Results of the 2017 ISEV Survey on EV and exRNA Science, April 13-28, 2017

1. Background and general information

ISEV prepared a survey on EV and extracellular RNA science in early April, 2017. The survey was opened and announced to the ISEV mailing list and social media sites on April 13, 2017. In addition to the 4/13 announcement, email reminders were sent on 4/24 and 4/28. Almost all responses were submitted within 24 hours of the three ISEV email blasts. The survey was closed after the deadline of April 28, 2017, after the 29th had begun in all time zones. A list of scientific and general interest questions is provided in Appendix 1.

Numbers and geographical distribution

192 responses were received. Four of five identical responses from the same IP address were eliminated, leaving 188 unique responses. Just under 14% of respondents worked in the Asia-Pacific region, 51% were in Europe or Africa, and 35.6% were located in the Americas (Figure 1).

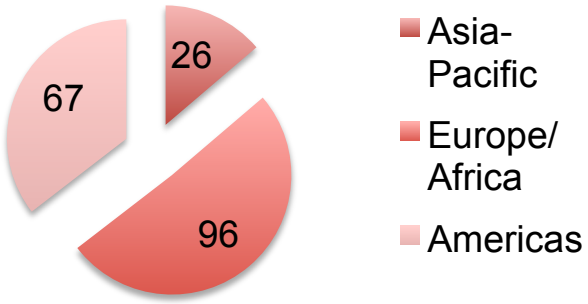

Figure 1. Geographical distribution of respondents

2. Summary of scientific responses

Question 1: State of scientific knowledge of EVs

The first scientific survey question asked respondents to rank current knowledge of the following topics, from 1 (most knowledge) to 5 (least knowledge): Biogenesis, Cargo Loading, Transport/Stability, Uptake, and Cargo Transfer of EVs. A different rank was required for each response, and a response was required for each topic. Summing up all scores, the rank from most to least knowledge was:

1) Biogenesis
2) Transport/Stability
3) Uptake
4) Cargo Loading
5) Cargo Transfer

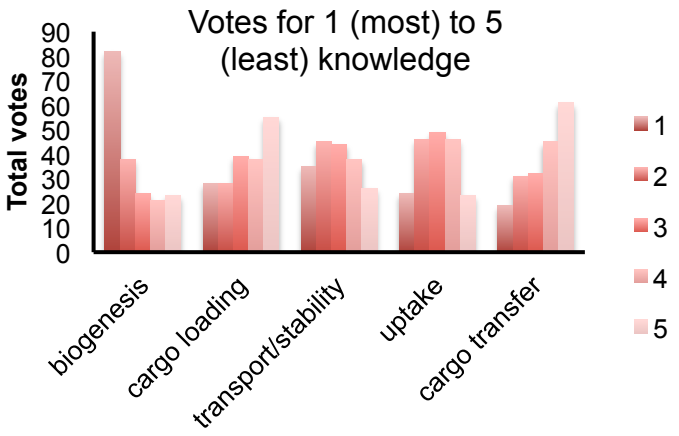

Figure 2. EV topics about which we know the "most" to the "least"

The distribution of votes is visualized in Figure 2 by plotting the total numbers of votes for each rank. Among substantial difference of opinion, several results stand out. Biogenesis received by far the most "1" (high level of knowledge) votes, nearly as many as all other categories combined, and results skewed towards the left (1). Cargo Transfer and Cargo Loading received the most and second-most "5" (least knowledge) votes, and results were otherwise skewed towards the right (5). Ambivalence was obvious around the transport and uptake categories, with most votes in the middle of the range. These results suggest perceived unmet needs across all topics, but especially in cargo loading and transfer.

