set-up) could be very informative for the therapeutic proof-of-concept. However, the absence of a functional immune system could bias the results, mainly if the treatment directly or indirectly acts on the immune system. In this case, subsequent tests in

immunocompetent animals, ideally using EVs from the same animal species (but produced by an equivalent method and cell type considering the EV-enriched secretome product of interest), may be valuable. Another point to be considered is interspecies cross-reactivity. Limited ligand-receptor interactions could lead to the absence of therapeutic benefits. In this regard, the use of humanized animal models is increasing, as it is clinically relevant. Efficacy studies in humanized models could provide more insights into the biological effects of EVs. This is particularly important for genetically modified EVs displaying a particular ligand or receptor with a defined *in vitro* mechanism of action. Humanized models would be a valuable strategy in the case of limited cross-reactive biological activity observed in more classical animal models.

The selection of a suitable dosing frequency or regime is a crucial parameter to establish EVs' efficacy. Particular attention should be paid to EVs' effect depending on the therapy timing considering the disease's evolution. Moreover, EV administration may be performed before the disease's symptoms (preventive effects) or during the acute/chronic phases of the disease (curative effect). The dosing regimen can be obtained from early acute PD. This type of study will further provide predictable power before investing time and resources for long-term studies. A dose-dependent response using an appropriate concentration should be established during the efficacy evaluation of EVs. If required, post-dose effects on the target tissue and target-mediated downstream pathway can be evaluated using proper *ex vivo* analysis such as cytokine measurement in serum, western blot for protein expression, qPCR for gene expression, and FACS for immune cell infiltration.

4.2. Pharmacokinetics

This section of the CTD Module 4 [112] addresses PK and toxicokinetics (TK) issues concerning absorption, excretion, tissue distribution, metabolism, and pharmacokinetic drug interactions. The analytical methods used and their validation should be documented. Pharmacokinetic drug interactions and other pharmacokinetic studies were also included. General guidance is provided in the guidelines ICH S3A (on toxicokinetics) [116], ICH S6 (R1) (on non-clinical safety of biotechnology-derived pharmaceuticals) [115] and ICH M3 (R2) (on non-clinical safety studies) [117].

Many of the tests listed in the CTD Module 4 [112] are required in a case-by-case basis after early phase clinical trials (phase I or Phase I/II). EMA/CAT/852602/2018 [20] guidance on the minimum non-clinical data needed before a clinical trial for ATMP indicates that data concerning the choice of the administration route and application procedure/devices should be provided. A biodistribution study is expected to provide information on the persistence, effect duration, and target organs of the product to design appropriate safety studies, including their duration. Importantly, biodistribution studies are required if the administration strategy leads to systemic exposure (EMA/CAT/852602/2018) [20]. According to this guideline, biodistribution studies are necessary for gene therapy before clinical trials.

Different methods may be used to perform biodistribution studies of EVs. Fluorescence imaging using lipophilic dyes (PKH26, PKH67, DiI) or near-infrared fluorescent dyes (DiR, DiD) is the most frequently used method because of its ease of use and low cost. Lipophilic dyes may induce EV aggregation, label lipoproteins, and form dye lipid micelles remaining in the EV preparations [121]. Negative controls consisting of lipophilic dye alone should be included to avoid misinterpretation. Other methods such as bioluminescence, nuclear imaging (PET/SPECT), and magnetic resonance imaging (MRI) are also used. However, none of these methods offer high penetration, high sensitivity, and high spatial/temporal resolution [122]. Therefore, the method's choice should consider the issues related to the accessibility of target organs and consider the structural preservation of labeled EVs. Indeed, EVs can be labelled by different methods including membrane integration, genetic integration, covalent binding, internalization, and metabolic labeling. Methods that modify EVs' surface by membrane integration or covalent binding may change the EV structure and alter their interactions with the target cells and, therefore, their biodistribution [123]. Unfortunately, more reliable methods currently used for ATMPs that rely on the detection of human proteins (by immunohistochemistry or ELISA) or nucleic acid (by PCR) in animals are less appropriate for EV biodistribution studies because of the limited amounts of proteins/nucleic acids administered as compared to cell therapy. Such reliable methods may be more relevant for the detection of locally administered EVs (enabling higher local concentration for detection). Recommendation R 4.4 takes into account these technical difficulties in the context of biodistribution studies.

