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TECHNICAL NOTE

A decision-making tool for navigating extracellular vesicle research and product development

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Abstract

Due to their intercellular communication properties and involvement in a wide range of biological processes, extracellular vesicles (EVs) are increasingly being studied and exploited for different applications. Nevertheless, their complex nature and heterogeneity, as well as the challenges related to their purification and characterization procedures, require a cautious assessment of the qualitative and quantitative parameters that need to be monitored. This translates into a multitude of choices and putative solutions that any EV researcher must confront in both research and translational environments. In this respect, decision-making tools may help assess various options, weigh pros and cons, and ultimately arrive at a thought-out decision that considers both the best fit-to-source and fit-to-scope EV application(s). Here, we present a multi-criteria EV decision-making grid (EV-DMG) as a novel, efficient, customizable, and easy-to-use tool to support EV research and innovation. By identifying and weighing key assessment criteria for comparing distinct EV-based preparations and related processes, our EV-DMG may assist any EV community member in making informed, traceable, and reproducible decisions regarding the management of EV sources or samples. Ultimately, this EV-DMG may guide the adoption of the most suitable EV production and analytical pipelines for targeting a defined aim or application.

KEYWORDS

decision-making tools, extracellular vesicles, multi-criteria decision-making, product development, quality control, research and development, responsible research and innovation

1 | INTRODUCTION

Extracellular vesicles (EVs) are nanosized (i.e.,˜50–150 nm, defined as "small EVs";˜200–800 nm, defined as "medium EVs") and micro-sized (i.e., ≥ 1000 nm, defined as "large EVs") membranous particles secreted and taken up by either eukaryotic or prokaryotic cells. By intrinsically conveying a multitude of biologically active components, including proteins, lipids, nucleic acids, and metabolites, EVs have been shown to mediate cell-to-cell and organism-to-organism communication, thus governing the functionality of organisms and their interplay with the surrounding environment (Buzas, 2023; Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015).

The complex and heterogeneous nature of EVs underlies both their enormous potential and the significant challenges related to their exploitation. The spotlight on EVs has raised high expectations, mostly relying upon the promise of EV-based technologies to revolutionize nanoscience, as well as offer competitive clinical and commercial solutions within broad industrial landscapes

(e.g., healthcare, personal care, and agrifood) (Zarovni et al., 2021). In line with this, substantial public funding, alongside corporate and venture investments, has supported the prominent academic and industrial engagement in Research and Development (R&D), paving the way to the first Product Development (PD) attempts. However, the shortage of reference materials, quality standards, and standardized methodologies for EV R&D and PD still challenges the ability to unravel the full spectrum of EV constituents and mechanisms of action. Consequently, this has posed a burden not only on comprehending the content and physio-pathological role of EVs, but also on the ability to manage and control their manufacturing reliably and sustainably.

Currently, EV bioprocessing in research and industrial settings is mostly dominated by non-standardized manufacturing systems. This enables researchers to keep up with the rapid innovations in the field. Nevertheless, it may also lead to the inevitable introduction of variability into EV-based products, resulting in inconsistencies in their structural identity (e.g., EV subset collection), quality, and safety, and accounting for reported discrepancies among different studies. By potentially and severely affecting data reliability and reproducibility, this overall lack of standardization and robustness has had a strong detrimental impact on the EV landscape. It also poses challenges to the rationale by which scientists select the most appropriate and tailored applications for any given EV source, as well as to the way they formulate and dose EVs. On top of that, the predominant EV bioprocessing procedures are still mostly research-compliant, characterized by considerable cost intensiveness and low-throughput performance (Agrahari et al., 2019; Clayton et al., 2019; Lener et al., 2015; Liguori & Kisslinger, 2021; Liguori & Kisslinger, 2022; Lötvall et al., 2014; Nieuwland et al., 2020; Paolini et al., 2022; Théry et al., 2018). Furthermore, so far, they have been able to address only small-scale preclinical development and/or integration into clinical trials featuring small patient cohorts (e.g., 10–500 enrollments), even at advanced study phases (i.e., phases 3 and 4) (clinicaltrials.gov, n.d.). Proper support for EV manufacturing and analytical assessment at an industrial scale, which requires extremely controlled standards of precision and has limited space for errors, remains an open issue.

In this challenging scenario, to prompt the translation of EV innovations into effective and marketable solutions, we likely need to make our efforts more streamlined and responsible. The delivery of Responsible Research and Innovation (RR&I) within the EV landscape should involve an increasing interconnection of R&D operations with supporting activities related to quality, data, and risk management, both beforehand and along the bioprocessing path (Liguori & Kisslinger, 2021; Owen et al., 2013). In this context, the strategic adoption of standardized and customizable working methodologies since early developmental phases could be crucial for ensuring reproducibility and reliability in compliance with Good Research Practice (GRP) and Good Manufacturing Practice (GMP) regulations (Hollmann et al., 2022).

Hereby, we describe the application of a multi-criteria decision-making (MCDM) matrix that can serve as an efficient, customizable, user-friendly, and transferable decision-making tool (DMT) model, specifically dedicated to EV features and related bioprocesses. In this manuscript, we showcase the adoption of our EV decision-making grid (EV-DMG) to streamline and improve the quality control (QC) of EVs derived from microalgae (i.e., nanoalgosomes). We demonstrate its effectiveness in promoting application-tailored EV classification, leading to the minimization of erroneous and cost-intensive misuses. Furthermore, we describe the great potential of implementing our EV-DMG to address the following: (i) maximization of EV bioprocess cost and time efficiency, targeting the achievement of desired quality and quantity performances; (ii) provision of traceable records of the whole EV PD life cycle, which can be later used for the compilation of the regulatory dossier relative to the final EV-based product. Overall, we underline the validity of the EV-DMG as a tool for steering complex decision-making throughout the entire EV value chain, from research to PD, with the ultimate goal of delivering responsible and sustainable EV R&I.

