

Peer Review File

Mapping bacterial extracellular vesicle research: insights, best practices and knowledge gaps



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Comments to the Authors

Major

1. The authors did not include a conflict of interest statement about EV-TRACK, which is crucial given that they are the founders of EV-TRACK. In the spirit of transparent reporting, we request the authors to add a conflict of interest statement to the manuscript.

2. We appreciate the authors' efforts to identify current challenges and knowledge gaps in BEV research, propose recommendations, and provide future outlooks. We fully acknowledge the importance of standardized and transparent reporting. However, we believe that the second aim of the manuscript is to promote EV-TRACK and to further advance the it for BEV research (integrate experimental parameters). We kindly ask the authors to add the second aim to the manuscript, as its omission appears to compromise transparency.

3. The authors state “We analyzed 845 BEV-related publications published between 2015 and 2021 using the EV-TRACK knowledgebase (Supplementary Table 1)”. How many BEV-related publications were published from 2015-2021? How many publications on eukaryotic EVs were published from 2015-2021? Please elaborate how representative the 854 BEV-related publications are for the years 2015-2021? Furthermore, we would like the authors to add a statement about this to the paper.

4. When reading the Introduction and Method sections (Screening, data extraction and quality assessment), it is unclear if the EV-TRACK tool was used by independent researchers/research groups that chose to use the tool themselves or if the authors added the studies to EV-TRACK.

The following statements indicate that independent research groups added the studies to EV-TRACK. “Full-text screening was conducted independently by the individual submitting to EV-TRACK and EV-TRACK administrator S.D.V.” (Lines 375-376).

However, the following statements indicate that the authors themselves added the studies to EV-TRACK. “All 845 studies were submitted to the EV-TRACK knowledgebase...” (Line 402). “Full-text screening, submission to the EV-TRACK knowledgebase and collection of additional data not included in the EV-TRACK knowledgebase was performed by N.D.L....” (Lines 437-438). Please comment on what has been done to conduct this study. Furthermore, in case that the authors added the studies themselves, please add a clear statement about this to the Introduction as well as the Method section.

Minor

5. For all Figures the font size of the axis titles and legends are extremely small. We kindly ask the authors to increase the font size for all to increase readability. The same applies to Supplemental Figures (e.g. Supplemental Figure 1).

6. EV-TRACK was published in 2017 to increase transparent reporting. The study presented in the manuscript contains BEV-related studies published between 2015 and 2021. How did transparent reporting regarding BEV research improve since EV-TRACK was launched?

7. Readability seems to be a recurring problem in this manuscript. Please try to improve readability of long sentences by e.g. splitting them.

Examples are:

- “However, the complexity and challenges associated with BEV research are considerable: 1) a plethora of methods are available for BEV preparation and characterization, 2) a diversity of bacterial strains releases heterogeneous BEV subsets, 3) sources from which BEVs are retrieved contain other extracellular particles with comparable biophysical and biochemical features, and 4) to date, no consensus exists on universal BEV markers 1,6.” (Lines 70-74)

- “BEVs from gram-negative bacteria are more abundantly analyzed (77%, 2560/3338), with *Escherichia coli* and *Pseudomonas aeruginosa* representing respectively 22% (742/3338) and 6% (201/3338) of experiments, compared to BEVs from gram-positive bacteria (16%, 526/3338), with *Staphylococcus aureus* representing 7% (235/3338) of experiments (Figure 2a; Supplementary Table 2).” (Lines 112-116). This is just one example from the Result section. Please address this.

8. In lines 110 – 111 the authors state: “The vast majority of experiments (94%, 3123/3338) analyzes BEVs in media conditioned by bacterial cells under laboratory conditions.” Please define laboratory conditions. Did all studies included in this manuscript that used BEVs from cell culture supernatant use the same conditions e.g. culture time, used media or chemical components, incubation time, temperature?

9. In Figure 1 the authors split the parameters into preparation and characterization: biophysical and characterization: biochemical. Please use the same structure in the Result section (pages 6 – 7).

10. In Figure 1 authors add “FC: bead size” as a biophysical characterization. The characterization of beads is not directly connected to BEV characterization. Please comment on why this has been added here.

11. The authors report that the size has been reported. “68% of experiments performing particle characterization (1216/1797) reports particle size (Figure 1), with the majority implementing NTA (45%, 546/1216), followed by EM (36%, 434/1216), and DLS (30% 361/1216) (Figure 1; Figure 2c). Average reported particle sizes of BEV preparations for frequently studied phyla Pseudomonadota (63%, 770/1216), Bacillota (14%, 174/1216) and Bacteroidota (3.5%, 43/1216) are respectively 118 nm ($\pm$ 78 nm), 158 nm ($\pm$ 277 nm) and 141 nm ($\pm$ 92 nm)...”(Lines 184-188). The authors switch between reported particle size and BEV size. Please comment on what is meant here. Furthermore, is there any proof that particles detected with e.g. NTA are indeed BEVs? Or are they non-BEV particles present in the samples? Please comment on this and add a clear limitation statement about this type of analysis to the manuscript.

12. The authors state “Of note, the reported particle size depends on the used method; smaller 197 particle sizes are reported using DLS and EM compared to NTA (Figure 3a)15,19” (Lines 196-197). While we agree that the reported particles size depends on the used method, we miss a statement that the reported particle size also highly depends on the sensitivity of the chosen method and the lower detection limits. Please add a clear statement about this to the paper.

13. Figure 3 a shows the average particle size (nm) per experiment for most abundant phylum Pseudomonadota measured with DLS, EM, NTA. In Figure 1 also includes flow cytometry for characterization. Why is this not included in Figure 3?

14. “Systematically cataloguing biochemical and biophysical characteristics, supports data validation and contributes insights into BEVs residing in diverse natural habitats. Furthermore, characterization of BEV preparations allows to identify what is present (presumably BEVs) and what is not (presumably other extracellular particles), supporting data interpretation and conclusion-making.”. We agree with the authors that this will help to promote reproducibility of BEV research. However, we lack a statement about assay controls to insure that researchers are indeed detecting BEVs and not other non-BEV particles.

15. In the list of key words a ; is missing behind “Plant” (Line 25).

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

The manuscript titled "Bacterial Extracellular Vesicle Analysis: Challenges and Opportunities" explores the complex and evolving field of bacterial extracellular vesicles (BEVs). It emphasizes BEVs' potential roles as biomarkers and therapeutic agents due to their ability to facilitate communication across species and environments. The manuscript critically examines the diversity of BEVs in terms of their biogenesis, composition, and functions, which vary significantly among bacterial species. Here is a detailed review of the manuscript :

1.The manuscript mentions the inconsistent use of terminology like "outer membrane vesicle" across the studies analyzed, which can be confusing and misleading. The authors should advocate for stricter guidelines on nomenclature in the discussion of their results. This could involve setting clearer definitions and usage examples for each term based on the biogenesis and composition of the vesicles. Providing a standardized glossary for all terms related to BEVs at the beginning of the manuscript could help ensure consistent use across the board.

2.The manuscript points out that many studies do not report critical experimental parameters, which hampers reproducibility. Strongly emphasize the need for increased data transparency in the manuscript. Recommend the inclusion of a supplementary section that lists all parameters that should be reported by future studies. This could help increase the EV-METRIC scores and improve the overall quality of BEVs research.

3.While the study compiles an extensive amount of data, there is a lack of discussion on the biological or clinical relevance of the findings. Expand the discussion of results to include more on the potential implications of the findings for clinical and environmental applications. This could involve a separate section that hypothesizes how variations in BEVs properties might influence their role in disease, treatment, or monitoring environmental health.

4.The manuscript would benefit from a more detailed explanation of the statistical methods used to analyze the data. Include information on the statistical tests performed, justification for their use,

and interpretation of results. This could involve the addition of a subsection on statistical methodology within the Materials and Methods section.

5. Review and improve the clarity and presentation of figures and tables. Ensure that all graphical data are easy to understand and adequately labeled. This may involve revising existing figures or adding new ones to better illustrate key findings.

6. The manuscript should provide a more detailed description of the sample collection and handling procedures, especially when discussing the collection of BEVs from natural habitats like ocean seawater, plants, and human body fluids. Specific details such as the volume of samples collected, the storage conditions before processing, and any pre-treatment steps should be clearly outlined. This will help ensure that other researchers can accurately replicate the study and assess the impact of sample handling on BEVs integrity and analysis.

The reviewer suggests the following articles related to the BEVs for the authors to refer to in order to revise the manuscript:

1. Bioact Mater. 2021 Dec 17;14:169-181. doi: 10.1016/j.bioactmat.2021.12.006.
2. Theranostics. 2022 Sep 11;12(15):6576-6594. doi: 10.7150/thno.78034.
3. Compos Part B-eng. 2023 Apr 15;110610. doi:10.1016/j.compositesb.2023.110610.
4. Compos Part B-eng. 2023 Dec 11;1047. doi:10.1016/j.compositesb.2023.111047.

Reviewer #4 (Remarks to the Author):

In the manuscript “Bacterial extracellular vesicle analysis: challenges and opportunities” De Langhe and colleagues provide an overview of the recent literature (from 2015 to 2023) on bacterial extracellular vesicles, highlighting the heterogeneity in methods and analytical techniques used to study them. The manuscript is detailed and well written and highlights certain areas where consistency of techniques would improve reproducibility and draws attention to the relative lack of studies on BEVs in natural environments.