Recommendation R 4.4:
Considering technical difficulties, we do not recommend systematic biodistribution studies.
In general, we recommend conducting a biodistribution study depending on the risk analysis. When applicable, it should be performed before the beginning of early phase clinical trials.
For gene therapy medicinal products, a biodistribution study must be performed in accordance with EMA/CAT/852602/2018 [20]*. These guidelines should be consulted for additional test requirements.*
For drug-loaded products, we recommend documenting the biodistribution of the drug molecule (drug quantification in organs after the administration of drug-loaded EVs). A biodistribution study of the free drug (not encapsulated in EVs) may be conducted as a control. A study of EV biodistribution may be conducted preferentially if such a need has been identified in a risk analysis.

4.3. Toxicology

One of the aims of this section is to evaluate the medicinal product's toxicological profile after a single administration or following repeated administrations. Another aim is to identify off-target organs concerned by toxicity, the determination of a no adverse effect level (NOAEL), the investigation of the relationship between exposure and response, and the eventual reversibility of toxic effects. Other tests include:

- genotoxicity tests including, for instance, genotoxicity in bacteria (Ames test), *in vitro* tests in mammalian cells, and *in vivo* tests for chromosomal damage (micronucleus test usually performed in the mouse)
- carcinogenicity tests to identify tumorigenic potential include a short-term study carried out in a transgenic model (6-months) and other long-term studies
- reproductive toxicity and developmental toxicity (i.e., effect on female and male fertility, embryonic development, and juvenile toxicity if pediatric use is considered)

Immunotoxicity tests evaluate unintentional immunosuppression or enhancement by evaluating parameters of the immunologic response in the above-mentioned repeated dose toxicity studies

- Local tolerance test performed at sites of the body that may be exposed to the product

General guidance is provided in the ICH S6 (R1) guidelines (on non-clinical safety of biotechnology-derived pharmaceuticals) [115], ICH S9 (on anti-cancer pharmaceuticals) [118], ICH M3 (R2) (on non-clinical safety studies) [117], EMA/CHMP/SWP/169215/2005 (tests in juvenile animals) [119] and ICH S2 (R1) (on genotoxicity) [120].

Toxicity tests should be performed in compliance with good laboratory practice (GLP) in certified laboratories. Not all tests are necessary before early phase clinical trials and even for phase III clinical trials. According to the ATMP guidelines EMA/CAT/852602/2018 [20], at minimum, safety/toxicity studies should support the selection of safe and biologically effective starting dose and appropriate safety margins before a clinical trial. The need for toxicity studies, such as genotoxicity, tumorigenicity, reproductive and developmental toxicity, and immunotoxicity studies, should be determined via a risk analysis and considering the intended clinical use. According to EMA/CAT/852602/2018 [20], before a clinical trial, it is necessary to have appropriate safety data on the following:

(i) Genotoxicity: For gene therapy products, insertional mutagenesis should be evaluated in relevant *in vitro* and/or *in vivo* models. The choice of other genotoxicity studies for gene therapy products involving host-DNA integration will depend on the route of administration and target organ/tissue according to EMA/CAT/852602/2018 [20].

(ii) Tumorigenicity: Standard studies on lifetime rodent carcinogenicity are usually not requested. Depending on the risk analysis, studies should be performed in relevant *in vitro/in vivo* models for neoplasm signals, cell proliferation index, or oncogene activation.

(iii) Immunogenicity and immunotoxicity: Evaluation should be performed both locally and systemically via histological analysis of immune system activation. Furthermore, the impact of the immune response on the product's fate should be investigated.

In an indicative basis, a selection of non-clinical tests is provided in the Recommendation R 4.5 to be completed case-by-case.