Question 2: Factors responsible for the effects of EVs

Respondents next, in scientific question 2, identified factors they felt were responsible for the effects of EVs:
1) Lipids
2) Nucleic acids
3) Proteins
4) Metabolites
5) The EV as a whole, not any one constituent molecular category
6) Particles/molecules associated with the outside of EVs, not the EVs themselves
7) Other (please specify)

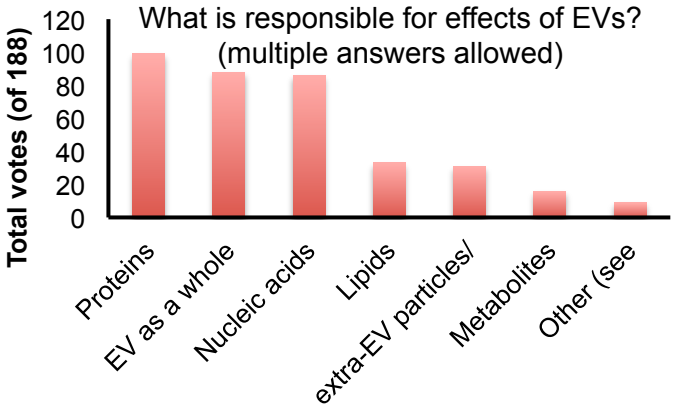

Figure 3. Factors most responsible for observed effects of EVs

Multiple selections were permitted, and an "other" box was included for comments. Only proteins were picked as a responsible factor by a majority of respondents (Figure 3). However, not far behind were "EV as a whole" and nucleic acids. Lipids, extra-EV particles, and metabolites received far fewer votes. Nine respondents provided "Other" comments, which are included in Appendix 2 at the end of this document.

Question 3: Factors with biomarker potential

The same choices as above were offered in scientific question 3 as factors with the greatest biomarker potential. This time, nucleic acids and proteins were chosen by a majority of respondents, while other factors were not highly rated (Figure 4). There were seven "other" comments (see Appendix 2).

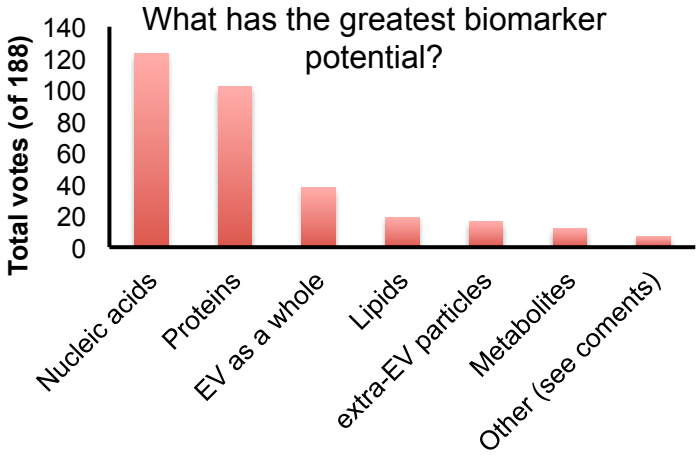

Figure 4. Factor(s) with the greatest biomarker potential

Question 4. Needs in international and other collaboration

The state of regional and international collaboration in the field was next queried. 66% of respondents suggested that more international collaboration was needed, while 62% asked for more national or regional collaboration. 38% indicated a need for both. 10% felt that the current levels of collaboration were appropriate. 22 respondents left "other" comments (see Appendix 2).

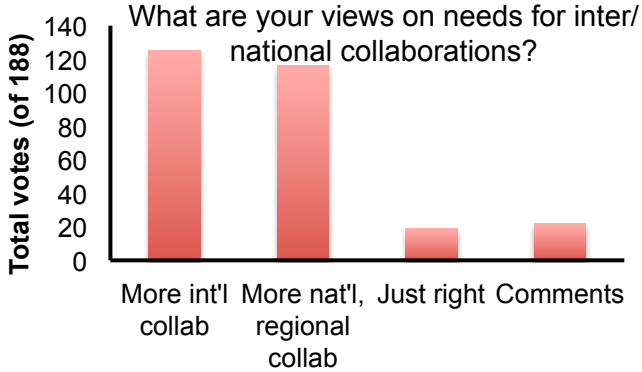

Figure 5. Collaboration needs

Question 5: Is the focus on RNA (in the last decade) justified?