Despite some interesting observations the manuscript is limited in scope and significance. The primary overall observation is that there exists a lot of variation in how BEVs are isolated and

studied, and does not go on, for example, to outline or suggest best practices for these procedures. I have additional concerns with the manuscript in its current form which are outlined below and need to be addressed.

The manuscript is submitted as a review article, however I'm not sure this is entirely appropriate. A large amount of data analysis was performed, and results generated (along with figures), making this feel more like a research paper than a review. For example it is reported that there is a large degree of variation in the methods used to isolate BEVs (and a number of figures provided to support this) however there is no discussion of the individual techniques, and the pros and cons of each....which are points I would expect to see in a review article. Instead, it is simply reported what percentage of studies use each technique. This makes the manuscript more of a survey than a review.

EV-TRACK and EV-METRIC are mentioned throughout the manuscript however the authors fail to properly introduce these terms, concepts and their history. This makes for a confusing read. The original publication that generated EV-TRACK and EV-METRIC should be discussed in detail in the introduction along with clearly stated goals for how this manuscript builds upon that work and integrates with it.

Throughout the manuscript the following term appears approx. 8-9 times (cfr. "Assess quality by evaluating the EV-METRIC"). What does this mean? No explanation is given.

COMMENTS FROM ECR:

The study by De Langhe et al. analyzed bacterial extracellular vesicle (BEV) studies and described their content using various metrics. They notably used the EV-TRACK database to score the different selected studies and outlined the needs and guidelines to improve future BEV research. This research provides helpful insights into BEV studies, particularly concerning the diverse methods used in the selected studies. This highlights the fact that researchers need to implement and agree on standardized protocols to study BEVs. I personally think that the research is sound, but it needs improvement to be published. You can see the details of my recommendations/comments below:

****Line 122:**** I would like the authors to clearly define their definitions of protocol, method, and experiment. It seems like these terms are used interchangeably throughout the manuscript, which can be confusing. Additionally, I am unsure what the authors consider as part of the BEV protocol. Does a BEV protocol start from cultivating bacteria in the medium of interest, pelleting bacterial cells, and filtering supernatants, and include BEV characterization (NTA, TEM, WB, etc.), or does it start after the cells have been removed and filtered out? If the former is true, it is normal to observe

that centrifugation is almost always used with filtration (as seen in Figure 1), and it is also normal to see density gradient being highly associated with ultracentrifugation (since high-speed centrifugation is mandatory for particle separation through the gradient).

****Figure 1:**** It is not clear why the authors chose to display these 102 parameters (out of 233). Additionally, some parameters, such as tangential flow filtration and precipitation (e.g., ExoQuick TC), are not shown. Is this because they are not commonly used? Also, can we really define centrifugation below 20,000xg (the usual max speed for a benchtop centrifuge) as ultracentrifugation, as seen in Figure 1? I would prefer to see all speeds defined under 20,000xg (or at least all centrifugation not performed in an ultracentrifuge) as standard centrifugation steps.

****Line 167:**** How is the lipid content evaluated? Is it by lipidomics or using different dyes to stain the lipid bilayer?

****Line 169:**** I think the authors should provide a percentage of studies performing 16S RNA within the studies analyzing RNAs. Also, indicate what experiments were done other than 16S RNA sequencing.

****Line 148:**** One-fourth of experiments report neither particle nor protein characterization. This means a significant number of studies did not perform characterization of particles or proteins within BEVs. This suggests that one-fourth of selected BEV studies cannot be trusted, as we are not sure whether vesicles were indeed isolated. Can the authors describe more (perhaps in the discussion) about those studies (e.g., percentage of Gram-negative vs. Gram-positive bacteria, culture media, or isolation from natural habitats)?

****Figure 2A:**** The variation of color for the phylum is not very distinct in the graph.

****Figure 2 legend:**** TFF (tangential flow filtration) is not mentioned in the graphs.

****Supplement Figure 2 and Figure 3:**** The DLS method seems to yield very large vesicle size estimations. Is this an intrinsic flaw of the DLS method, or is it related to the materials studied (e.g., BEVs from explosive lysis)?

****Line 191-193:**** It is very difficult to see these results in the graph in Supplementary Figure 2A due to the very large BEV size. I suggest either breaking the y-axis into two or removing these outliers.

****Line 194-196:**** There is no graph supporting these data.

****Supplement Figure 2B:**** The graph showing NTA from other sources is not meaningful unless there is another method displayed for comparison.

****Supplement Figures 2C and 2D:**** The treemaps showing the enriched and depleted associated BEV proteins are, in my opinion, not useful. *E. coli* and *S. aureus* are the most studied BEV species, so the proteins shown belong to *E. coli* and *S. aureus*. For example, alpha-toxin (Hla) is only found in Staphylococcal species (mostly *S. aureus*). Consequently, the authors cannot claim that Hla is enriched in all Gram-positive bacteria.

****Figure 3D:**** The same comment applies here. Enriched/depleted proteins for all species do not make sense, as all species do not share the same protein repertoire (e.g., OmpA is not present in Gram-positive bacteria).

****Figure 3 legend:**** Change “(e) Molecules” to “(d) Molecules”.

****Figure 4A:**** The number of dots does not match the number of studies per year indicated in parentheses on the x-axis unless stacked dots were used. If so, it might be a good idea to depict the number of studies by the size of each dot.

****Figure 4 legend:**** Change “(b-d) Radar charts” to “(b-e)”.

****Table 2:**** BEV-enriched proteins OmpA/C/F/T are only relevant for Gram-negative bacteria, not Gram-positive bacteria. Again, the BEV-enriched proteins would be specific to a species or genus.

****Line 435-442:** Are EV-Track administrators S.D.V. and S.D.D. the same person or two different contributors? Additionally, some authors listed in the publication do not appear in the author contributions (e.g., QR, ODV, LV, HD, PT, and both SD).

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-by-point response to the reviewers' comments

We thank the reviewers for their time and constructive evaluation which allowed us to further improve our manuscript. We agree with the editor and reviewers that the manuscript better adheres to the content type of a Perspective and thus adjusted the manuscript accordingly. In the point-by-point answers we clarify the scope of our manuscript, further address the comments raised and provide extended clarifications.

Reviewer: 1

Major

1. The authors did not include a conflict of interest statement about EV-TRACK, which is crucial given that they are the founders of EV-TRACK. In the spirit of transparent reporting, we request the authors to add a conflict of interest statement to the manuscript.

We thank the reviewer to point out the potential conflict of interest. We added the following paragraph on competing interests in the “End Notes section” (page 23-24, line 547-550):

“The authors declare no competing financial interests. N.D.L., S.V.D., N.G., A.V.C., Q.R., S.D., S.D., O.D.W. and A.H. are affiliated to the Laboratory of Experimental Cancer Research, which coordinated the development of the EV-TRACK knowledgebase¹⁹. EV-TRACK can be accessed freely at <https://www.evtrack.org/>.”

2. We appreciate the authors’ efforts to identify current challenges and knowledge gaps in BEV research, propose recommendations, and provide future outlooks. We fully acknowledge the importance of standardized and transparent reporting. However, we believe that the second aim of the manuscript is to promote EV-TRACK and to further advance the it for BEV research (integrate experimental parameters). We kindly ask the authors to add the second aim to the manuscript, as its omission appears to compromise transparency.

We agree with the reviewer that further expanding the EV-TRACK knowledgebase towards BEV research, further promotes its use. We have expanded the section on EV-TRACK in the “Introduction section” as follows (page 4, line 73-80):

“Over the past years the International Society for Extracellular Vesicles (ISEV) developed minimal information guidelines^{17,18} and endorsed the use of EV-TRACK (<https://evtrack.org/>), a freely accessible knowledgebase that facilitates transparent reporting of experimental parameters and centralizes knowledge for EV research¹⁷⁻¹⁹. To enable best practices, it is essential to encourage awareness and to stimulate implementation, but also to ensure the continuous development of tailored guidelines and tools to meet the needs of particular subdomains such as BEV research^{2,20}. Hereto, we record experimental parameters of BEV-related studies published between 2015 and 2021 using the EV-TRACK knowledgebase.”

And in the “Discussion section” as follows (page 17, line 384-390):

“We enable researchers to easily query all BEV-related publications released between 2015 and 2021 in the EV-TRACK knowledgebase for source, preparation protocols and BEV characteristics (size, density, concentration, BEV-enriched and BEV-depleted molecules) while familiarizing them with experimental guidelines. This effort overall aims to raise awareness on the available guidelines and tools and steer an

informed dialogue with BEV researchers to ensure their implementation, since transparency and reproducibility will be indispensable for the development and translation of BEV-based applications.”

3. The authors state “We analyzed 845 BEV-related publications published between 2015 and 2021 using the EV-TRACK knowledgebase (Supplementary Table 1)”. How many BEV-related publications were published from 2015-2021? How many publications on eukaryotic EVs were published from 2015-2021? Please elaborate how representative the 854 BEV-related publications are for the years 2015-2021? Furthermore, we would like the authors to add a statement about this to the paper.