Recommendation R 4.5:
We recommend considering the following tests before a clinical trial:
- single dose toxicity,
- repeated dose toxicity in case multiple dose administrations are envisaged,
- genotoxicity studies if EV-enriched secretome products are classified as gene therapy products,
- tumorigenicity studies (short-term studies of 6 months) in case their need is evidenced by a risk analysis
- immunogenicity and immunotoxicity studies - after local (if applicable) and systemic administration.

A selection of relevant literature information and examples of toxicological studies for EV-enriched secretome products is provided in **Supplementary Box 3**.

5. Clinical aspects

Clinical evaluation is a crucial step in the development of new medicinal products. The general principles apply for EV-enriched secretome products as for any medicinal product, the purpose of which is to draw attention to specific questions raised for EV products.

This section focuses on early clinical trials (phase I, phase I/II, or phase II). In Europe, clinical trials have to comply with Directive 2001/20/EC [124] on clinical trials, which will soon be repealed by Regulation 536/2014 [125]. General principles and guidance are available in the ICH guideline E8 (R1) [125] on general considerations for clinical studies.

For investigational EV products, general principles that apply to any investigational medical product should be used. They are described in Annex I of Regulation 536/2014 [126], including guidance on the protocol's content (**Supplementary Box 4).** It is stated that the IMPD contains a protocol with a description of the objective, design, methodology, statistical approach, purpose, and organization of the trial. If relevant, it also contains data from previous clinical trials or human experience, in a logical structure such as that described in the CTD module 5 from ICH M4E (R1) [127] (**Supplementary Box 5**).

In early clinical trials, when the product is used for the first time in humans, safety is the primary concern. Regulatory frameworks demand GxP standards (Good Manufacturing, Good Laboratory, Good Distribution, Good Clinical, Good Scientific Practice, or GMP/GLP/GDP/GCP/GSP) for production and quality control. EV products share essential characteristics with ATMP, such as the complexity of the product, limited extrapolation from animal data for PK, PD, or immunogenicity, uncertainty about the adverse effects, or the need for long-term efficacy and safety follow-up. Therefore, as recommended for ATMPs, it is necessary to build a safety plan for a robust evaluation of the risks and their mitigation. Although most of the time, EVs do not fulfill the definition of an ATMP, the guidelines on quality, non-clinical, and clinical requirements for investigational advanced therapy medicinal products in clinical trials EMA/CAT/852602/2018 [20] states that general scientific principles may be applicable. Compliance with existing regulatory frameworks will increase the confidence of the pivotal stakeholders involved in clinical trial regulation.

Although most of the ATMP early clinical trials are phase I/II trials, things are different for EV products, where both phase I and phase I/II are performed. The targeted disease may explain this difference. Also, because of the potential risk of tumorigenicity, ectopic tissue formation, or immune response, ATMP are used directly in patients rather than healthy volunteers. EV-enriched secretome products might pose risks to patients that could be considered ''high'' since they compile three main criteria defining "high-risk medicinal product" according to the guideline EMEA/CHMP/SWP/28367/2007 [60]: i) uncertainties

exist related to the mechanisms of action; ii) the nature of the target may be unclear; iii) the relevance of animal models used to confirm the mechanisms of actions may be limited. That said, the classification in the "high risk" category can only be established on a case-by-case basis. In this case, investigators decide that their research falls under the high-risk definition that would augment preclinical safety testing requirements. However, the ISEV position paper on clinical trials [45] proposed several justifications that might mitigate the high-risk aspects of EV-based therapies: (a) Autologous EVs naturally occur in the human body. (b) All cell types physiologically produce EVs, and their production and uptake in target cells is a natural process. (c) Substances contained within native EVs are physiological body constituents (unlike synthetic molecules). (d) Increasing evidence indicates that DCs and (autologous and allogeneic) MSCs show good safety profiles in clinical trials; therefore, it is expected that their EVs will not cause more harm to patients than their parent cells. Finally, (e) there is no evidence that allogeneic EVs massively co-transfused with blood products cause adverse events. Altogether, these arguments support the assumption that EVs generated from a human cellular source do not carry higher risks than the cells and, thus, are not automatically classified as high-risk medicinal products.