The final question with specified answer choices was about the focus on RNA in EV studies: is it justified or not? A majority (almost 62%) felt that an emphasis on RNA was justified, while 21% did not. Around 18% disagreed that there was an emphasis on RNA. In optional comments (Appendix 2), several respondents observed that the emphasis on RNA has been a relatively recent development.

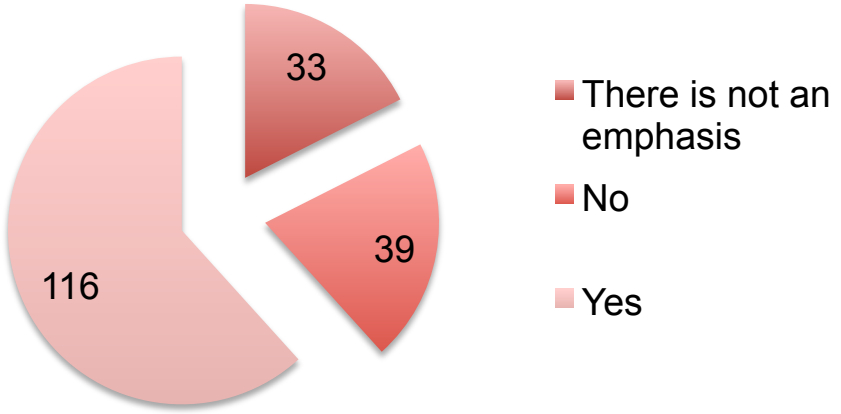

Figure 6. Is the recent emphasis on RNA justified?

Open response questions

Four open response questions completed the survey. Respondents were asked for input on needs in the field of EVs and exRNA (open question 1), including fundamental aspects/mechanisms (2). Third, respondents were invited to indicate standards/protocols that are needed. Fourth was a final comment box for any thoughts that were not captured in the earlier parts of the survey. The first two questions, responses were mandatory, although some provided placeholders only, and these were eliminated. 175, 178, 129, 23 answers were provided to the four questions.

Because of the wide-ranging nature of these responses, we include the raw answers here (Appendix 3) in their entirety and leave any conclusions to the reader.

Appendix 1. ISEV 2017 EV and exRNA Survey Questions (scientific and general interest)

Scientific and general interest questions from the survey are included below. Questions specific to ISEV or to the individual respondents are not included. Answers have been de-coupled from any personal identifiers.

Required-answer questions. Multiple choices were provided, and 2, 3, 4, and 5 included an "other" response and/or comments box.

1. (Required) Please rank the following in order of what you feel we know the MOST about to what we know the LEAST about (MOST knowledge is "1," LEAST is "5"):
2. (Required) In your opinion, the effects of EVs are mediated mostly by (choose at least one):
3. (Required) Which component of EVs offers the richest biomarker potential (choose at least one)?
4. Regarding collaboration in the field (choose one or more):
5. Is the historical focus on RNA in the EV field justified?

Open Response questions. 1 and 2 were required, but not all provided substantive answers (N/A, ?, etc.). 3 and 4 were optional.

1. RNA has been a strong focus in the EV field during the last decade. This interest was reflected, among others, in the decision of the National Institutes of Health in the United States to sponsor an Extracellular RNA Communication Consortium (ERCC) for five years of funding. What are the remaining obstacles and challenges in advancing extracellular RNA and EV research? What is needed to overcome these obstacles and challenges?
2. What fundamental aspects/mechanisms/characteristics of extracellular RNA or EV biology remain to be determined in order for the field as a whole to flourish?
3. What standardized protocols or resources, if any, would you like to see developed for the EV community?
4. Please use this space to add any comments about EV or exRNA science that were not captured by the questions above.