Supplementary Fig. 3 illustrates the study selection. BEV-related publications were selected from two databases (EMBASE and PubMed), implementing the search strategy as shown in supplementary methods. To not exclude any relevant articles, we implemented a broad search strategy, resulting in an initial 2919 publications (after deduplication and removal of 2022 publications). After title and abstract (n=1060) and further in depth screening (n=6), studies on non-bacterial origin EV were eliminated (total n= 1066). Other BEV-related publications were excluded due to several reasons: wrong publication type (n=173), no BEV preparation performed (n=822), failure to obtain full-text (n=5) or English-written text (n=8). Hence, all applicable 845 BEV-related publications published between 2015 and 2021 have been analysed in this manuscript, thus enabling a fully representative evaluation of BEV research. We have further clarified this in the “Results section” as follows (page 5, line 87-88):

“We selected all 845 studies reporting BEV preparation from any source between 2015 and 2021 (Supplementary Table 1)¹⁹.”

Between 2015 and 2021 EEV 3499 experiments (accounting for 1116 publications) were submitted to EV-TRACK. These studies were analyzed for comparison of EV-METRIC scores of BEV-studies to EEV-studies (Fig. 4a, Fig. 4e).

4. When reading the Introduction and Method sections (Screening, data extraction and quality assessment), it is unclear if the EV-TRACK tool was used by independent researchers/research groups that chose to use the tool themselves or if the authors added the studies to EV-TRACK. The following statements indicate that independent research groups added the studies to EV-TRACK. “Full-text screening was conducted independently by the individual submitting to EV-TRACK and EV-TRACK administrator S.D.V.” (Lines 375-376). However, the following statements indicate that the authors themselves added the studies to EV-TRACK. “All 845 studies were submitted to the EV-TRACK knowledgebase...” (Line 402). “Full-text screening, submission to the EV-TRACK knowledgebase and collection of additional data not included in the EV-TRACK knowledgebase was performed by N.D.L....” (Lines 437-438). Please comment on what has been done to conduct this study. Furthermore, in case that the authors added the studies themselves, please add a clear statement about this to the Introduction as well as the Method section.

All 845 included studies underwent full-text screening. 18 studies were already submitted to the EV-TRACK knowledgebase by independent researchers. For these studies, full-text screening was performed

by N.D.L. and the data revised by EV-TRACK administrator S.D.V. The remaining 827 studies were submitted by the authors.

To assist with manuscript clarification, we adapted the text to better illustrate how the study was conducted:

In the “Results section” (page 5, line 98-103):

Next, we submitted all studies that were not yet available (98%, 827/845) to the EV-TRACK knowledgebase¹⁹. Hereto, studies that reported multiple sources or preparation protocols (i.e. a combination of methods) were divided in multiple entries, resulting in a total of 3338 experiments (with a unique source and/or preparation protocol). For each experiment a checklist of 233 parameters related to source, preparation protocol and characterization method was completed (Supplementary Table 2).

In the “Methods section”:

- *Page 18-19, line 410-419: “The selection process is illustrated in Supplementary Fig. 3. All publications captured by the search were collected in Rayyan. After deduplication, 2919 records were identified and screened by title and abstract by two reviewers (N.D.L. and S.V.D.) before full-text screening. In case of doubt, studies were included for full-text screening. Full-text screening was conducted independently by the individual submitting to EV-TRACK (N.D.L., S.V.D., N.G., A.V.C., M.B., S.C., J.M., P.V.V., M.V.H., R.V.) and EV-TRACK administrator S.D.V. to identify studies fulfilling the inclusion criteria. Full-text screening of studies already submitted to EV-TRACK by independent researchers (n= 18) was performed by N.D.L. When full-text articles could not be retrieved, authors were contacted to provide this. Failure to establish contact or to receive full text in a timely manner resulted in exclusion. In total 845 publications were included.”*
- *Page 19, line 421-427: “All 845 studies were submitted to the EV-TRACK knowledgebase (<https://www.evtrack.org/>). Studies that included multiple sample types or preparation methods were divided into multiple entries, resulting in 3338 experiments. For each experiment a checklist of 233 parameters related to source, preparation protocol and characterization method was completed (Supplementary Table 2). EV-TRACK submission was revised by N.D.L. or S.V.D. and by EV-TRACK administrator S.D.V. For studies that were already submitted to EV-TRACK by independent researchers (n= 18), data was revised by EV-TRACK administrator S.D.V.”*

In the “End Notes section (author contributions)” (page 23, line 537-541):

“A.H. and S.V.D. designed the study. S.V.D. performed the database search. Titles and abstracts were screened by N.D.L. and S.V.D. Full-text screening, submission to the EV-TRACK knowledgebase (if the publication was not yet submitted by independent researchers), and collection of additional data not

included in the EV-TRACK knowledgebase was performed by N.D.L., S.V.D., N.G. and A.V.C., revised by N.D.L. or S.V.D. and by EV-TRACK administrator S.D.V.”

Minor

5. For all Figures the font size of the axis titles and legends are extremely small. We kindly ask the authors to increase the font size for all to increase readability. The same applies to Supplemental Figures (e.g. Supplemental Figure 1).

We thank the reviewer to point out potential issues with figure readability. We have addressed this and increased the font size of axis titles and legends for all figures.

6. EV-TRACK was published in 2017 to increase transparent reporting. The study presented in the manuscript contains BEV-related studies published between 2015 and 2021. How did transparent reporting regarding BEV research improve since EV-TRACK was launched?

Figure 4a illustrates the evolution of the average EV-METRIC (%) for EEV (cyan) and BEV (purple) studies from 2015-2021. The MISEV2018 guidelines recommend use of the EV-TRACK knowledgebase to maximize transparency and support reproducibility (Théry et al., JEV, 2018). Upon release of these guidelines, the average EV-METRIC for EEV studies distinctly increases. In contrast, the average EV-METRIC of BEV studies plateaus in 2018. This highlights that while general principles of the existing guidelines and tools are applicable, research on BEVs encounters specific challenges and requires tailored recommendations to further mature.

Furthermore, of all 845 BEV-related studies, only 18 were already submitted by an individual researcher to EV-TRACK. Hence underlining the lack of reporting of BEV studies in the EV-TRACK knowledgebase. With our study we therefore present a unique overview of all BEV-related studies between 2015 and 2021 thereby building a stepping-stone for future BEV-research.

7. Readability seems to be a recurring problem in this manuscript. Please try to improve readability of long sentences by e.g. splitting them.

Examples are:

- “However, the complexity and challenges associated with BEV research are considerable: 1) a plethora of methods are available for BEV preparation and characterization, 2) a diversity of bacterial strains releases heterogeneous BEV subsets, 3) sources from which BEVs are retrieved contain other extracellular particles with comparable biophysical and biochemical features, and 4) to date, no consensus exists on universal BEV markers 1,6.” (Lines 70-74)
- “BEVs from gram-negative bacteria are more abundantly analyzed (77%, 2560/3338), with *Escherichia coli* and *Pseudomonas aeruginosa* representing respectively 22% (742/3338) and 6% (201/3338) of experiments, compared to BEVs from gram-positive bacteria (16%, 526/3338), with by *Staphylococcus aureus* representing 7% (235/3338) of experiments (Fig. 2a; Supplementary Table 2).” (Lines 112-116). This is just one example from the Result section. Please address this.

To improve readability, we have addressed this throughout the manuscript. We illustrate this with the adapted examples from the reviewer's comment in the "Introduction section":

- Page 4, line 69-73:

"However, the complexity and challenges associated with BEV research are considerable. A plethora of methods are available for the preparation and characterization of BEVs². A diversity of bacterial strains releases heterogeneous subsets of BEVs^{3,4}. The sources from which BEVs are retrieved contain other extracellular particles with comparable biophysical and biochemical features^{2,6}. Lastly, to date no consensus exists on universal BEV markers^{1,6}.

And in the "Results section":

- Page 6, line 111-114:

"BEVs from gram-negative bacteria are more abundantly analyzed (77%, 2560/3338) compared to BEVs from gram-positive bacteria (16%, 526/3338). Top three studied bacterial species are Escherichia coli (22%, 742/3338), Staphylococcus aureus (7%, 235/3338) and Pseudomonas aeruginosa 6% (201/3338) (Fig. 2a; Supplementary Table 3)."

We thoroughly screened the entire manuscript and made additional adaptations if needed.

8. In lines 110 – 111 the authors state: "The vast majority of experiments (94%, 3123/3338) analyzes BEVs in media conditioned by bacterial cells under laboratory conditions." Please define laboratory conditions. Did all studies included in this manuscript that used BEVs from cell culture supernatant use the same conditions e.g. culture time, used media or chemical components, incubation time, temperature?

With "BEVs in media conditioned by bacterial cells under laboratory conditions" we indicate BEVs prepared from bacterial cell culture. Not all studies use the same conditions, partly due to differences between bacterial species, requiring different laboratory conditions. We agree with the reviewer that experimental parameters such as culture time, media type and chemical components, incubation time and temperature are crucial parameters and should be reported to increase transparency. Additionally, specifically for bacterial cell culture we highlight the importance of reporting antibiotic use, OD600 and bacterial growth phase at harvest. In Table 2 we hence recommend integration of these parameters in the EV-TRACK knowledgebase.