2 | METHODOLOGY

The workflow for the design of the EV-DMG is displayed in Figure 1. Overall, the process comprises four main steps: (i) identification of "alternatives," which are the EV-based preparations or processes to compare for evaluation and decision-making; (ii) definition of EV-related alternative assessment criteria and relative importance weights; (iii) determination of EV-related acceptance criteria and relative scoring system; iv) implementation of the MCDM method to integrate EV-related alternative assessment into a weighted sum model (WSM) matrix to rank the alternatives. The elements and concepts at the basis of our MCDM approach are elucidated in the following sections.

2.1 | Step 1–Identification of EV-related alternatives

An MCDM approach aims at generating preferences from different options or conditions under comparison (i.e., "alternatives") by considering and integrating more than one assessment criterion in the selection process (Triantaphyllou et al., 1998). Therefore, the first step defines the decision to be made and requires the listing of the alternatives to choose from. In both EV R&D and PD settings, there are some cautious decisions to make that are of core importance for the conduction of any upstream and downstream EV bioprocess. These can be allocated into the following categories: (i) EV sources; (ii) EV production, handling, characterization, and QC methods; (iii) EV applications. To maximize the performance of any EV-based product and process, optimal decision-making within each of these categories needs to be driven by specific assessment criteria set in advance.

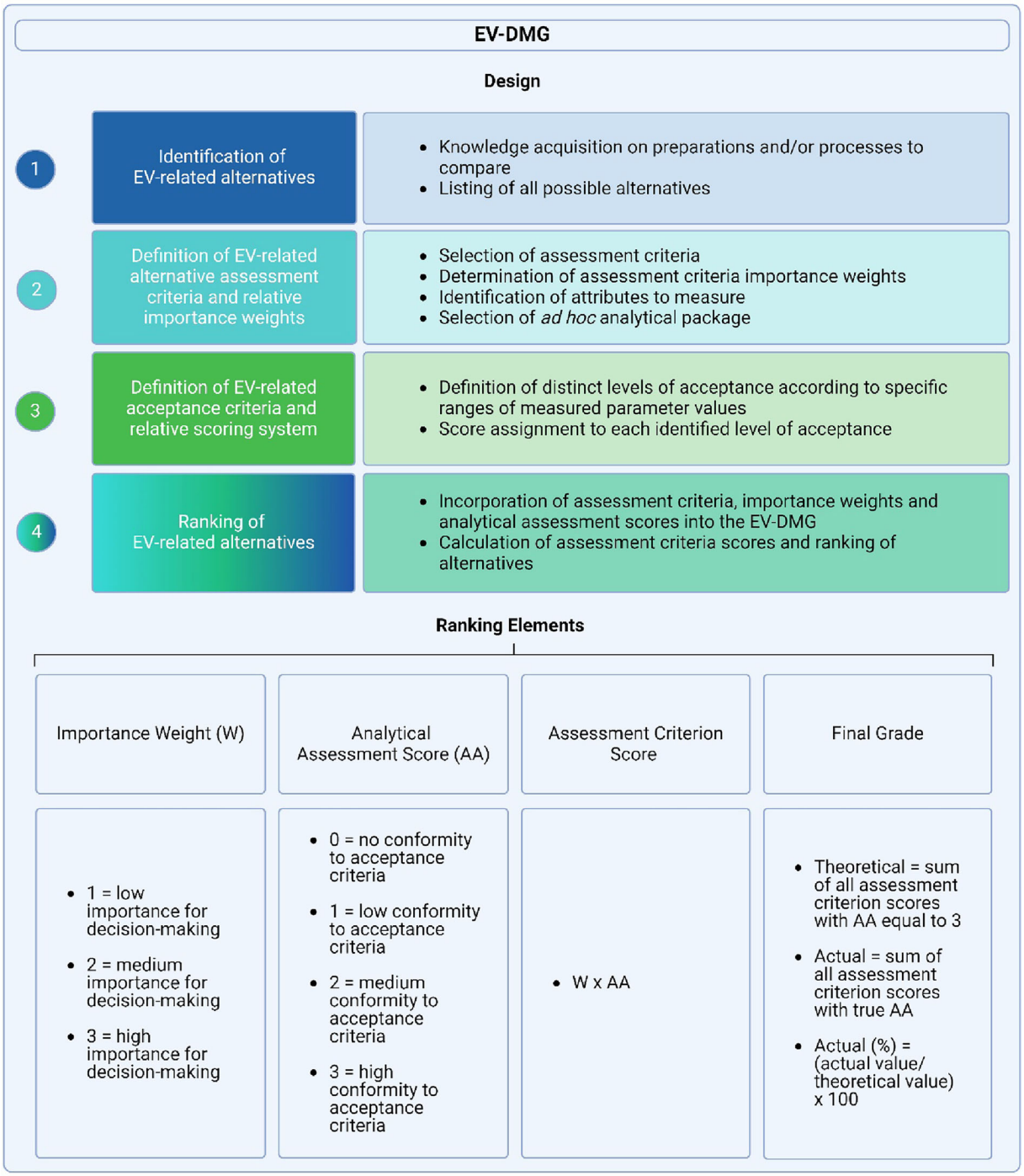

FIGURE 1 Proposed workflow for EV-DMG design and implementation. This figure was created with BioRender.com. EV-DMG, extracellular vesicledecision-making grid.