Appendix 2. ISEV 2017 Survey on EVs and exRNA: comments associated with multiple-choice questions

Question 2, "In your opinion, the effects of EVs are mediated mostly by (choose at least one)." Responses in the "Other" comments section:

1. I think its too early to say
2. impossible to answer this question: depending on the experimentalmodel and question, it could be either one of these molecules, or the combination
3. Leukotriène: PLoS Biol 14(1): e1002336
4. In some metabolic diseases carbohydrates, glycans, mostly complexed with proteins (i.e. glycoproteins)
5. Not enough known to make this choice
6. Nucleic acids are most studied, but not sure if that means they are the primary mediators.
7. The EVs and associated molecules
8. the target cell status and the surrounding environment
9. unprocessed RNAs

Question 3, "Which component of EVs offers the richest biomarker potential (choose at least one)?" "Other" comments:

1. Cellular origin of EVs
2. I think for proteins and nucleic acids the field is more advanced
3. I think its too early to say
4. impossible to answer this question: depending on the experimentalmodel and question, it could be either one of these molecules, or the combination
5. metabolites should be stably transferred without EVs
6. miRNA
7. This is a disease by disease situation.

Question 4, on perceived needs for international or regional collaboration. Comments:

1. We need to stop this race over publication to ensure good quality work and sefety in the project : we need to put specialists on the same fields of research around a table to decide what are the objectives and what would be the best way to answer those.
2. We need more opportunities to interact on the local level routinely and yearly on the national international level.
3. We need more disease-driven collaborations in EV research, including international and national.
4. We need collaborations that enhance productivity regardless of other factors. Those tend to be international due to larger pool of compatible expertse and the absence of competition for local resources.
5. Unfortunately, the way science is organized and success judged on a personal level, you better invest in changing the way we publish and get funding.
6. there are many international collaborations between EV veterans, novices tend to be out of the loop

7. The field is on a good way, internationally and nationally
8. the collaboration among academic, industry and clinic
9. publish more specifics regarding isolation in order to find the most suitable one for each application
10. Notably absent is a strong network and society in the USA
11. It would be great to get some regional networks going that can then link in with other regional networks to set up national and international collaborations. The advancments in digital communications is making this a lot easier.
12. it starts to work out :-)
13. It is vital to foster collaborations between groups with expertise in complementary areas to promote sinergies
14. Include research groups from undeveloped countries. We have access to clinical samples with a rich clinical data from different stages of the diseases. In our country, there is a high interest of institutions in stablish research centers in traslational medicine.
15. in an attempt to solving potential issues or having plausible answers; some tasks might be devided among international groups involving regional teams. Involving young researchers might be a positive addition since they are much enthusiastic and motivated.
16. I don't have a real opinion about that
17. I am not sure how ISEV can foster international collaborations, apart from making people meet at the meetings and workshops, which is already good!
18. I am not recognised though I have several original research papers with IF>10 ;-)
19. hard to say too much competition and probably not so much discussion
20. Fresh field requires collaboration in order to promote the progress
21. e.g. Germany
22. Basically non-existant in my region

Comments on "Is the historical focus on RNA in the EV field justified?":

1. ...although I don't think we have full proof that it is indeed the RNA component of EV that is functionally active in target cells
2. All the components of EVs to be studied extensively along with its functions.s
3. Also a lot of protein work done! RNA is of course fantastic marker molecule since there are numerous cell type, organ and cancer specific transcripts that can easily and specifically be amplified for analysis. Small RNAs are stable; what more do you want?
4. But in my opinion it is not the only aspect that should be in focus.
5. but other components deserve focus as well (lipids, proteins)
6. but that shouldn't detract from looking to explore other markers
7. But the focus should slowly shift from RNA only
8. DNA seems to be left out because of difficulties to make sense of it. RNA diversity is great and a big effort is still needed to go beyond miRNA
9. do not know exactly what is meant
10. First seminal study by Valadi et al on miRNA in exosomes stimulated EV-RNA studies, but we should also encourage first studies which reported functional RNA transfer. historically speaking study by Ratajczak is less recognized among community in particular people who are new in the field. also functional transfer studies by Pegtel,

Skog and initial decade of EV-RNA field might be given a historical coverage among comunity, when delivering talks, suggeting these studies to new ones.