9. In Figure 1 the authors split the parameters into preparation and characterization: biophysical and characterization: biochemical. Please use the same structure in the Result section (pages 6 – 7).

We have adapted this throughout the manuscript, as illustrated here in the “Results section” (page 7, line 145-148):

“Over one fourth of experiments (29%, 956/3338) report neither biophysical nor biochemical characterization (Fig. 1; Figure 2c). 29% (981/3338) and 18% (585/3338) of experiments perform solely biophysical or biochemical characterization, respectively. Only 25% of experiments (816/3338) perform both biophysical and biochemical characterization.”

In addition, we have adapted Fig. 2c to implement the same structure.

10. In Figure 1 authors add “FC: bead size” as a biophysical characterization. The characterization of beads is not directly connected to BEV characterization. Please comment on why this has been added here.

Figure 1 illustrates the reporting of 102 parameters regarding BEV preparation and characterization. The parameters for implemented characterization methods, as shown in Fig. 2c, were included in this heatmap. For each method, a series of experimental parameters should be reported to ensure full transparency. For flow cytometry, one of these parameters is the bead size. To keep the same structure as for other methods (DLS, NTA, TRPS), we have changed this to “FC: relevant variables”.

11. The authors report that the size has been reported. “68% of experiments performing particle characterization (1216/1797) reports particle size (Figure 1), with the majority implementing NTA (45%, 546/1216), followed by EM (36%, 434/1216), and DLS (30% 361/1216) (Figure 1; Figure 2c). Average reported particle sizes of BEV preparations for frequently studied phyla Pseudomonadota (63%, 770/1216), Bacillota (14%, 174/1216) and Bacteroidota (3.5%, 43/1216) are respectively 118 nm ($\pm$ 78 nm), 158 nm ($\pm$ 277 nm) and 141 nm ($\pm$ 92 nm)...”(Lines 184-188). The authors switch between reported particle size and BEV size. Please comment on what is meant here. Furthermore, is there any proof that particles detected with e.g. NTA are indeed BEVs? Or are they non-BEV particles present in the samples? Please comment on this and add a clear limitation statement about this type of analysis to the manuscript.

We agree with the reviewer that methods that measure particles such as NTA are not able to distinguish BEVs from other particles. Consequently, throughout the manuscript we implement the terms “particle size” or “particle concentration”.

In the above excerpt of the manuscript we refer to “the particle size of BEV preparations”. Of note, with BEV preparation we refer to “the sample” which is the end-result of implementing a BEV preparation protocol on a particular biofluid. The specificity of the implemented BEV preparation protocol will define the ratio of BEVs versus other particles that are present in this BEV preparation (Hendrix et al., Nat Rev Methods Primers, 2023).

In addition, we have included the follow sentence in the “Results section” to highlight the limitations of such characterization methods (page 9, line 190-194):

“Of note, the reported particle size inherently depends on the settings, the sensitivity and the detection limits of the selected instrument; smaller particle sizes are reported using DLS and EM compared to NTA (Fig. 3a)^{17,21}. Furthermore, methods such as DLS and NTA are not able to distinguish BEVs and other extracellular particles. Overall, results should be interpreted with caution¹⁷.”

12. The authors state “Of note, the reported particle size depends on the used method; smaller particle sizes are reported using DLS and EM compared to NTA (Figure 3a)^{15,19}” (Lines 196-197). While we agree that the reported particles size depends on the used method, we miss a statement that the reported particle size also highly depends on the sensitivity of the chosen method and the lower detection limits. Please add a clear statement about this to the paper.

We agree with the reviewer that the chosen characterization method undeniably impacts reported particle size. We have clarified this in the “Results section” with the highlighted sentence as follows (page 9, line 190-192):

“Of note, the reported particle size inherently depends on the settings, the sensitivity and the detection limits of the selected instrument; smaller particle sizes are reported using DLS and EM compared to NTA (Fig. 3a)^{17,21}.

13. Figure 3a shows the average particle size (nm) per experiment for most abundant phylum Pseudomonadota measured with DLS, EM, NTA. In Figure 1 also includes flow cytometry for characterization. Why is this not included in Figure 3?

Flow cytometry is indeed reported as a method for BEV characterization. However, only two size measurements with flow cytometry are reported. Therefore we excluded this method from Fig. 3a.

14. “Systematically cataloguing biochemical and biophysical characteristics, supports data validation and contributes insights into BEVs residing in diverse natural habitats. Furthermore, characterization of BEV preparations allows to identify what is present (presumably BEVs) and what is not (presumably other extracellular particles), supporting data interpretation and conclusion-making.”. We agree with the authors that this will help to promote reproducibility of BEV research. However, we lack a statement about assay controls to insure that researchers are indeed detecting BEVs and not other non-BEV particles.

We concur with the reviewer that inclusion of correct assay controls is indispensable to confirm that findings are indeed attributed to BEVs and not other non-BEV particles. We integrated this in the “Results section” as follows (page 13-14, line 311-315):

“Systematically cataloguing biochemical and biophysical characteristics, supports data validation and contributes insights into BEVs residing in diverse natural habitats. Furthermore, characterization of BEV preparations, using adequate assay controls, allows to identify what is present (presumably BEVs) and what is not (presumably other extracellular particles), in turn supporting data interpretation and conclusion-making².”

To highlight the importance of such assay controls, we have included this as an additional recommendation in Table 1 (recommendations on characterization):

- *Characterize BEVs with multiple, complementary methods*
- *Catalogue biochemical characteristics: BEV-enriched and BEV-depleted molecules*
- *Catalogue biophysical characteristics*
- *Implement additional PAMP characterization methods*
- *Include adequate assay controls*

15. In the list of key words a ; is missing behind “Plant” (Line 25).

We have replaced the comma with a semicolon (page 1, line 25).

Reviewer: 2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer: 3

The manuscript titled "Bacterial Extracellular Vesicle Analysis: Challenges and Opportunities" explores the complex and evolving field of bacterial extracellular vesicles (BEVs). It emphasizes BEVs' potential roles as biomarkers and therapeutic agents due to their ability to facilitate communication across species and environments. The manuscript critically examines the diversity of BEVs in terms of their biogenesis, composition, and functions, which vary significantly among bacterial species. Here is a detailed review of the manuscript :

1. The manuscript mentions the inconsistent use of terminology like "outer membrane vesicle" across the studies analyzed, which can be confusing and misleading. The authors should advocate for stricter guidelines on nomenclature in the discussion of their results. This could involve setting clearer definitions and usage examples for each term based on the biogenesis and composition of the vesicles. Providing a standardized glossary for all terms related to BEVs at the beginning of the manuscript could help ensure consistent use across the board.

To assist with consistency and clarity throughout the manuscript, we have added a box (Box 1: Nomenclature) in the beginning of the manuscript, summarizing the current knowledge on nomenclature:

Box 1. Nomenclature

Applying certain nomenclature to BEVs, reflects the underlying biogenesis pathways^{3,4}. As much remains to be explored in terms of BEV biogenesis across bacterial species, the term "BEV" is recommended when multiple subsets are studied, or if biogenesis remains unclear or not specified, especially when exploring natural habitats composed of diverse bacterial species. This term can be contemplated with additional operational terms (e.g. "engineered BEVs" for BEVs that are modified as treatment vehicle)¹⁷.

- **Bacterial extracellular vesicle (BEV):** overarching term to denote EVs released by bacteria
- **Outer membrane vesicle (OMV):** BEV released through blebbing of the outer membrane of gram-negative bacteria, typically free of cytosolic cargo
- **Outer-inner membrane vesicle (OIMV):** BEV released from gram-negative bacteria through endolysin-triggered explosive cell lysis, including the inner membrane and encompassing cytosolic cargo
- **Explosive outer-membrane vesicle (EOMV):** BEV released from gram-negative bacteria through endolysin-triggered explosive cell lysis and encompassing cytosolic cargo
- **Cytoplasmic membrane vesicle (CMV):** BEV released from gram-positive bacteria through blebbing or bubbling cell death

We furthermore highlight the importance of the correct implementation of this nomenclature in the "Discussion section" (page 15, line 348-352):

"Heterogeneous nomenclature is inconsistently implemented, often reflecting a certain underlying biogenesis pathway. As much remains to be explored in terms of BEV biogenesis across bacterial species, a more general term, "BEV", is recommended. This is especially the case when multiple subsets are studied, or if biogenesis remains unclear or not specified or natural habitats are explored (Box 1)."

2. The manuscript points out that many studies do not report critical experimental parameters, which hampers reproducibility. Strongly emphasize the need for increased data transparency in the manuscript. Recommend the inclusion of a supplementary section that lists all parameters that should be reported by future studies. This could help increase the EV-METRIC scores and improve the overall quality of BEVs research.