Some substantial modifications of the cell source or the EV can be made to overexpress a specific molecule in the EV or use EVs as vectors for chemical drugs. In such cases, the EV cargo becomes the primary active substance, and the expected mode of action is known. In particular, the clinical safety plan will primarily focus on cargo molecules.

It should be made clear that the benefit-risk assessment made in clinical development is different from the risk-based approach used for developing the product and writing the IMPD, as described in Annex I, Part IV of Directive 2001/83/EC [43] applied to ATMPs. The benefit-risk evaluation should focus on the clinical trial protocol to protect the trial subjects and future patients; however, many aspects of the risks and their mitigation will be found in the IMPD.

5.1 Safety and tolerability

Safety and tolerability are the main objectives of early clinical trials. To design a clinical safety plan, investigators will need to tailor it to the pharmaceutical category of EV preparation, with the product specifications determining the pharmaceutical classification, its biological effect (i.e., potency), and non-active components (excipients).

The safety plan should also depend on the donor, the type of parent cells, the disease in which an EV-enriched secretome product is being tested on the route of administration dosage and dosage regimen. All these variable factors require the specificities to be evaluated in terms of clinical safety.
Regulation 536/2014 [126] states that clinical trials should anticipate both known and potential risks and benefits for trial subjects or patients. The benefit and risk evaluation should include classical points (the expected effects, other medical options) and risks related to the product itself (native EV, or modified EV containing a drug or the product of a transgene). It also considers all aspects of manufacturing (such as donor, type of parent cells, and isolation methods) and the data obtained from the non-clinical study phase or clinical data from similar products. Because of the innovative characteristics of EV-enriched secretome products, the theoretical risks should be evaluated. The action mode is crucial; it may not be wholly understood even after the non-clinical studies (which is a risk), but can involve various physiological functions or organs (immune response). Long-term effects should be considered, even though numerous potential risks inherent to ATMP (ectopic tissue formation and malignant transformation) are not relevant to EV-based products that contain no cells. Global interventions, including the administration mode, should then be discussed, especially if invasive procedures (surgery, invasive vascular access, etc.) are necessary to deliver the EV product. As for ATMP, the population chosen for the first in human could be patients rather than healthy volunteers. This type of population may be at higher risk; the risk should be thoroughly examined as per the disease or disease state. The investigator should undertake all relevant actions to mitigate the risks.

5.2 Dose finding strategy

Classically, early studies in humans aim to determine a starting dose that could be considered the minimal dose required to obtain a pharmacological effect and induce no harm to the trial subjects/patient. Then, dose escalation is expected to determine the optimal dose range required to obtain the intended effect, if

the maximum tolerated dose (MTD) can be determined.

To date, four fragile lessons emerge from partial data available that may be of interest for EV dose-finding: (i) The expected EV dose and sometimes the way to quantify it are mainly unknown. (ii) The general findings that can be extrapolated from clinical data obtained on cell therapies as well as from non-clinical and early clinical data on EV therapies tend to show that EVs are well tolerated. (iii) PK/PD is challenging in humans. (iv) No significant therapeutic efficacy reduction or anaphylaxis due to immune adverse events is expected based on the data available on repeated MSC cell injections in patients, which cannot be extrapolated to EVs from cell sources other than MSCs.

Therefore, the starting dose should be proposed and discussed based on all available information, including non-clinical and clinical data and from the literature. It should be adapted to the product composition, including key attributes such as allogeneic/autologous, presence within the product of a transgene or loaded molecules, etc.

Once the initial dose is defined, phase I escalation studies design is usually done using the classical 3+3 design, that is, administering the first dose to three subjects/patients and escalating to the next dose in three other subjects if no adverse effects are found. This allows MTD findings based on toxicity. According to EMA/CAT/852602/2018 [20], a very progressive inclusion of the patients (one by one with a waiting period between them) and intense monitoring of the first one are recommended in ATMP trials with a particular focus on acute and delayed adverse effects. However, it may not be well adapted to most EV-enriched secretome products because of their limited expected toxicity. Other protocols could be considered and proposed based on strong non-clinical safety arguments or previous trials in humans with similar products. A detailed discussion on phase I escalation dose designs has been reviewed elsewhere [128, 129].