11. Historical focus on miRNA in particular
12. historical focus?
13. I am more focused on proteins.
14. I do not know well.
15. I do not understand the question, what do you mean by historical?
16. I don't get the question. Is there a historical focus on RNA?
17. I don't know
18. I favour proteins
19. I think all fields of EV researches are going further
20. I think it was one of the first to be show changes in a recipient cell phenotype, and the excitement began there. But other molecular components have emerged since that are likely just as important - it may be the whole package is required for signaling.
21. I think that perhaps other areas have been neglected, because of the focus of many on RNA. There should be encouragement to look at other aspects of EVs such as lipids, metabolites and proteins.
22. I think we should also focus on the role of RNA-binding proteins.
23. It is only part of the story and can not be viewed apart from other extracellular molecules.
24. It needs to be part of the mix. More needs to be known about lipid and protein function and holistic functions of all acting in concert
25. It was just easier to persue
26. justified due to the instability of RNA yes, but the marriage of these two concepts has made the field too exclusive
27. mRNA in particular
28. Much ado about not as much done...
29. Not sure 'justified' is a good term ...
30. Nucleic acids just happen to be simpler to analyze
31. proteins and lipids are as interesting and relevant
32. RNA holds the most exciting therapeutic and diagnostic potential, plus the coolest biological implications (i.e. connections to viruses)
33. RNA in EV is quick to act, easy to be transferred and abundant- so it serves a specific purpose that we have little understanding.
34. RNA is probably important, but it's the lipid and proteins that will have first actions. RNA are slower activities and quantities to have a real effect.
35. The fascination about RNA reflects early discoveries and the need to better understand post-transcriptional regulation (hence focus on miRNA, for example).
36. the focus is not historical, it started in 2006-2008, several decades after historical discovereies/descriptions of EVs
37. The focus on RNA has mainly been driven by strong research groups in the RNA field and their interest, as well as applicability into translating into clinical use, but the protein/lipid content is also worth studying
38. The focus on RNA is recent and "historical" only for newcomers to the field. It may be justified from the biomarker perspective, but we shall see.
39. The focus on RNA seems to be on the rise, but for the right reasons?
40. The importance of proteins has been shown, not for RNA.
41. tunnel vision on microRNAs, which are minority of all RNA classes present in EVs

42. Why RNA only? This is a very biased view.
43. Yes and no. I think that too much emphasis has been given to any one component instead of all together.

Appendix 3. Open response answers to the 2017 ISEV EV and exRNA survey

For the open response questions 1 and 2, an answer was required, while 3 and 4 were optional. Some respondents used placeholders (-, ?, N/A, etc.); these answers were eliminated.

Open Response 1 (required). RNA has been a strong focus in the EV field during the last decade. This interest was reflected, among others, in the decision of the National Institutes of Health in the United States to sponsor an Extracellular RNA Communication Consortium (ERCC) for five years of funding. What are the remaining obstacles and challenges in advancing extracellular RNA and EV research? What is needed to overcome these obstacles and challenges?

1. bioinformatics is just now the biggest limitation, miRNA are easy but for more complex small RNAs one needs special training. We also need single vesicle study and for this maybe digital pcr offers good perspectives.
2. Understanding physiological function of exRNA. Need to use mouse model or other in vivo model.

3. - Accurate RNA quantification - EV reference genes for qPCR normalization (and methods to identify and validate these per disease setting / experimental questions) - Better understanding of the RNA content of individual vesicles - EV targeting, uptake, RNA cargo delivery
4. 1. Identifying suitable controls (and guidelines on isolation methods) for RNA analysis 2. guidelines on blood/plasma/tissue isolation of EVs and EV-derived RNA 3. Downstream tissue-specific functional assays. 4. demonstration of EV and EV material (RNA) transfer- development of mouse models 5. study of in vivo EV half life, transfer
5. 1. most of current work focus on small RNA (more abundant) not enough on mRNA 2. discrepancy of data due to technical issues (ways to collect EV, library prep systems, enrichment schemes...etc)
6. 1. Seeking EV donor tissues in body 2. Non-disease physiological function of EVs 3. Effect of exogenous RNA as a functional food component
7. 1) to optimize and standardize more 2) make results better comparable; especially the isolation procedures 3) to bring all types of data together (morphology, size, RNA, protein, lipid, metabolite) => integrative data analysis
8. A non-biased platform for quantification of exRNAs. Commercially available platforms are not able to provide this, and the companies do not have the resources to develop this very important tool.
9. A reliable method in obtaining RNA for RT-PCR and such techniques
10. A similar consortium/collaborative effort in Europe, as well as continuous meetings on the topic
11. Acceptance from some areas of the scientific community that EVs are not artifacts, particularly with respect to funding individual grants.
12. Access to technology in undeveloped countries that have more access to clinical samples. The high prices for reagents and methods for RNA analysis. Is necesary to find and acqurate method for isolation, normalization and quantification of the RNAs from EVs, that combined with the improvement of standarized methods of isolation of EVs could allow the comparison of the data obtained by different groups.
13. Answer the question how RNAs can be functional when taken up by the target cell via a vesicle. Better technologies to specifically isolate EV subpopulations from biofluids