In the manuscript we indeed highlight the importance of transparent reporting of experimental parameters, in agreement with the MISEV guidelines (Théry et al., JEV, 2018; Welsh et al., JEV, 2024), which in return recommend the implementation of EV-TRACK as a reporting tool. We have included an additional Supplementary Table (Supplementary Table 2), which includes all parameters that should be reported (Supplementary data):

“SUPPLEMENTARY TABLES

Supplementary Table 1: Included studies

Supplementary Table 2: Experimental parameters included in EV-TRACK

Supplementary Table 3: Bacterial taxonomy

Supplementary Table 4: Additional experimental parameters: storage, PAMP characterization, 16S rRNA sequencing, bacterial contamination and study aim

Supplementary Table 5: BEV-enriched and BEV-depleted proteins and other molecules”

We underline that this list is dynamic, as it is updated based on the newest MISEV guidelines and current knowledge on EV research. We furthermore suggest BEV-specific parameters to integrate into the EV-TRACK knowledgebase (Table 2).

To further emphasize the need for increased data transparency, we have added following statements on this in the “Discussion section”:

- Page 15-16, line 357-360:

“Transparent reporting will be key to ensure reproducibility and further advance BEV research, and to achieve this, tailored guidelines and tools are fundamental. Hereto, we integrated a set of BEV-specific experimental parameters into the EV-TRACK knowledgebase (Table 2).”

- Page 17, line 388-390:

“This effort overall aims to raise awareness on the available guidelines and tools and steer an informed dialogue with BEV researchers to ensure their implementation, since transparency and reproducibility will be indispensable for the development and translation of BEV-based applications.”

3. While the study compiles an extensive amount of data, there is a lack of discussion on the biological or clinical relevance of the findings. Expand the discussion of results to include more on the potential implications of the findings for clinical and environmental applications. This could involve a separate section that hypothesizes how variations in BEVs properties might influence their role in disease, treatment, or monitoring environmental health.

We concur with the reviewer that it is important to highlight the potential implications for clinical and environmental applications. We have therefore added the following paragraph to the “Discussion section” (page 16, line 371-381):

“We furthermore catalogued particle size and particle concentration of BEV preparations, revealing source-dependent biophysical characteristics, while the density of BEV preparations remains largely unexplored. Cataloguing biophysical and biochemical characteristics of BEVs will be essential to further advance our insights in the presence, function and application potential of BEV subsets across natural habitats. Inherent characteristics of BEVs and their parental bacterium significantly influences their impact on the surrounding natural habitat. BEVs from various origin can either induce immunosuppression or stimulate immune activation^{1,8,28–30}. BEV biogenesis is further affected by several aspects, including medium composition, antibiotics and bacterial growth phase, which leads to variations in their molecular cargo^{3,29,31}. For instance, the levels of virulence factors within BEVs can vary depending on the bacterial growth phase, eliciting either a robust pro-inflammatory response or prolonged low-level immune activation^{3,8,31–33}.”

4. The manuscript would benefit from a more detailed explanation of the statistical methods used to analyze the data. Include information on the statistical tests performed, justification for their use, and interpretation of results. This could involve the addition of a subsection on statistical methodology within the Materials and Methods section.

We agree with the reviewer that statistical analysis should be performed to support the data. We have therefore added this information in the “Methods section” (page 19-20, line 435-443):

*“Data were synthesized and analyzed by N.D.L. and S.D.D. using Excel (Microsoft), Graphpad Prism (version 8.4.0), R (version 4.2.2), and R studio (version 2022.07.2) using following R packages: “readxl”, “circulize”, “glue”, “stringr”, “fmsb”, “ggtree”, “pheatmap”, “taxonomizr”, “ggplot2”, “ape”, “phytools”, “ggnewscale”, “ggtreeExtra”, “RColorBrewer”, “dplyr”, “ggpubr”, “gridExtra”, “grid”, “readr”, “treemap”. Statistical calculations were performed using Graphpad Prism (version 8.4.0). Mann-Whitney U tests were performed to compare non-normally distributed continuous variables. P values smaller than 0.05 were considered statistically significant (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001). Data in text are*

represented by mean value ($\pm$ standard error). Illustrations were made with Adobe Illustrator (version 28.5)."

We furthermore included visualization of significant p-values in Fig. 3 and Supplementary Fig. 2.

5. Review and improve the clarity and presentation of figures and tables. Ensure that all graphical data are easy to understand and adequately labeled. This may involve revising existing figures or adding new ones to better illustrate key findings.

We thank the reviewer to point out potential issues with clarity and presentation of the figures and tables. We have addressed this by e.g. increasing the font size in all figures and tables and made sure to adequately label all figures to improve clarity and readability.

6. The manuscript should provide a more detailed description of the sample collection and handling procedures, especially when discussing the collection of BEVs from natural habitats like ocean seawater, plants, and human body fluids. Specific details such as the volume of samples collected, the storage conditions before processing, and any pre-treatment steps should be clearly outlined. This will help ensure that other researchers can accurately replicate the study and assess the impact of sample handling on BEVs integrity and analysis.

We concur with the reviewer that reporting of sample collection and handling procedures should be thoroughly reported. However, these parameters are only limitedly included in the current version of the EV-TRACK knowledgebase. Therefore, we opted to focus our analysis on BEV nomenclature, source, preparation, characterization and reporting. We do however acknowledge that this information should be reported, for each type of EV (not only BEVs). More detailed parameters regarding sample collection will therefore be included in the updated version of the EV-TRACK knowledgebase, in collaboration with the dedicated ISEV task force "EV-TRACK". Following parameters will be included in the scheduled updated version of EV-TRACK:

Sample type	Experimental parameter
Plasma/serum	- <i>Blood collection tube (already included)</i> - Time between blood collection and centrifugation - Centrifugation speed and time - Volume used for EV preparation - Storage temperature
Urine	- Pre-treatment prior to sample collection - Collection procedure, time and device - Volume used for EV preparation - Urine void - Storage temperature

Tissue	- Tissue mass - Preprocessing of tissue: mechanically dissociated or enzymatically digested - Volume used for EV preparation - Storage temperature
Cerebrospinal fluid (CSF)	- Details collection of CSF - Volume used for EV preparation - Storage temperature
Saliva	- Collection of saliva - Volume used for EV preparation - Storage temperature
Synovial fluid	- Details collection of synovial fluid - Volume used for EV preparation - Storage temperature
Milk	- Details collection of milk - Volume used for EV preparation - Storage temperature
Faeces	- Bristol stool chart - Preprocessing of faeces - Volume used for EV preparation - Storage temperature
Cell culture	- <i>Cell type, number and viability (already included)</i> - <i>Medium (already included)</i> - <i>Preparation of EV-depleted serum (EDS) (already included)</i> - Serum heat inactivation - Volume used for EV preparation - Storage temperature

7. The reviewer suggests the following articles related to the BEVs for the authors to refer to in order to revise the manuscript:

1. Bioact Mater. 2021 Dec 17;14:169-181. doi: 10.1016/j.bioactmat.2021.12.006.

2. Theranostics. 2022 Sep 11;12(15):6576-6594. doi: 10.7150/thno.78034.

3. Compos Part B-eng. 2023 Apr 15;110610. doi:10.1016/j.compositesb.2023.110610.

4. Compos Part B-eng. 2023 Dec 111047. doi:10.1016/j.compositesb.2023.111047.

We thank the reviewer for these suggestions. We have read all four publications and included the references that are applicable.

Reviewer: 4

In the manuscript “Bacterial extracellular vesicle analysis: challenges and opportunities” De Langhe and colleagues provide an overview of the recent literature (from 2015 to 2023) on bacterial extracellular vesicles, highlighting the heterogeneity in methods and analytical techniques used to study them. The manuscript is detailed and well written and highlights certain areas where consistency of techniques would improve reproducibility and draws attention to the relative lack of studies on BEVs in natural environments. Despite some interesting observations the manuscript is limited in scope and significance. The primary overall observation is that there exists a lot of variation in how BEVs are isolated and studied, and does not go on, for example, to outline or suggest best practices for these procedures. I have additional concerns with the manuscript in its current form which are outlined below and need to be addressed.

- The manuscript is submitted as a review article, however I’m not sure this is entirely appropriate. A large amount of data analysis was performed, and results generated (along with figures), making this feel more like a research paper than a review. For example it is reported that there is a large degree of variation in the methods used to isolate BEVs (and a number of figures provided to support this) however there is no discussion of the individual techniques, and the pros and cons of each....which are points I would expect to see in a review article. Instead, it is simply reported what percentage of studies use each technique. This makes the manuscript more of a survey than a review.

We agree with the reviewer that the current manuscript does not entirely fit the review content type. The publication will be considered as a Perspective in Nature Communications, which is *“intended to provide a forum for authors to discuss models and ideas from a personal viewpoint. They are more forward looking and/or speculative than Review articles and may take a narrower field of view. They may be opinionated but should remain balanced and are intended to stimulate discussion and new experimental approaches.”* We agree that our publication better fits this content type and have adapted the manuscript as such. Discussion of all individual preparation and characterization techniques and the pros and cons of each, is however very elaborate and has already very thoroughly been outlined by us and others (Hendrix et al., Nat Rev Methods Primers, 2023; Théry et al, JEV, 2018; Welsh et al., JEV, 2024). As the aim of our publication is to map current research practices, highlight knowledge gaps and provide recommendations with a focus on BEVs, we are convinced such an overview does not fall within the scope of this manuscript.

- EV-TRACK and EV-METRIC are mentioned throughout the manuscript however the authors fail to properly introduce these terms, concepts and their history. This makes for a confusing read. The original publication that generated EV-TRACK and EV-METRIC should be discussed in detail in the introduction along with clearly stated goals for how this manuscript builds upon that work and integrates with it.