Clinical, medical imaging or biological follow-up after treatment need to be based on the results of preclinical toxicology studies. To date, no specific toxicity has been reported; therefore, a general and relatively broad clinical and biological follow-up is recommended until new data are available.

Lessons from the MSC field show that the pitfall in regenerative medicine is not on demonstrating the limited toxicity but rather demonstrating the efficacy in phase II, based on limited data from dose escalation without clear preliminary dose-related efficacy.

In order to limit long and dose-unsuitable phase II studies, if toxicity is not reached, a preliminary phase I trial with extensive biological characterization (biopsy with immune profiling, histology, biomarkers, etc.) may be critical to select the phase II dose that has at least shown some efficacy. Another option to detect preliminary signs of efficacy to select an appropriate dose is to run a study in a selected population with severe or resistant diseases. This can be proposed when usual therapeutic strategies have failed or are expected to fail, and when any improvement may be sufficient to emerge from biological and statistical noise. When known PK or IC50 parameters exist for a particular molecule loaded in EVs, a pharmacologically guided dose escalation based on this parameter should be considered.

The development of biomarkers would, of course, be of interest in selecting an EV responding population.

Finally, the dose regimen is a crucial question for EV products. Their half-lives are considered to be very short in systemic administrations (about 5–10 min in the blood) [130] (much shorter than most cells or tissue ATMP), although the efficacy is probably not related to the blood concentration but to the concentration in tissues of interest. Repeated administration is a potential treatment option. This could be slightly different in local administration. Thus, the dose regimen must be extensively studied in non-clinical development and thereafter tested post a single dose study in humans.

5.3 Pharmacodynamics and pharmacokinetics study plan (selecting suitable PD markers to predict the efficacy)

Pharmacodynamics and pharmacokinetics are classical secondary but potentially fundamental goals in exploratory trials. As for ATMP, the typical pharmacokinetic approach (absorption, distribution, metabolism, excretion, ADME) is generally not applicable. As mentioned above, non-clinical data on

EVs suggest a limited EV half-life in the blood [130]. Therefore, half-life assessment, distribution volume, and clearance measurement in humans are challenging to perform. An exception could be made for EV-enriched secretome products with loaded molecules where PK/PD study plan should be based on PK/PD of the drug of interest using classical designs—keeping in mind that the EV-loading strategy could, and sometimes is meant to, modify PK and/or PD. According to EMA/CAT/852602/2018 [20], in the case of gene therapy products, PD assessments are performed to study the expression and function of the gene expression product (e.g., as a protein). Besides, the plasma concentration and half-life should be determined for the therapeutic transgene product. Unmodified EVs' metabolization is not expected to produce any unphysiological degradation products, rendering EV metabolization and elimination measurement difficult or even impossible. Our general suggestion for PK studies is indicated in the Recommendation R 5.1.

Recommendation R 5.1:
When PK is not feasible for technical reasons, we recommend adopting the general principles that apply to Advanced Therapy Medicinal Products.

The mode of action of EV products is not well known. The PD assessment should be designed based on robust non-clinical and literature data and highly dependent on the indication. Clinical, paraclinical (biology, imaging), functional or histological analyses, for example, could be proposed; they should be reproducible and as far as possible quantitative. As for several ATMPs, relevant PD markers should be studied and chosen as soon as possible during the development of an EV product to determine the most relevant dose and assess its biological activity in the clinical phase. Robust PD markers are vital to the success of both ATMP- and EV-enriched secretome products.
A data and safety monitoring board (DSMB) is convened to serve as a committee for monitoring safety data. All missions and the precise operating methods of the DSMB should be described in the DSMB's charter. This board's primary mission is to monitor the trial to protect the study participants by constantly evaluating the product's safety and efficacy.

5.4. General considerations for phase III trials

Because safety remains a crucial goal in phase III trials, adverse effects should be anticipated based on the product's knowledge, closely monitored, and reported.