to study diversity in EV RNAs How best to load EVs with specific RNAs
14. Better isolation Better sequencing
15. Better isolation techniques
16. Better methods for the purification of EVs
17. better methods: instruments and reagents; better use of flow cytometric methods
18. better pre-analytic: stabilization of samples
19. Better science and more rigorous peer review will improve quality and respectability of the field.
20. Better Screening Better Databases
21. better/more robust assay techniques
22. Cargo Loading and Deletion Better understanding of how EVs are loaded with their content
23. catalogue of specific reagents able to work on minimally purified EV samples
24. Cell and environment specific RNA content. Purification of EV and separation. Solution: more integrated across the board research.
25. cell/tissue specific EV isolation
26. challenge: the small amounts of RNA in circulating EVs. Solution: better/more affordable technology for detecting small amounts
27. Challenges: - understand/overcome biases in non-coding RNA analysis - optimization + standardization in all steps taken from acquiring the EV/exRNA containing fluid to bioinformatic analysis of the data - develop methodologies to determine heterogeneity in RNA content of EV populations - develop more advanced methods to prove that EV-induced effects on target cells are caused by the RNA associated with these EV
28. Characterization of EV identify, RNA purification kits, RNA integrity status, mRNA sequencing of EV cargo from human body fluids
29. Collaboration and interaction Database open resource
30. Collaboration, sharing of samples to pin down the reproducibiliy of data
31. Collaboration. Testing and investment in new technologies.
32. Concensus on methods
33. Consesus. How we define, isolate and work with exosomes. Why the exosomal RNA yield is that much variable among samples.
34. Continuing to identify and clarify all mechanisms of exRNA transfer; developing further standards to increase research reproducibility
35. Define initial isolation approaches and scale-up
36. Defined set of standards for the field.
37. Defining standards and classes for EVs Rigorous standardization of EV isolation protocols
38. Detection (verifying platforms for detection, i.e. microarrays, RNAseq, drop digital PCR), determining the contribution of protein/liproprotein associated RNA.
39. Detection limits when working with body fluids, quantification, normalization. All this influences reproducibility and reliability of obtained results. Dedicated technologies are developing rapidly and we are confident that these problems will be quickly overcome. Another main point remains to understand how extracellular RNA is selected for vesicle loading and which kind of vesicle represents major targets / exosomes, microvesicles etc. for the cell to load the RNA. The distinction between the different vesicle types remains a major challenge influencing also extracellular RNA research.
40. Development of new methodology for sophisticated delivery system based on exosomes for future therapy