To assist with manuscript clarification, we added a paragraph to the “Introduction section” explaining more thoroughly the EV-TRACK knowledgebase and added clearly stated goals for how this manuscript builds upon that work and integrates with it (page 4, line 73-80):

“Over the past years the International Society for Extracellular Vesicles (ISEV) developed minimal information guidelines^{17,18} and endorsed the use of EV-TRACK (<https://evtrack.org/>), a freely accessible knowledgebase that facilitates transparent reporting of experimental parameters and centralizes knowledge for EV research¹⁷⁻¹⁹. To enable best practices, it is essential to encourage awareness and to stimulate implementation, but also to ensure the continuous development of tailored guidelines and tools to meet the needs of particular subdomains such as BEV research^{2,20}. Hereto, we record experimental parameters of BEV-related studies published between 2015 and 2021 using the EV-TRACK knowledgebase.”

We furthermore included a paragraph in the “Discussion section” (page 17, line 384-390):

“We enable researchers to easily query all BEV-related publications released between 2015 and 2021 in the EV-TRACK knowledgebase for source, preparation protocols and BEV characteristics (size, density, concentration, BEV-enriched and BEV-depleted molecules) while familiarizing them with experimental guidelines. This effort overall aims to raise awareness on the available guidelines and tools and steer an informed dialogue with BEV researchers to ensure their implementation since transparency and reproducibility will be indispensable for the development and translation of BEV-based applications.”

In addition, we included a box (Box 2. EV-TRACK and EV-METRIC) to provide the reader with a concise introduction to EV-TRACK and the EV-METRIC:

Box 2. EV-TRACK and EV-METRIC

EV-TRACK is a freely accessible knowledgebase (<https://evtrack.org/>) developed to improve transparency and reproducibility through increasing reporting of relevant experimental parameters in EV analysis²⁰.

Each experiment submitted to the EV-TRACK knowledgebase, receives an **EV-METRIC**, a measure for the level of transparency in the reporting of experimental parameters. The EV-METRIC currently encompasses nine different METRIC components imperative for unambiguous interpretation and independent reproduction of EV experiments and is displayed as a percentage of fulfilled components²⁰. The parameters can be divided into three sections:

A. Preparation method

1. **(Ultra-)centrifugation specifics:** reporting of g-forces, duration and rotor type of (ultra-)centrifugation steps (**conditional*)
2. **Density gradient:** density gradient performed, at least as validation if described results are attributed to BEVs
3. **BEV density:** reporting of obtained EV density (**conditional*)

B. Biophysical characterization

1. **Qualitative and quantitative analysis:** implementation of both qualitative (example: EM, AFM) and quantitative methods (example: NTA, DLS, TRPS, high resolution flow cytometry); for the quantitative method, reporting of particle concentration is expected
2. EM images: inclusion of a wide-field and a close-up electron microscopy image

C. Biochemical characterization

1. **BEV-enriched proteins:** analysis of three or more BEV-enriched proteins
2. **Non-BEV enriched protein:** assessment of at least one non-BEV-enriched protein
3. **Antibody specifics:** antibody clone/reference number and dilution (**conditional*)
4. **Lysate preparation:** lysis buffer composition provided, or reference number for commercial lysis buffers (**conditional*)

The EV-METRIC for each study is calculated by expressing the proportion of reported parameters as a percentage. While a high EV-METRIC implies well-annotated published data, it should not be seen as a stand-alone 'score' that directly relates to the quality of any particular study²⁰.

**Conditional: these parameters can be considered 'not applicable' depending on the implemented characterization methods in a particular study*

- Throughout the manuscript the following term appears approx. 8-9 times (cfr. “Assess quality by evaluating the EV-METRIC”). What does this mean? No explanation is given.

With this term we referred to the section of the manuscript with the title “Assess quality by evaluating the EV-METRIC”. Each of the mentioned parameters of the EV-METRIC is explained in the corresponding paragraphs. In order to avoid unnecessary repeating, we referred to this section to summarize this data. As we now included a box (Box 2. EV-TRACK and EV-METRIC) providing the reader with a concise introduction to EV-TRACK and the EV-METRIC, we have adjusted this, to refer to this box instead. We illustrate this with an example from the “Results section” (page 7, line 156-158):

“15% (513/3338) of experiments thus implement both qualitative and quantitative biophysical characterization (Figure 2c, Box 2).”

COMMENTS FROM ECR

The study by De Langhe et al. analyzed bacterial extracellular vesicle (BEV) studies and described their content using various metrics. They notably used the EV-TRACK database to score the different selected studies and outlined the needs and guidelines to improve future BEV research. This research provides helpful insights into BEV studies, particularly concerning the diverse methods used in the selected studies. This highlights the fact that researchers need to implement and agree on standardized protocols to study BEVs. I personally think that the research is sound, but it needs improvement to be published. You can see the details of my recommendations/comments below:

1. ****Line 122:**** I would like the authors to clearly define their definitions of protocol, method, and experiment. It seems like these terms are used interchangeably throughout the manuscript, which can be confusing. Additionally, I am unsure what the authors consider as part of the BEV protocol. Does a BEV protocol start from cultivating bacteria in the medium of interest, pelleting bacterial cells, and filtering supernatants, and include BEV characterization (NTA, TEM, WB, etc.), or does it start after the cells have been removed and filtered out? If the former is true, it is normal to observe that centrifugation is almost always used with filtration (as seen in Figure 1), and it is also normal to see density gradient being highly associated with ultracentrifugation (since high-speed centrifugation is mandatory for particle separation through the gradient).

A preparation method denotes one method (e.g. density gradient centrifugation) that can be used for BEV preparation. A preparation protocol is a combination of methods implemented for BEV preparation (e.g. size-exclusion chromatography followed by density gradient centrifugation). An experiment within a study is defined through implementation of one specific source, of which BEVs are prepared with a unique preparation protocol (i.e. a combination of methods). Thus, within one study, multiple experiments can be included with for example multiple source types and/or multiple different preparation methods. To improve comprehension of these terms, we have adapted the text in the “Results section” to clarify the implementation of these terms (page 5, line 98-105):

“Next, we submitted all studies that were not yet available (98%, 827/845) to the EV-TRACK knowledgebase. Hereto, studies that reported multiple sources or preparation protocols (i.e. a combination of methods) were divided in multiple entries, resulting in a total of 3338 experiments (with a unique source and/or preparation protocol). For each experiment a checklist of 233 parameters related to source, preparation protocol and characterization method was completed (Supplementary Table 2). Data were curated and integrated into the EV-TRACK knowledgebase. In-depth analysis of this data for all 845 BEV-related studies reveals widespread methodological heterogeneity and variable reporting of experimental parameters (Fig. 1).”

The “BEV protocol” indeed starts from cultivation of bacteria in the medium of interest, however it does not include BEV characterization. We agree with the reviewer that it is to be expected that filtration and low-speed centrifugation are often combined. In a similar reasoning, combination of a density gradient and ultracentrifugation is to be anticipated. However, the (ultra-)centrifugation steps subsequent to the preparation of the density gradient are included in the preparation method “density gradient

centrifugation". In other words, in case the methods "density gradient" and "DUC" are combined into a preparation protocol, this indicates that a density gradient was prepared followed by ultracentrifugation; and in another part of the preparation protocol, the sample (e.g. the collected density gradient fractions containing the BEVs) was again (ultra-)centrifuged.

2. **Figure 1:** It is not clear why the authors chose to display these 102 parameters (out of 233). Additionally, some parameters, such as tangential flow filtration and precipitation (e.g., ExoQuick TC), are not shown. Is this because they are not commonly used? Also, can we really define centrifugation below 20,000xg (the usual max speed for a benchtop centrifuge) as ultracentrifugation, as seen in Figure 1? I would prefer to see all speeds defined under 20,000xg (or at least all centrifugation not performed in an ultracentrifuge) as standard centrifugation steps.

We have indeed made a selection of 102 out of the 233 parameters. With the heatmap displayed in Fig. 1, we aim to highlight experimental parameters regarding preparation, biophysical and biochemical characterization. Experimental parameters regarding for example source type or nomenclature were thus not included. In order to maintain a clear overview, we opted to include detailed parameters on the most implemented methods, such as differential (ultra-)centrifugation or filtration. For this reason, tangential flow filtration was excluded from this graph. ExoQuick is however included under "commercial methods", which can be found between the methods ultrafiltration and size-exclusion chromatography.

We agree with the reviewer that the term "differential ultracentrifugation" might be misleading and imply ultracentrifugation with speeds higher than 20,000g. A more correct term might be "sequential/differential centrifugation" to indicate these centrifugation steps with a lower centrifugation speed. In line with the terminology used throughout the MISEV guidelines, we aim to give an overview of all centrifugation steps, both centrifugation and ultracentrifugation, under "DUC". To this purpose we have slightly adapted this term throughout the manuscript: "differential (ultra-)centrifugation". We have altered Fig. 1 in accordance with this term used throughout the manuscript.

3. **Line 167:** How is the lipid content evaluated? Is it by lipidomics or using different dyes to stain the lipid bilayer?