2.2 | Step 2–Definition of EV-related alternative assessment criteria and relative importance weights

To drive the selection of the best "alternative(s)" under evaluation, a set of qualitative and quantitative EV-related assessment criteria needs to be formulated. Each assessment criterion addresses a specific question and ultimately yields clear and measurable assessment results. Figure 2 outlines six major assessment criteria considered in R&D contexts to qualify EV-based products and processes, most of them adapted from the "checklist" reported in the "Minimal Information for Studies of EVs" (MISEV) 2018 guidelines (Théry et al., 2018). Newly released MISEV2023 guidelines do not re-propose an analogue of this checklist, but

Assessment Criteria	Assessment Sub-criteria	Importance Weights	Analytical Methods
Quantity	Particle number [per volume unit or original (tissue) mass/number of cells]	+++	(F-)NTA, (high resolution) FC, TRPS, EM, AFM, etc.
	Total protein content [per volume unit or original (tissue) mass/ number of cells]	+++	BCA, Bradford assay, SDS-PAGE, etc.
	(Total) lipid amount [per volume unit or original (tissue) mass/number of cells]	++	Sulfophosphovanilin assay, FTIR, TLC, phospholipid assay, cholesterol assay (depending on the source), etc.
	Ratios of two quantification indicators (*e.g.*, total particle number/total protein amount)	+++	/
Identity	Particle size distribution	+++	(F-)NTA, (high resolution) FC, DLS, EM, AFM, SRM, FCS, AF4-MALS, TRPS, etc.
	Structure/morphology	+++	(F-)NTA, (high resolution) FC, DLS, EM, AFM, SRM, FCS, AF4-MALS, TRPS, SAXS, etc.
	EV plasma membrane-derived or endosomal transmembrane or GPI anchored protein	+++	WB, ELISA, FC, etc.
	EV cytosolic protein with membrane binding or association capacity	+++	WB, ELISA, FC, PCR, etc.
	Presence/absence of expected contaminants	+++	WB, ELISA, FC, PCR, etc.
	Topology of the relevant functional components	+	Mild digestions, detergent permeabilization, antibody studies, etc.
	Surface charge	/	ZP measurement, etc.
Purity/Enrichment	Ratios of two quantification indicators (*e.g.*, total particle number/total protein amount)	+++	Assays for determining quantity and identity
	Presence/absence of expected contaminants	+++	
Potency/Functionality	Dose-response assessment	+++	*In vitro* [cell-based, cell-free (enzymatic assays] assays, *in vivo* assays
	Negative control assessment (conditioned medium, biofluid/ tissue from control donors, as applicable)	+++	
	Quantitative comparison of functional activity of total input material vs EV-depleted control vs EVs	+++	
	Quantitative comparison of functional activity of EV subtypes	+	
Integrity/Stability	Testing changes in quantity or quality attributes (identity and potency/functionality)	+++	Assays for determining quantity, identity and potency/functionality
Sustainability	Environmental footprint	+++	Life cycle assessment study
			Market analysis
	Costs of goods		Evaluation of economic feasibility
			Regulatory assessment and guidelines

FIGURE 2 Setting the EV-DMG metrics for EV R&D—alignment of assessment criteria, sub-criteria, importance weights, and analytical methods inspired by and adapted from MISEV2018. AF4-MALS, asymmetric flow field-flow fractionation-multiangle light scattering; AFM, atomic force microscopy; BCA, bicinchoninic acid assay; DLS, dynamic light scattering; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle; EV-DMG, extracellular vesicle decision-making grid; FC, flow cytometry; FCS, fluorescence correlation spectroscopy; (F-)NTA, (fluorescence) nanoparticle tracking analysis; FTIR, Fourier transform infrared spectroscopy; PCR, polymerase chain reaction; SRM, selected reaction monitoring; SAXS, small-angle x-ray scattering; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TRPS, tunable resistive pulse sensing; WB, western blot; ZP, Z-potential.

maintain the continuity with recommendations on the key qualitative and quantitative criteria to consider for upgrading EV study design and execution (Welsh et al., 2024). These criteria include quantity, identity, purity/enrichment, potency/functionality, stability/integrity, and sustainability. Each of these may be further subdivided into various sub-criteria addressed by different analytical methods and can be given a specific importance weight depending on its impact on the decision-making process. MISEV2018 proposed the following system for criteria scoring: +++ for mandatory criteria; ++ for mandatory criteria, if applicable to follow; + for encouraged criteria (Figure 2). We assumed that this weighing system would be equivalent to numerically assigning an importance weight ranging from 3 to 1, as later applied in our showcase and reported in Figure 3. It is important to specify that the list of assessment sub-criteria presented in Figure 2 is not exhaustive; rather, it includes some of the assessment sub-criteria that we deemed most relevant and commonly considered by the EV community.

2.3 | Step 3–Definition of EV-related acceptance criteria and relative scoring system

Formatting an EV-DMG based on MCDM principles necessarily requires the establishment of appropriate acceptance criteria and correspondent scoring. Acceptance criteria represent "numerical limits, ranges, or other suitable measures" (Elder, 2017), such as those based on non-numerical, qualitative assessments (e.g., visual analysis), that are relative to the qualitative and quantitative features of the alternative options under assessment. These criteria define the requirements that the products or processes under evaluation must meet to satisfy stakeholders' expectations and needs for an intended use. In an EV R&D environment, for each EV-related assessment (sub-)criterion, the acceptance value range needs to be empirically determined through specific, pre-validated analytical procedures. The performance of the samples or processes under assessment can be then ranked through a systematic scoring system, reflecting the different degrees to which the performance conforms to the pre-established requirements. The description of the acceptance criteria and respective scoring system selected for showcasing the application of our EV-DMG are reported in Section 3.3.

2.4 | Step 4–Ranking of EV-related alternatives

In our study, the relevant criteria selected for assessing alternatives had been systematically incorporated into a WSM matrix (Triantaphyllou et al., 1998). The general construction of a WSM matrix satisfies the following mathematical equation:

$$A^*_{\mathrm{WSM}} = \max_i \sum_{j=1}^{N} q_{ij} w_j \text{ for } i = 1, 2, 3, \ldots, M \quad (1)$$

in which: A^*_{WSM} is the WSM of the final score of the (best) alternative; M is the number of the alternatives to assess; N is the number of decision criteria; i is a single alternative; j is a single criterion; q_{ij} is the actual value relative to the ith alternative in terms of the jth criterion; W_j is the importance weight of the jth criterion (Triantaphyllou et al., 1998). Upon generation of the WSM matrix, for all alternatives under evaluation, an analytical assessment score (corresponding to q_{ij}) is given to each assessment criterion, based on its conformity to acceptance criteria. This is subsequently multiplied by its relative importance weight (W_j) to obtain an overall assessment criterion score. The sum of all assessment criterion scores provides a final, cumulative grade or score (A^*_{WSM}) relative to each alternative evaluated, which leads to the selection of the best alternative for a specific downstream application or to discarding. In the following subsections, we present an illustrative example demonstrating the generation and utilization of the WSM matrix, showcasing the EV-DMG.