The clinical efficacy plan summarizes all the relevant data to prove the drug's efficacy for specific indications in the desired population. According to ICH M4E (R1) [127], these data need to be provided in a technical document for seeking approval as a Summary of Clinical Efficacy.

Phase-III trials are designed to demonstrate efficiency. The endpoints should be carefully selected to reflect drug effects. The primary endpoint should be based on the intended effect of the product. For example, for EV used for wound healing, the decrease in wound size can be evaluated. The main primary endpoint can involve evaluating a patient's clinical outcome, such as events, symptoms, or function changes. However, composite endpoints can be selected based on the disease's complexity, especially when one of the endpoints' rates of occurrence is low. However, it is advisable to choose as few primary endpoints as possible because selecting multiple endpoints may cause a type-I error - when the product is considered to be practical or more effective but it is not. Secondary endpoints can be selected to further strengthen the efficacy of EVs. Moreover, they can help identify the underlying mechanism. For example, comparing the cytokine levels in GVHD patients with and without EV therapy can suggest a possible pathway involved in EV modulation of GVHD.

If EVs are used as a delivery system, there is a possibility of interaction between EVs and the loaded particles; therefore, a comparative efficacy plan may be planned to provide comparative data to already licensed formulations. Moreover, EVs are obtained from the cells; therefore, a comparative trial to the cell, for example Stem-cell therapy, could be proposed to show the higher or non-inferior efficacy.

An independent data and safety monitoring board (DSMB) can be created. This board's primary mission is to monitor the trial with the primary goal of protecting the study participants by constantly evaluating the product's safety and efficacy.

6. Conclusions

In this position paper, we attempted to overview the IMPD content and related guidelines taking into consideration specificities of EV-based therapeutics. We focused here on current European legislation, but since there is an international commitment to converge toward common rules, most of the general principle proposed here may apply to other regulatory frameworks. Strategic choices when developing EV- medicinal products were commented. When we considered valuable, recommendations were highlighted concerning quality, non-clinical and clinical investigation issues. A discussion and justification on method selection for the quality part was provided. Besides, a global strategy for control was suggested including in-process control, control of the drug substance, final product and control for stability investigation. A content comparison in relation to the ISEV position paper [45] is provided in Table 6.

Table 6: Comparison between the ISEV position paper Lener et al. 2015 [70] and ours to highlight agreement, disagreement and the issues that were not previously addressed.

Sub-section / Figure / Table/ Recommendation	Agreement with Lener et al. 2015	Different from Lener et al. 2015	Not addressed in Lener et al. 2015	Comments
Figure 2			X	Although the classification is addressed in Lener et al. 2015 [70], the complexity and cost-saving aspects indicated in this figure were not previously featured
Subsection 2.6.	X		The classification shift from biological medicine to a Class III medical device for EVs formulated with biomaterials responsible for the main therapeutic effect	Although it is very unlikely that developers make this classification choice, it is an option.
Subsection 2.6.		Updated		The EMA/CAT considered that EVs containing recombinant RNA fall within the definition of gene therapy products We think that EVs containing recombinant peptides or proteins could be considered biotechnological products (as recombinant proteins)
Table 2 and 5			X	Several of these guidelines are cited by Lener et al. 2015 [70]. However, we

				present herein a selection considering the structure of CTD Module 3 and 4 content
Recommendations R 3.1-3.26			X	
Table 3			X	
Box 1			X	Although information on methods for quality control is provided in Lener et al 2015 [70], herein we provide rather a selection with the related justification
Table 4			X	Lener et al 2015 [70] addressed the need for quantity, identity and purity test as well as potency assay and impurity evaluation. Herein, we suggested tests in this regard that we consider relevant. Besides, we presented their integration in the overall control strategy.
Recommendation R 4.1; 4.3-4.5			X	
Recommendation R 4.2	X			
Recommendation R 5.1	X			
Conclusion	X		The need for consensus is rather highlighted herein	We fully agree with Lener et al 2015 [70] that "Regulatory frameworks for manufacturing and clinical trials exist in Europe, Australia and United States, but special guidelines targeting EV-based therapeutics may be needed."