None of included studies evaluated lipid content through lipidomics.

4. **Line 169:** I think the authors should provide a percentage of studies performing 16S RNA within the studies analyzing RNAs. Also, indicate what experiments were done other than 16S RNA sequencing.

We concur with the reviewer that it is more relevant to indicate the percentage of studies performing 16S rRNA sequencing within the studies analyzing RNA, rather than the percentage of all studies. It is however important to make a clear distinction between overarching RNA sequencing (included into the EV-TRACK knowledgebase) and 16SrRNA sequencing, specifically evaluated for taxonomic evaluation of the bacteria and their BEVs. For this reason, we aimed to highlight these studies performing 16S rRNA sequencing and

evaluated these numbers separately from the existing data in EV-TRACK. We have adapted the numbers in the text of the “Results section” in compliance with the reviewer’s comment, including 16S rRNA sequencing in the overall analysis (page 8, line 161-169):

“Lipids or nucleic acids are evaluated in 11% (363/3338) and 3.9% (133/3338) of experiments, respectively. BEV-associated nucleic acids are predominantly analyzed using RT-qPCR assays (34%, 45/133) or RNA sequencing (52%, 69/133), with 30% (40/133) performing 16S rRNA sequencing (Supplementary Table 4). Confirmation of luminal nucleic acid localization is performed in 30% of experiments (i.e. RNase (22%, 29/133), in combination with proteinase (10%, 13/133) or not), with the majority reporting enzyme (83%, 24/29 and 100%, 13/13) and concentration (69%, 20/29 and 54%, 7/13), but only 24% reporting treatment time. Data sharing through online databases is rarely reported (proteomics (13%, 89/670) or transcriptomics (7.2%, 5/69)).”

Other RNA analysis performed are e.g. RT-qPCR, as indicated in the manuscript (page 8, line 162-163): *“BEV-associated nucleic acids are predominantly analyzed using RT-qPCR assays (34%, 45/133)...”* In the current version of EV-TRACK the type of RNA sequencing is not evaluated. As we are convinced 16S rRNA sequencing is especially of interest for BEVs, we recorded and analyzed this as an additional parameter (Supplementary Table 4). RNA sequencing subtype, including 16S rRNA sequencing will be included in the updated version of EV-TRACK.

5. ****Line 148:**** One-fourth of experiments report neither particle nor protein characterization. This means a significant number of studies did not perform characterization of particles or proteins within BEVs. This suggests that one-fourth of selected BEV studies cannot be trusted, as we are not sure whether vesicles were indeed isolated. Can the authors describe more (perhaps in the discussion) about those studies (e.g., percentage of Gram-negative vs. Gram-positive bacteria, culture media, or isolation from natural habitats)?

More than one fourth of BEV experiments indeed do not perform any biophysical or biochemical characterization. These experiments evaluate BEVs from bacterial cell cultures (89%, 850/956) and other sources (11%, 106/956), both from gram-negative (75%, 713/956) and gram-positive bacteria (15%, 140/956). As this reflects the overall findings in the manuscript (94% of experiments on BEVs from bacterial cell cultures and 77% gram-negative) and does not bring to light lack of characterization in one particular subgroup, we have decided not to include the information on these studies in the discussion.

6. ****Figure 2A:**** The variation of color for the phylum is not very distinct in the graph.

We thank the reviewer to point out potential issues with figure readability. We have addressed this and altered the colors of the phyla in Fig. 2a.

7. ****Figure 2 legend:**** TFF (tangential flow filtration) is not mentioned in the graphs.

We thank the author for thorough revision of the figure legends and have adapted them according to the comments.

8. ****Supplement Figure 2 and Figure 3:**** The DLS method seems to yield very large vesicle size estimations. Is this an intrinsic flaw of the DLS method, or is it related to the materials studied (e.g., BEVs from explosive lysis)?

It is indeed a striking observation that DLS yields very large particle size estimations. The observed large particle sizes might be due to the materials, specifically the bacterial strain, studied, as suggested by the reviewer. The largest particle sizes, respectively 2806nm and 2765nm, are both observed in BEV preparations of *Lactobacillus reuteri*, a gram-positive bacterium, with the third largest particles (1740nm) in *Helicobacter pylori*. As the underlying biogenesis of BEVs is not fully understood, we are careful to state this is due to the mode of BEV formation, however it is possible that the underlying mechanism, such as explosive lysis or bubbling cell death, might indeed impact the size of released BEVs. Other than the materials studied, the implemented characterization method can impact size measurements. Reported particle size inherently depends on the sensitivity and detection limits of the chosen method (Welsh et al., JEV, 2024; Arab et al., JEV, 2021). We have clarified this in the “Results section” as follows (page 9, line 190-194):

“Of note, the reported particle size inherently depends on the settings, the sensitivity and the detection limits of the selected instrument; smaller particle sizes are reported using DLS and EM compared to NTA (Fig. 3a)^{17,21}. Furthermore, methods such as DLS and NTA are not able to distinguish BEVs and other extracellular particles. Overall, results should be interpreted with caution¹⁷.

Due to these limitations, methods such as DLS are for example not able to differentiate large particles from particle clusters, thus potentially overestimating particle size.

9. ****Line 191-193:**** It is very difficult to see these results in the graph in Supplementary Figure 2A due to the very large BEV size. I suggest either breaking the y-axis into two or removing these outliers.

We have adapted Supplementary Fig. 2a, removing size values of 1000 nm and above (n=4) to increase comprehension and clarity. Furthermore, to clearly show the difference between gram-positive and gram-negative BEVs, we have grouped all methods.

10. ****Line 194-196:**** There is no graph supporting these data.

We agree with the reviewer that this information is currently not supported by a graph. We have therefore adapted Supplementary Fig. 2a to include information on all BEVs prepared under laboratory conditions, in order to compare this to other sources (“Results section”, page 9, line 187-190):

“The average particle size of 193 BEV preparations obtained under laboratory conditions ($132\text{ nm} \pm 144\text{ nm}$, $n=1300$ experiments) is 194 lower compared to BEV preparations from other sources ($182\text{ nm} \pm 127\text{ nm}$, $n=74$ experiments) ([Supplementary Fig. 2a](#)).”

11. ****Supplement Figure 2B:**** The graph showing NTA from other sources is not meaningful unless there is another method displayed for comparison.

We concur that it is not meaningful to only display NTA from other sources. We have therefore adapted Supplementary Fig. 2b to include information on all BEVs prepared under laboratory conditions, in order to compare this to particle concentration of other sources.

12. ****Supplement Figures 2C and 2D:**** The treemaps showing the enriched and depleted associated BEV proteins are, in my opinion, not useful. *E. coli* and *S. aureus* are the most studied BEV species, so the proteins shown belong to *E. coli* and *S. aureus*. For example, alpha-toxin (Hla) is only found in Staphylococcal species (mostly *S. aureus*). Consequently, the authors cannot claim that Hla is enriched in all Gram-positive bacteria.

The treemaps in Supplementary Fig. 2c indeed highlight the most studied BEV-enriched and BEV-depleted proteins and other molecules for gram-positive and gram-negative bacteria and other sources. However, we do not claim that these proteins are present in all bacteria belonging to this category. In contrast, we aim to highlight: 1) low reporting of proteins and other molecules and 2) that reported proteins are specific for certain bacterial species. In order to underline these findings, we have added a statement in the “Discussion section” (page 16, line 362-371):

“Besides experimental parameters, biophysical and biochemical characteristics of BEVs remain largely underreported which hampers the understanding of BEV heterogeneity across different sources. To advance the reporting of biochemical characteristics, we integrated BEV-specific research tools (e.g. PAMP reporter assays) into the EV-TRACK knowledgebase and catalogued commonly explored BEV-enriched and BEV-depleted proteins across abundantly studied phyla and species (Table 2, Supplementary Table 5). Most studied proteins are however often specific to one bacterial species and cannot be implemented as a universal marker for BEVs (Fig. 3d, Supplementary Fig. 2c). Additionally, the observation that some proteins are reported as both BEV-depleted and BEV-enriched underpins how little we know. It is pertinent that further research is required to expand and fine-tune this catalogue.”

13. ****Figure 3D:**** The same comment applies here. Enriched/depleted proteins for all species do not make sense, as all species do not share the same protein repertoire (e.g., OmpA is not present in Gram-positive bacteria).

The treemaps in Fig. 3d indeed highlight the most studied BEV-enriched and BEV-depleted proteins and other molecules for for all species studied to recover BEV from bacterial cells under laboratory conditions, and for the top three studied species: *E. coli*, *S. aureus* and *P. aeruginosa*. As for the treemaps in Supplementary Fig. 2c we do not claim that these proteins are present in all bacterial species. In contrast,

we aim to highlight: 1) low reporting of proteins and other molecules, 2) reported proteins are specific for certain bacterial species and 3) there is no universal BEV-enriched or BEV-depleted “marker”. In order to underline these findings, we have added a statement in the Discussion section” (page 16, line 362-371):

“Besides experimental parameters, biophysical and biochemical characteristics of BEVs remain largely underreported which hampers the understanding of BEV heterogeneity across different sources. To advance the reporting of biochemical characteristics, we integrated BEV-specific research tools (e.g. PAMP reporter assays) into the EV-TRACK knowledgebase and catalogued commonly explored BEV-enriched and BEV-depleted proteins across abundantly studied phyla and species (Table 2, Supplementary Table 5). Most studied proteins are however often specific to one bacterial species and cannot be implemented as a universal marker for BEVs (Fig. 3d, Supplementary Fig. 2c). Additionally, the observation that some proteins are reported as both BEV-depleted and BEV-enriched underpins how little we know. It is pertinent that further research is required to expand and fine-tune this catalogue.”