41. Discovering more biomarkers
42. Do not work in the RNA fields of EVs.
43. Ease of access to enough material for research e.g. enough volume of plasma to isolate enough rna for ngs
44. EV Imaging and metabolite profiles
45. EV purification
46. Fidelity of function of RNA. Among RNA product, there still have not 100% functional RNA.
47. Figuring out what RNA is inside vs. outside EVs and figuring out how to study whether RNA is transferred.
48. Findings that apparently no RNA is "junk" RNA--how do we deal with that in informatics and biology?
49. For people interested in exosomes but working outside the field it is difficult to network and gain access to training or information about oppportunities
50. Funding, and getting on top of issues in methodology and heterogeneity.
51. How best to selectively incorporate specific RNAs into exosomes. Should also focus on non-RNA components of EVs
52. How RNA would be transferred into cells and how often this occurs in comparison to degradation, etc.
53. how to normalize RNA level in EVs
54. I am a beginner in the field and do not work with RNA so I cannot answer this question
55. I am an expert on surface antigens on large EVs...
56. I am not sure the consortia (U-type) grants will help us get to the next level
57. I believe more gatherings where professionals can share their experiences and information is an essential part of advancing the field that is currently lacking.
58. I believe that good stable controls to normalize rna data are necesary in order to have more accurate results
59. I do not engage in research in the field of EVs RNA and thus cannot comment on this specific question.
60. I do not have have any expertise in this field
61. I do not work on RNA in EV.
62. I don't know
63. I don't know
64. I got No sufficient expertise on this research field to answer
65. I think lack of training and funding are considered the basic obstacle to take this field into the next level. I , myself, am searching for training on exosomes and I cannot find a place or lab. to train me. I applied for EMBEL course and seats was so limited and I was rejected for such reason.
66. Improvements in isolation methods and emphasize to the clinical research community on the CRITICAL IMPORTANCE of knowing that they are REALLY working with EV! Too many rely on sales pitches and "kits" from companies that have been proven over and over to NOT isolate purified EVs! Yes, this is an exploding increase of clinical publications using the failed kits!
67. In vivo detection of specific population of exosomes and the effect on target cell exosomes classification
68. Increase funding for more reserch
69. Increase the RNA yield obtained from ultracentrifugation.
70. Isolating EVs effectively for downstream analysis. Obtaining sufficient RNA to

analyze.

71. Isolation of EVs from plasma without isolating lipoproteins which can also contain RNA.
72. It is important to establish the mechanisms whereby RNAs are selectively sorted in to the EVs. Is this a planned and orchestrated action to modulate the protein expression in target cells? How are the different RNAs conveyed in EVs "selected" to modulate protein expression in recipient cells? Is this a non-selective random process?
73. It is published several fold that RNA is transported via micro vesicles, but there is little knowledge about the import of specific forms of RNA into Microparticles or exosomes and also about the release into the target cells.
74. It remains a question of how many EVs contain eRNA and we have to address the heterogeneity of EVs in more detail. For sure eRNA is important, however, focusing mainly on eRNA provides the risk in neglecting other essential aspects in EV me.diated intercellular signalling
75. knowledge on mechanisms of RNA cargo loading to the different types of EVs, which requires to analyze separately the different EVs (exosomes, other small EVs, larger EVs etc), rather than as a bulk mixed population
76. Larger studies required to better define the function of the RNA in the EVs
77. Little material available from patients High costs for the analysis (RNA seq)
78. Mains questions remaining are which mechanisms are underlying the selective selection of RNA, to be incorporate, and which mechanisms of uptake promote an effective RNA incorporation in target cells
79. Many challenges including clinical applications and the potential for medical tourism
80. Many studies focus on miRNA and have explored everything from expression (arrays and sequencing) to transfer, so the main obstacle is to standardise techniques between studies and invest more interest in other EV biomolecules
81. Many would argue that there is little proof that EV-RNA has a biological effect at physiological concentrations.
82. Method of isolation
83. Methods to more effectively isolate and characterize subpopulations. What is needed are both better wet lab methods and "standardized" informatics tools to decipher/compare cargo to existing datasets and repositories.
84. micro RNA "encyclopedia"
85. More effective and contaminent free EV isolation
86. more funding and educating the fund giving bodies on the importance of EVs
87. More research is needed into ev biogenesis
88. more sensitive methods to analyze poor quantity of vesicular RNA
89. More work needs to be done on exRNA biogenesis, not just in EVs, but other complexes. Biogenesis and function is just beginning to be elucidated
90. My main concern with extracellular RNA research is one of biological relevance. It's difficult to address how many EVs or how much exRNA might be enough to exert a biological effect in vivo, despite interesting findings in vitro.
91. Need patient samples, bodily fluids and big data analysis
92. Networking among ERCC funded investigators and non-ERCC funded investigators. The ERCC seems to be a club where only these members collaborate and are given access to databases/software and it is not available to the public.
93. New advances in “interactome profiling” focusing on network-centred strategies for EV analysis to highlight protein interactions and the influence of protein structures

during biogenesis, trafficking and function Integrated proteomic, genomic, lipidomic, and metabolomic investigations to provide an integrative systems biology approach of EV biology