3 | ILLUSTRATIVE EXAMPLE OF EV-DMG DESIGN AND APPLICATION FOR EV R&D

In this manuscript, we exemplify the setting, adoption, and utility of our EV-DMG to streamline the QC assessment and grading of microalgae-derived EVs (*viz.*, nanoalgosomes) (Adamo et al., 2021), chosen as model EVs. Its result addresses the consequent destination of our EV preparations to different uses within an exploratory scenario.

3.1 | Identification of nanoalgosome-related alternatives

Nanoalgosomes were extensively characterized and evaluated in several lines of research, exploring their potential as intrinsic effectors or drug delivery vehicles (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021; Picciotto et al., 2022). Our EV-DMG is suitable for a QC evaluation that allows EV batches of different quality to be shunted towards the most appropriate application, or to be discarded if failing to reach the desired standards. In this work, we showcase the application of the EV-

Assessment Criteria	Assessment Sub-criteria	Importance Weights	Analytical Methods
Quantity	Scattering particle number per volume unit	3	NTA
Identity	Scattering particle size distribution	2	NTA
	Morphology		AFM
	Membrane H+/ATPase expression		ELISA
	Surface charge		ZP measurement
Purity	Scattering particle number-to-protein content ratio	3	NTA/BCA
	Fluorescent particle number-to-scattering particle number ratio		(F-)NTA
	Aggregation of gold nanoparticles		CONAN assay
Functionality	Esterase activity	2	Esterase activity assay

FIGURE 3 Setting the EV-DMG metrics for nanoalgosome QC—alignment of assessment criteria, sub-criteria, importance weights, and analytical methods. AFM, atomic force microscopy; BCA, bicinchoninic acid assay; CONAN, colorimetric nanoplasmonic assay; ELISA, enzyme-linked immunosorbent assay; EV-DMG, extracellular vesicle decision-making grid; (F-)NTA, (fluorescence) nanoparticle tracking analysis; QC, quality control; ZP, Z-potential.

DMG for the comparative analysis and classification of three distinct nanoalgosome batches. Our case study was conducted within the frame of the Biogenic Organotropic Wetsuits (BOW) project [funded within the Horizon 2020 Future and Emerging Technologies (FET) Proactive Program in 2021], which aims at exploiting nanoalgosomes as scaffolds for the production of engineered EV membrane-coated magnetic bead devices (CORDIS—EU research results, n.d.). In this context, we have chosen (i) engineering and (ii) *in vivo* functional testing as primary nanoalgosome applications, tailored to meet the immediate needs of the BOW project Partners/users.

3.2 | Definition of nanoalgosome assessment criteria and relative importance weights

Our QC system was based upon a minimal but informative analytical package, comprising a feasible number of EV assessment methods, all having the characteristic of being user-friendly, time-effective, mostly transferable, and requiring low sample consumption. The following EV properties were assessed: (i) EV quantity [determined and expressed in terms of particle concentration by nanoparticle tracking analysis (NTA)]; (ii) identity [assessing scattering particle size distribution by NTA, morphology by atomic force microscopy (AFM), expression of nanoalgosome biomarkers (i.e., the plasma membrane protein H+/ATPase) by enzyme-linked immunosorbent assay (ELISA), and surface charge by particle Z-potential measurement]; (iii) purity [labelling samples with an EV-specific lipidic dye (i.e., di-8-ANEPPS) and measuring the percentage of fluorescent particles over the total number of scattering particles by Fluorescence NTA (F-NTA); determining the ratio between total particle number and total protein content (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021; Picciotto et al., 2022; Sverdlov, 2012); estimating purity from soluble co-isolated proteins by Colorimetric NANoplasmonic (CONAN) assay (Maiolo et al., 2015; Zendrini et al., 2020)]; iv) functionality [determining EV esterase activity as a proxy of EV functionality and membrane integrity by a proprietary EV functional enzymatic activity assay (Adamo et al., 2023)]. Considering the importance of the weighing system described in Section 2.2, we provided an importance weight equivalent of 3 out of 3 to EV quantity and purity, whereas an importance weight of 2 out of 3 was assigned to EV identity and functionality (Figure 3).

3.3 | Definition of nanoalgosome acceptance criteria and determination of relative scoring

To assign each nanoalgosome batch a specific quality score, a set of acceptance criteria was defined based on the published MISEV-inspired checklist of minimal information for studies of nanoalgosomes, which identified characteristics of

Assessment Criteria	Assessment Sub-criteria	Acceptance Criteria			
		Score 0	Score 1	Score 2	Score 3
Quantity	Scattering particle number/mL	x < 5E+10	5E+10 < x < 1E+11	1E+11 < x < 5E+11	x > 5E+11
Identity	Scattering particle size distribution (mode; nm)	x < 60	60 < x < 80	80 < x < 90	90 < x < 120
		x > 200	130 < x < 200	120 < x < 130	
	Morphology (number of rounded object(s)/10 total objects)	0	1-2	3-5	> 6
	Membrane H+/ATPase expression (signal-to-noise ratio/1E+10 particles)	x < 2	2 < x < 3	3 < x < 4	x ≥ 5
	Surface charge (ZP; mV)	x < -45	/	/	-45 < x < -10
		x > -10			
Purity	Scattering particle number-to-protein content (μg) ratio	x < 1E+8	x<2E+8	x<2E+9	2E+9 < x < 1E+10
		x > 1E+11	x > 5E+10	x > 1E+10	
	Fluorescent particle number/ scattering particle number (%)	0	0.5 < x < 3	3 < x < 10	x > 10
	Aggregation of gold nanoparticles	/	NO	Proportional to sample dilution with aggregation index above the 20% threshold	Proportional to sample dilution with aggregation index below the 20% threshold
Functionality	Esterase activity (nmol/min/ 5E+10 particles)	x < 0.5	0.5 < x < 1	1 < x < 2	x > 2