14. ****Figure 3 legend:**** Change “(e) Molecules” to “(d) Molecules”.

We thank the author for thorough revision of the figure legends and have adapted them according to the comments.

15. ****Figure 4A:**** The number of dots does not match the number of studies per year indicated in parentheses on the x-axis unless stacked dots were used. If so, it might be a good idea to depict the number of studies by the size of each dot.

The number of dots indeed does not match the number of studies per year indicated in parentheses, as stacked dots were used. However, the width of the overlying violin plot indicates the number of studies.

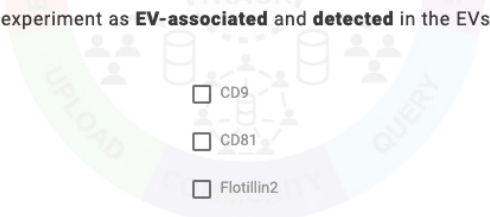
16. ****Figure 4 legend:**** Change “(b-d) Radar charts” to “(b-e)”.

We thank the author for thorough revision of the figure legends and have adapted them according to the comments.

17. ****Table 2:**** BEV-enriched proteins OmpA/C/F/T are only relevant for Gram-negative bacteria, not Gram-positive bacteria. Again, the BEV-enriched proteins would be specific to a species or genus.

We agree with the reviewer that OmpA/C/F/T are only relevant for gram-negative bacteria. However, we propose to integrate these proteins into EV-TRACK as BEV-enriched protein as these are not specific to only one bacterial species. Currently no bacterial proteins are included, only allowing to select markers for eukaryotic EV, or to be added in the “other” textbox (allowing for different possible annotations). To illustrate, we include an example of the EV-TRACK submission platform:

Proteins regarded in the experiment as **EV-associated** and **detected** in the EVs



☐ Alix	☐ CD9
☐ CD63	☐ CD81
☐ Flotillin1	☐ Flotillin2
☐ HSP70	☐ HSP90
☐ MHC1	☐ MHC2
☐ TSG101	☐ Other
☐ Other	☐ Other

Ideally, additional BEV-enriched and BEV-depleted proteins will be included. For this however, further research will be required to expand our catalogue and identify such proteins.

18. **Line 435-442: Are EV-Track administrators S.D.V. and S.D.D. the same person or two different contributors? Additionally, some authors listed in the publication do not appear in the author contributions (e.g., QR, ODV, LV, HD, PT, and both SD).

EV-TRACK administrators S.D.V. and S.D.D. are two different contributors and represent both S.D. from the author list. In order to make a distinction, these abbreviations were used. Some authors were indeed not explicitly mentioned in the author contributions. For full transparency, we have altered the author contributions as followed in the “End Notes section” (page 23, line 537-544):

A.H. and S.V.D. designed the study. S.V.D. performed the database search. Titles and abstracts were screened by N.D.L. and S.V.D. Full-text screening, submission to the EV-TRACK knowledgebase (if the publication was not yet submitted by independent researchers), and collection of additional data not included in the EV-TRACK knowledgebase was performed by N.D.L., S.V.D., N.G. and A.V.C., revised by N.D.L. or S.V.D. and by EV-TRACK administrator S.D.V. Data was extracted from the EV-TRACK knowledgebase by EV-TRACK administrator S.D.D. Data were synthesized and analyzed by N.D.L. and S.D.D. The manuscript was written by N.D.L. and A.H. All authors (N.D.L., S.V.D., N.G., A.V.C., S.D.D., S.D.V., Q.R., O.D.W., L.V., H.D., P.T and A.H.) reviewed and approved the final manuscript.

Reviewer: 5

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

My questions and concerns from the original submission have all been answered very well. Overall this version of the manuscript is much clearer and easier to understand.

Reviewer #6 (Remarks to the Author):

I think the authors have done a great job in improving the manuscript. I can imagine the hard efforts the authors have put into gathering the information, which will support in advancing the field. Below are several comments:

1. The authors assess the quality of manuscripts with EV-METRIC by using several criteria, including the details of the experimental conditions. I think many manuscripts cite other papers if the same protocol was used for sample preparation. In those cases, how is it reflected in the assessment? If a reference was cited and the condition was not rephrased, does it mean that the condition used was not provided? The authors should mention how these types of manuscripts were evaluated.
2. Supplemental Fig. 2c: While the “Enriched” figure contains bacterial proteins/components, the figure showing “Depleted” displays non-bacterial proteins such as CD9. I couldn’t understand why non-bacterial proteins are shown here.
3. Please check the list of “included studies” that is provided as a supplemental file, as there are several typos. For example, the author for PMID30962079 is listed as “NULL,” and the title is "NA." Additionally, there are several manuscripts with the same title, "Digitoxin metabolism by rat liver microsomes," in this file.
4. After reading the abstract and conclusions, I feel the title is not appropriate. While the authors mention the “challenges and opportunities” in the manuscript, it was not the main subject.

Point-by-point response to the reviewers' comments (second round)

We thank the reviewer for the time and constructive evaluation which allowed us to further improve our manuscript, as outlined in the point-by-point answers below. We also adjusted the title as suggested by the reviewer and editors: "Mapping bacterial extracellular vesicle research: insights, best practices and knowledge gaps".

Reviewer: 6

I think the authors have done a great job in improving the manuscript. I can imagine the hard efforts the authors have put into gathering the information, which will support in advancing the field. Below are several comments:

1. The authors assess the quality of manuscripts with EV-METRIC by using several criteria, including the details of the experimental conditions. I think many manuscripts cite other papers if the same protocol was used for sample preparation. In those cases, how is it reflected in the assessment? If a reference was cited and the condition was not rephrased, does it mean that the condition used was not provided? The authors should mention how these types of manuscripts were evaluated.

A selection of included papers indeed refer to another paper if the same preparation protocol was used. If this is the case, applicable parameters (e.g. preparation method “density gradient”) are included in the EV-TRACK checklist. However, if the protocol is incorrectly cited (e.g. referring to a method for a different sample type), this information will not be included. In order to clarify this, we have added a statement in the “Methods section” (page 19, line 413-427):

“All 845 studies were submitted to the EV-TRACK knowledgebase (<https://www.evtrack.org/>). Studies that included multiple sample types or preparation methods were divided into multiple entries, resulting in 3338 experiments. For each experiment a checklist of 233 parameters related to source, preparation protocol and characterization method was completed (Supplementary Table 2). For studies implementing and referring to the preparation protocol of other studies, this information was included in the checklist, if correctly cited. EV-TRACK submission was revised by N.D.L. or S.V.D. and by EV-TRACK administrator S.D.V. For studies that were already submitted to EV-TRACK by independent researchers (n= 18), data was revised by EV-TRACK administrator S.D.V. Data was extracted from the EV-TRACK knowledgebase EV-TRACK administrator S.D.D. Additional data regarding BEV preparation or characterization not included in the EV-TRACK knowledgebase, was collected in a standardized manner in a spreadsheet matrix (Supplementary Table 4). For all bacterial species identified in the EV-TRACK knowledgebase, bacterial taxonomy was complemented (through taxonomy ID) according to the NCBI taxonomy database (Supplementary Table 3)³⁴. Quality regarding EV preparation and characterization was assessed by the EV-METRIC score of the experiments within the individual studies¹⁹.”

2. Supplemental Fig. 2c: While the “Enriched” figure contains bacterial proteins/components, the figure showing “Depleted” displays non-bacterial proteins such as CD9. I couldn’t understand why non-bacterial proteins are shown here.

The term “BEV-depleted” is implemented in this manuscript to address molecules related to other extracellular particles. Absence of these molecules is examined to confirm removal of other extracellular particles from the BEV preparation, “the sample” which is the end-result of implementing a BEV preparation protocol on a particular biofluid. Well-established markers for eukaryotic EVs (EEVs), such as CD9, are typically implemented to confirm their absence in BEV preparations.

3. Please check the list of “included studies” that is provided as a supplemental file, as there are several typos. For example, the author for PMID30962079 is listed as “NULL,” and the title is "NA." Additionally, there are several manuscripts with the same title, "Digitoxin metabolism by rat liver microsomes," in this file.

We thank the reviewer for thorough revision of the supplementary data file and have checked the file and adapted it to contain the correct information.

4. After reading the abstract and conclusions, I feel the title is not appropriate. While the authors mention the “challenges and opportunities” in the manuscript, it was not the main subject.

We agree with the editor and reviewer that the title of the manuscript not fully adheres to the purpose of this work and thus adjust the title as suggested: “Mapping bacterial extracellular vesicle research: insights, best practices and knowledge gaps”.

REVIEWERS' COMMENTS

Reviewer #6 (Remarks to the Author):

The authors have cleared my concerns and I do not have further comments. Congratulations!