An important consideration in the translation of EV-based preparations into medicinal products will be the product definition not only by its manufacturing process (in a “the process is the product” basis) but also by its quality attributes in terms of quantity, identity, purity and biological activity. Although some overall recommendations are outlined herein, the next steps would be to reach an international consensus on the metrics for such quality attributes and the validation of related tests. Additionally, developers should consider that each new product will be evaluated on its own. Therefore, a risk-based approach as a function of the strategic manufacturing choices and implemented processes seems a valuable strategy. In this regard, the current regulation of ATMP provides at some extent valuable guidance. As current international guidelines may require special interpretation to be applied to EV-based products, our work group EVOLVE-France considers that an EV-dedicated guideline would be highly important.

The perspective of an international scientific consensus gathering efforts of all current group initiatives would be meaningful, including in case a future guideline draft dedicated to EV-based medicinal

products will be open for public consultation. In this regard, it is important to highlight the role of the ICH on the achievement of scientific consensus and harmonization. The ICH process for developing a new guideline takes place in several stages. It starts with the development of a consensus on the new topic by the relevant Expert Working Group (EWG - nominated from the regulatory and industrial bodies). The draft consensus from the EWG is then released for wider consultation. After the comments are received and consolidated, the final guideline is issued for adoption and implementation. We believe that such a cross-talk initiative gathering efforts of all current translational EV task forces, committees and work groups would help meet researchers, developers, and regulatory agencies' expectations. We anticipate that the joint contribution of the current group initiatives and the prospect of a dedicated guideline would facilitate the development of EV-based medicinal products to make the EV field's promises come closer to patients while maintaining quality, safety, and efficacy.

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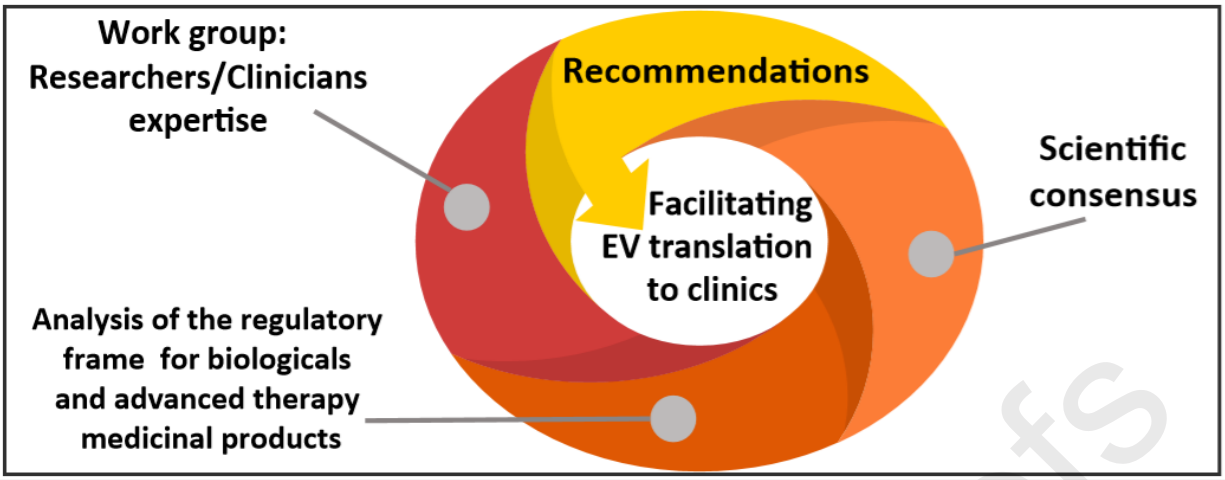
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Declaration of interest:

Florence Gazeau, Amanda Karine Andriola Silva, Claire Wilhelm and Gabriel Rahmi are co-founders of the spin-off Evora Biosciences. Amanda Karine Andriola Silva and Claire Wilhelm are co-founders of the spin-off EverZom. Max Piffoux is consultant and owns stocks in the spin-off Evora Biosciences and in the spin-off EverZom.

Journal Pre-proofs



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