FIGURE 4 Setting the EV-DMG metrics for nanoalgosome QC—alignment of assessment criteria, sub-criteria, and acceptance criteria with relative scoring. EV-DMG, extracellular vesicle decision-making grid; QC, quality control; x, the measure of the corresponding parameter; ZP, Z-potential.

nanoalgosome preparations (Adamo et al., 2021). Next, we empirically classified nanoalgosome preparations as either high-quality (positive) or low-quality (negative), according to their performance and suitability for our desired applications. The set of acceptance criteria relative to each nanoalgosome assessment sub-criterion was complemented by a scoring system. This system assigned a specific analytical assessment score to the obtained measurement results, describing to what extent the sample met the defined requirement for a specific assessment criterion and sub-criterion. Specifically for the work presented here, we defined a scoring system featuring an evaluation scale as follows: 0 = null; 1 = low; 2 = medium; 3 = high (Figure 4).

Assessment Criteria	Assessment Sub-criteria	Importance Weights (W)	Nanoalgosome Batch 1		Nanoalgosome Batch 2		Nanoalgosome Batch 3	
			AA	AA x W	AA	AA x W	AA	AA x W
Quantity	Scattering particle number per volume unit	3	3	9	3	9	3	9
Identity	Scattering particle size distribution	2	3	6	3	6	3	6
	Morphology		3	6	2	4	3	6
	Membrane protein H+/ATPase expression		2	4	0	0	1	2
	Surface charge		3	6	3	6	3	6
Purity	Scattering Particle number-to-protein content ratio	3	3	9	1	3	3	9
	Fluorescent particle number/ scattering particle number (%)		2	6	1	3	1	3
	Aggregation of gold nanoparticles		3	9	1	3	3	9
Functionality	Esterase activity	2	2	4	1	2	1	2
Actual Final Grade				59/66		36/66		52/66
Actual Final Grade/ Theoretical Final Grade (%)				89.4		54.5		78.8

FIGURE 5 EV-DMG for nanoalgosome QC. EV-DMG, extracellular vesicle decision-making grid; QC, quality control.

3.4 | Ranking of nanoalgosome-related alternatives

Figure 5 represents the EV-DMG compiled for the three analyzed nanoalgosome batches. The results obtained from the NTA analysis on nanoalgosome Batch 1, Batch 2, and Batch 3 showed differences in terms of particle concentration (Batch 1 = 9.1E+11 ± 4.1E+10 particles/mL; Batch 2 = 8.6E+12 ± 4.6E+11 particles/mL; Batch 3 = 7.2E+11 ± 2.2E+10 particles/mL; Figure S1-A), but a quite overlapping size distribution, peaking at approximately 100 nm (Figure S1-B1). No significant differences between the three batches were revealed by Z-potential measurements, indicating an analogous particle membrane surface charge (Figure S1-B4). However, the expression of nanoalgosome plasma membrane protein H+/ATPase, measured by ELISA, showed a greater signal-to-noise ratio (SNR) per particle in Batch 1 and Batch 3, with Batch 1 displaying the highest readout (Figure S1-B2). Batch 1 and Batch 2 exhibited a particle-to-protein ratio (Figure S1-C1) that deviated the least and the most, respectively, from the theoretical optimal ratio suggested by Sverdlov (2012) [Batch 1 =˜ 3.9E+9 particles/protein content (μg); Batch 2 =˜ 7.3E+10 particles/protein content (μg)]. This ratio states that 1 μg of EV preparation proteins corresponds to 2E+9 EVs and served as the basis for our nanoalgosome purity grading system. In line with these observations, F-NTA of fluorescently labelled samples demonstrated the presence of an inferior content of non-EV particles in Batch 1, compared with the other batches. This was highlighted by the different percent ratio of fluorescent particles over total particles detected in scattering mode, which corresponded to 3.3% for Batch 1, 1% for Batch 2, and 1.3% for Batch 3 (Figure S1-C2). This was further validated by the CONAN assay (Figure S1-C3), showing that Batch 1 and Batch 3 could be considered pure from protein co-isolates within the limit of detection (LOD) of the assay (20–50 ng/μL). Indeed, the Aggregation Index (AI) % of Batch 1, which is initially

Nanoalgosome QC Classification System						
Level	Description	Actual/Theoretical Grade (%)	Use(s)			
			No	Not Critical	Engineering	Functional Study
A	High Quality	70 ≤ x ≤ 100		x	x	x
B	Medium Quality	30 < x < 70		x	x	
C	Low Quality	< 30	x	x		

FIGURE 6 EV-DMG-based QC classification of nanoalgosomes. EV-DMG, extracellular vesicle decision-making grid; QC, quality control; x, the relationship indicator of two corresponding parameters.

above the threshold, decreased below assay LOD upon dilution. This behaviour is typical in pure samples rich in EVs (dilution is needed to match the assay working range). In Batch 3, the EV concentration was already in the CONAN assay optimal range, with the AI% stabilizing below the LOD even without diluting the sample. On the contrary, Batch 2 displayed a constant AI%, regardless of the dilution tested. Therefore, such samples either (i) did not contain enough EVs to trigger complete pristine gold nanoparticle aggregation or (ii) carried a significant amount of protein co-isolates. To verify option (i), increased amounts of sample volume (up to 5X the amount normally used to run the assay) were tested, leading to the same result. Therefore, Batch 2 could be considered contaminated by co-isolated proteins. In support of these results, AFM highlighted elongated objects in Batch 2, potentially originating from microalgal cells or the detachment of tangential flow filtration (TFF) cartridge fibres (Figure S1-B3). Consistently with these highlights, Batch 1 showed the highest esterase activity per particle (1.36 ± 0.01 nmol/min/5E+10 particles), indicating its superior functionality and membrane integrity, compared with the other two batches. Nevertheless, the particle enzymatic activity assessed in Batch 2 and Batch 3 revealed a comparable readout, with a slightly higher value detected in Batch 2 (Batch 2 = 0.80 ± 0.07 nmol/min/5E+10 particles; Batch 3 = 0.67 ± 0.00 nmol/min/5E+10 particles; Figure S1-D).

As shown in Figure S2, the maximum theoretical final grade, corresponding to 66, was calculated by summing up all assessment criterion scores obtained by multiplying the importance weight of each assessment criterion by the maximum assignable analytical assessment score (i.e., 3). The observed experimental discrepancies between the three tested batches translated into different analytical scores on our EV-DMG (Figure 5). The sum of all assessment criterion scores resulted in an actual final grade equivalent to 59/66 for Batch 1 (89.4% of the theoretical final grade), 36/66 for Batch 2 (54.5% of the theoretical final grade), and 52/66 for Batch 3 (78.8% of the theoretical final grade). As illustrated in Figure 6, depending on the final grade obtained, each batch could be classified into one of three QC classification levels: level A, for batches of good quality, appropriate for all uses, once confirmed to be sterile (actual final grade equal to 70%–100% of the theoretical final grade); level B, for batches of medium quality, for engineering and other uses, excluding functional testing (actual final grade equivalent to a value ≥ 30% up to < 70% of the theoretical final grade); level C, for batches of poor quality, to be discarded or used only for non-critical analyses (actual final grade inferior to 30% of the theoretical final grade). Accordingly, the use of our EV-DMG to streamline our nanoalgosome QC assessment highlighted, straightforwardly and objectively, the suitability of Batch 1 and Batch 3 to be exploited for any downstream uses, including *in vivo* applications, and the potential for Batch 2 to be used only for engineering and not functional analyses. The accuracy and actionability of this ranking system have been proven through feedback from the BOW project Partners/users, who had an opportunity and a mandate to test the three batches, and report the results that were in line with the EV-DMG-predicted nanoalgosome performance.

4 | DISCUSSION

Decision-makers have historically adopted computational or mathematical DMTs to manage and mitigate the technical, logistic, and economic risks associated with the complex set of factors affecting the dynamics of systems in numerous industrial manufacturing contexts (i.e., from aerial and automotive vehicles to biotherapeutics) (Chhatre et al., 2007; Rao, 2007; Rekhi et al., 2015; Sutton et al., 2020; Tan et al., 2014). DMTs have also been applied in public research environments to help identify the most promising project aims, directions, and activities (Bongiovanni et al., 2015; Digilio et al., 2016). In early 2019, the EV landscape first embraced the notion of implementing DMTs for assisting research- and clinical-grade EV-based PD. Specifically, Ng and co-authors developed a novel computational DMT to identify combinations of technologies for upstream cell expansion and downstream EV harvesting to promote upscaling, while addressing the minimization of the cost of goods (COGs) (Ng et al., 2019). More recently, Picciotto et al. (2021) have adopted a WSM-based DMT for addressing the selection of the microalgal sources best suited to produce microalgae-derived EVs (*viz.*, nanoalgosomes) (Picciotto et al., 2021). Nevertheless, a comprehensive and straightforward decision-support framework to drive and streamline complex decision-making at each stage of EV R&D and PD has not been proposed yet.

Assessment Criteria	Assessment Sub-criteria	Degree of Recommendation		
		R&D (MISEV2018)	PD of MSC EV-based medicinal products (Witwer et al., 2019)	PD of EV-based medicinal products (Silva et al., 2021)
Quantity	Particle number [per volume unit or original (tissue) mass/number of cells]	+++	+++	+++
	Total protein content [per volume unit or original (tissue) mass/number of cells]	+++	+++	+++
	(Total) lipid amount [per volume unit or original (tissue) mass/number of cells]	+	+	+
	Ratios of two quantification indicators (*e.g.*, total particle number/total protein amount)	+++	+++	+++
Identity	Particle size distribution	+++	+++	+++
	Structure/morphology	+++	+++	+++
	Plasma membrane-derived or endosomal transmembrane or GPI anchored protein	+++	+++	+++
	Cytosolic protein with membrane binding or association capacity	+++	/	/
	Presence/absence of expected contaminants	+++	+++	+++
	Topology of the relevant functional components	+	/	+++
	Surface charge	/	/	+/++
Purity/Enrichment	Ratios of two quantification indicators (*e.g.*, total particle number/total protein amount)	+++	+++	+++
	Presence/absence of expected contaminants	+++	+++	+++
Potency/Functionality	Biological activity testing	+/++/+++	+++	+++
Integrity/Stability	Testing changes in quantity or quality attributes (identity and potency/functionality)	+++	+++	+++
	Biophysical destabilization tests	/	/	++
Sterility	Microbiological security check and clearance	/	+++	+++
Sustainability	Environmental footprint	/	+++	+++
	Costs of goods			

FIGURE 7 Setting the EV-DMG metrics for EV PD—alignment of assessment criteria, sub-criteria, and importance weights deduced from the MISEV2018 guidelines (Théry et al., 2018), Silva et al. (2021), and Witwer et al. (2019). EV-DMG, extracellular vesicle decision-making grid; PD, product development.

To address this need, in the current work, we targeted the development and implementation of a DMT suitable for managing the multidimensionality of the decision-making issues challenging EV R&D and PD. Specifically, we focused our attention on a DMT based on the MCDM approach, which is known to provide a valid, reliable, and straightforward RR&I-tailored approach to make unbiased selections and classifications within complex sets of alternative options. This strategy is also noted for creating opportunities for cost and time savings, besides contributing to increased data reliability and reproducibility. Overall, it allows decision-making to take the lead over decision-control activities, enhancing project performance and simplifying project administrative work (Rao, 2007; Silva et al., 2021; Stilgoe et al., 2020; Taherdoost & Madanchian, 2023; Zarghami et al., 2011).

The EV-DMG outlined in this work was conceived to offer a user-friendly and transferable MCDM matrix model for addressing any uncertainty related to EV bioprocessing decisions. The design and working principles of the EV-DMG (described in Section 2) are applicable to any EV type, use, and bioprocessing context. However, the selection of the specific assessment criteria and importance weights to base the construction of the EV-DMG upon must be adjusted to the specific context of use, as

well as be built on empirical observations related to the key features of the products or processes that are targeted for evaluation (showcased in Section 3).

To demonstrate the implementation of the EV-DMG in a practical working scenario, we decided to present a case study illustrating its use as a straightforward and effective QC-support tool. This work was conducted within the frame of a multicentric and multidisciplinary research project at an early developmental phase, in which we were projected to tackle several potential usage venues for the EV types that were under investigation, ranging from their employment as substrates for engineering to their application as nano-vehicles or intrinsic effectors. Among the EV types studied in this early-stage research context, we selected nanoalgosomes as a less explored model EVs, suitable to validate our approach. Our findings showed that the adoption of the EV-DMG system to streamline nanoalgosome QC allowed us to go beyond the simple nanoalgosome grading for a single intended use. Rather, this method demonstrated the potential to maximize the full potential of the QC process by sorting the tested nanoalgosome preparations towards different potential applications that best suited their quality and quantity. Furthermore, this study exhibits the potential of the EV-DMG-based approach to tailor EV production design, scale, and performance to evolving goals and priorities concerning specific products and/or uses to target.

The design of the EV-DMG exemplified in this study was originally conceived taking into consideration the MISEV2018 guidelines. In a summary "checklist", reported in the final section of the document, MISEV2018 piloted the first attempt to assign specific importance weights to a comprehensive set of methodological criteria addressing the key EV features to study for conducting good EV science (Hendrix et al., 2023; Théry et al., 2018; Van Deun et al., 2017). However, it did not diversify the importance of each methodological criterion according to chosen study scopes, adopted EV sources, and envisaged EV-based application(s). Rather, it proposed a "one-scoring-fits-all-or-most" model, where the importance weights associated with the proposed methodological framework were averaged based on prevailing opinions within the EV community (Théry et al., 2018). Thus far, these metrics have served as a cornerstone for improving the quality of EV R&D results and provided an excellent benchmark to early industrial EV innovators. An updated version of the MISEV guidelines has been recently published, mostly focusing on strengthening recommendations for upgrading EV study design and execution (Welsh et al., 2024). MISEV2023 did not offer a checklist as the one reported by MISEV2018. Nevertheless, its basic principles appear in agreement with the fundamental notions contemplated in MISEV2018. Despite being inspired by the MISEV guidelines, we realized that the methodological criteria and weighing systems are likely to be determined in a case-by-case manner. Certainly, it would be ideal and wise to include as many EV assessment criteria as possible in a research-grade EV-DMG, especially at an early developmental stage (Silva et al., 2021). However, it is intuitive that their selection, weighing, and technical feasibility would still depend on the specific research objectives, study context, and logistics. For instance, it is more likely that preserving EV integrity and potency/functionality is more critical for researchers intending to use their model EVs as intrinsic effectors, than for those who are going to engineer them with heterologous moieties or cargos. Conversely, quantity is likely to be critical for dosing EVs in general and might be universally weighted as a highly impactful parameter. Furthermore, high purity and the absence of any other non-vesicular contaminants may have a very different degree of importance in distinct applications. Finally, the selection of technologies is often guided by pragmatic criteria of accessibility, cost, or time restraints, which can considerably vary across the research community. This supports the conduction of exploratory studies within multidisciplinary consortia with pooled expertise and infrastructure.

The integration of the EV-DMG into routine EV bioprocessing practices may arise as a strategical move to dynamically frame PD and manufacturing, with early reference to the later submission of a product Common Technical Document (CTD) or dossier. The EV-DMG could be aligned with and merged into the Target Product Profile (TPP) of the prospective product to be developed. The TPP is an evolving document, the formulation of which is recommended from the product design phase to guide benchmarking, provide a comprehensive recapitulation of the targeted product's attributes, and improve regulatory dialogue for more efficient regulatory review and approval times (Breder et al., 2017). National Regulatory Authorities (NRAs), including the European Medicines Agency (EMA) and the United States Food and Drug Administration (US FDA), have been increasingly exploiting Multi-Criteria Decision Analysis (MCDA) to regulate approval decisions to optimize trade-offs between risks and benefits (Chisholm et al., 2022). This suggests that the adoption of MCDM-based DMTs to target clinical validation and acceptance by the NRAs could improve communication and understanding between innovators and regulatory bodies about product value propositions. This would ultimately enhance the likelihood of shortening the PD life cycle and speeding up market approval. Moreover, the EV-DMG template that we propose may be easily handled by common software, and its associated numerical values could be turned into algorithms, providing an interface for unbiased and clear gatekeeping decisions.

5 | LIMITATIONS OF THE STUDY

In this study, we propose the EV-DMG as a flexible and evolving tool, which not only can, but must be adjusted and empirically validated in the context of the specific application scope that is envisaged. Therefore, we prompt its integration into additional case studies, with a view to illustrating its applicability to other, possibly any EV type, use, and bioprocessing setting, both in R&D and PD environments. Verifying reproducibility was not included as part of the current work, and translating the EV-DMG into a PD context will likely require the collection of empirical data from a larger number of replicates from independent production cycles. The selection of our analytical package for QC testing was minimal and specifically tailored to nanoalgosome

Considerations for setting up the EV-DMG metrics for EV product development

MISEV2018 principles have been a cornerstone for early EV-DMG design. However, recent position papers focusing on the development of next-generation EV-based therapeutics have identified a need to comply with regulatory and economic requirements to be met in clinical and industrial settings (Silva et al., 2021; Witwer et al., 2019). These prompt new considerations regarding the blueprint for a clinical- or industrial-grade EV-DMG (Figure 7), many of which have been included in our nanoalgosome specifications for increasing the maturity of nanoalgosome-based PD.

Purity

The requirement of high purity at the expense of potency should be revisited when targeting EV-based therapeutics, as some "co-isolates", possibly associated with the EV "protein corona", are known to potentially correlate with desired sample biological activity. Furthermore, it is widely recognized that a completely pure EV preparation is unlikely to be produced (Palviainen et al., 2020; Tóth et al., 2021). Therefore, in the construction of an EV-DMG, this may translate into the reduction of the importance weight relative to purity (e.g., importance weight of 2/3 instead of 3/3) or into the delineation of alternative acceptance criteria and relative scoring systems.

Functionality

If the evaluation of EV functionality is not imperative in a research-grade scenario, depending on the investigational purposes, fulfilling specific potency requirements represents a very critical aspect when developing products for in-human use (Silva et al., 2021; Witwer et al., 2019). This could require the adjustment of the importance weight attributed to the assessment of EV functionality in research settings, raising it to 3/3 in an EV-DMG for use in clinical environments (e.g., therapeutics, vaccines, and drug delivery).

Sterility and sustainability

The EV community is currently encouraging to encounter sterility and economic sustainability from the premature research phases to minimize safety concerns and avoid developmental setbacks at more advanced therapeutic development and manufacturing stages (Committee for Medicinal Products for Human Use, 2012; European Medicines Agency, n.d.; Guideline IH, 2011). In this scope, early adding microbiological security, cost, and time management to the panel EV assessment criteria configuring the EV-DMG (potentially with a high importance weight score) could help allocate resources into fair and best-suited categories of use, enabling us to make financial trade-offs and prevent cost-intensive misuses.

assessment, as well as to the needs and requirements of our project Partners. Although in basic and discovery research it is useful to include diverse alternative and orthogonal methodologies—and the design of the EV-DMG lends itself to the addition of any new emerging ones—the adoption of a comprehensive set of analytical tools to perform nanoalgosome QC did not fall within the scope of this study. To select our panel of EV assessment methodologies, we aimed at balancing the requirement for proper evaluation of nanoalgosome characteristics with method accessibility and cost-effectiveness. Specifically, we opted for a concise but informative analytical panel that meets the following criteria: (i) robustness; (ii) sensitivity; (iii) affordable sample consumption, in order not to allocate more than 30% of the total produced EV batch to QC; (iv) ease of use; (v) medium-to-low turnaround time; (vi) cross-site transferability. This rationale was in line with recommendations diffused and accepted across the EV community, suggesting to upscale the analytics along with EV manufacturing development (Paolini et al., 2022).

6 | CONCLUSIONS

The EV-DMG proposed in this work offers a user-friendly MCDM matrix model for addressing a wide range of uncertainties inherent in EV bioprocessing decisions. These include, but are not limited to, selecting the best "fit-to-the-purpose" EV sources and starting materials, choosing the most suitable methods for optimal EV sample extraction, purification, handling, and QC, as well as determining the best-fitting EV applications based on sample properties. Our approach emphasizes the significance of integrating RR&I principles into routine EV R&I practices. Specifically, we propose early conception, development, and adoption of multi-dimensional methodological strategies, such as our EV-DMG, for responsible EV R&I decision-making. This is intended to promote robust, sustainable, ethically acceptable, and socially desirable outcomes of EV R&I. On the one hand, the EV-DMG is presented as a tool that facilitates accountable and impartial qualification of EV-based products and bioprocessing decisions. On the other hand, we highlight its potential for tailoring resources and products towards *ad hoc* applications based on specific requirements, enabling early adjustments in designs and scales to maximize performance and cost-effectiveness. This is especially relevant for enhancing the prospects of successfully translating R&D efforts into regulatory-compliant marketable

solutions. Indeed, by providing traceable records throughout the EV PD life cycle, the EV-DMG will support the compilation of comprehensive regulatory dossiers for EV-based products.

AUTHOR CONTRIBUTIONS

Francesca Loria: Conceptualization (lead); data curation (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing—original draft (lead). **Sabrina Picciotto**: Conceptualization (supporting); data curation (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing—original draft (supporting); writing—review and editing (supporting). **Giorgia Adamo**: Conceptualization (supporting); data curation (equal); investigation (equal); methodology (equal); validation (equal); writing—original draft (supporting); writing—review and editing (supporting). **Andrea Zendrini**: Investigation (equal); validation (equal); writing—review and editing (supporting). **Samuele Raccosta**: Investigation (equal); validation (equal). **Mauro Manno**: Investigation (equal); resources (equal). **Paolo Bergese**: Funding acquisition (lead); resources (equal); writing—review and editing (supporting). **Giovanna L. Liguori**: Conceptualization (supporting); writing—review and editing (supporting). **Antonella Bongiovanni**: Conceptualization (lead); methodology (lead); resources (equal); supervision (equal); writing—review and editing (lead). **Nataša Zarovni**: Conceptualization (lead); methodology (lead); resources (equal); supervision (equal); writing - original draft (supporting); writing—review & editing (lead). Nataša Zarovni and Antonella Bongiovanni equally contributed to this work.

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CONFLICT OF INTEREST STATEMENT

The authors declare the following financial competing interests: Antonella Bongiovanni and Mauro Manno have filed a patent (PCT/EP2020/086622) related to microalgae-derived extracellular vesicles. Antonella Bongiovanni, Sabrina Picciotto, and Giorgia Adamo have filed an Italian patent (Patent: No. 102023000004503) related to the Esterase Activity Assay. Antonella Bongiovanni and Mauro Manno are co-founders, and Antonella Bongiovanni is CEO, of EVEBiofactory s.r.l. Nataša Zarovni is an independent researcher that currently acts as CTO of EVEBiofactory s.r.l. The remaining authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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