94. not competent enough to give ad educated answer
95. not sure
96. Not sure about focus of RNA
97. Nothing comes to mind.
98. Obtaining high quality samples of greater quantity
99. Overcoming noise to emphasize reproducible results.
100. Perhaps more collaboration between researchers and labs.
101. precise knowledge on loading, qunatification
102. Proof that RNA in EVs mediates disease pathogenesis in vivo.
103. Proper consultation mechanism among scientists working in this area supported by experimental evidences will help to overcome the problem to some extent.
104. Proteomics and lipidomics. Cargo uptake. Specificity
105. Publish more paper so people start believing
106. Purification of EV RNA is difficult if working with primary cells e.g. blood cells. We need better purification methods without compromising yield.
107. qRT analysis requires a reliable housekeeping gene. This has to be defined for ev research.
108. Quality control and Standards, Quantification measures
109. Quantitative data are required, in which EV RNAs incorporated into EVs, EVs in particles, and EV RNAs delivered to target cells, are measured precisely.
110. Reference ranges for RNA content based on the cell of origin in different body fluids
111. Reliable separation of EV RNA from non-EV RNA when needed. (Not necessarily prerequisite for e.g. biomarker discovery)
112. reliable technique to characterise EVs and identify EV cargo
113. Removal of RNA in EVs will determine their role.
114. Reproducible results and agreement in the RNA profiles: small example 18S and 28s are or not in the EVs cargo in the same bofluid/cell culture type media etc?
115. Requires funding for junior investigators
116. Researchers would like to purify the RNA only from EVs. However, we just known some of RNA are not from EVs. How to purify the RNA only from EVs not from lipoproteins or Ago2 complex?
117. Rigor, reproducibility and lack of standardization are major problems in the EV RNA field. The ISEV guidelines published recently go someway to address these issues and will hopefully help shape the future of EV RNA research.
118. RNA is secreted to extracellular space; however, we do not yet know exactly what determines what species of RNA is secreted, and in what particles, and if they have real function in recipient cells. We need more sequencing of EVs and throughout screening of biodistribution of the RNAs.
119. RNA loading into EV
120. RNA sorting mechanisms.
121. Sample handling and exosome purity tests
122. Samples
123. Single cell response to EV-RNA

124. Single EV analysis, including single EV cargo analysis, to increase clinical sensitivity and specificity of translational studies
125. Single EV analysis. --> Single EV isolation device and procedures --> Sensitive analysis devices
126. single-vesicle RNA analysis to identify biologically meaningful subpopulations
127. Small amount Optimal endogenous control for gene expression studies
128. stabilitty of EVs and safety of EVs
129. stability of RNA and also EVs
130. standard procedure
131. Standardisation of methodologies for isolation and analysis; validation studies; obtaining insights into the roles of other EV associated RNA's besides miRNA
132. standardization is the biggest challenge and the most important
133. standardization of protocols, reproducibility of results
134. Standardization of techniques and methods across studies. Influence of media for cell culture experiments. As always: more precise terminology (EV subclasses) and differential analysis of molecules enriched in these populations
135. Standardization, improved methods for detection and isolation
136. Standardization, standardization, standardization.
137. standardizations used for experiments. publishing according to recommendations: Mateescu et al. 2017
138. standardized methods for RNA isolation from EVs and possibility to sequence both very short (25-50 nt) and short (up to 150 nt) RNAs in same sequencing run.
139. Still many things such as: 1. Finding an appropriate endogenous control (housekeeping gene) for study of gene expression analysis with qPCR. 2. Establishing a good protocol for removing EV-binding exogenous RNA to be sure about specificity of RNA inside EVs. The current effort using RNase and proteinase K treatment of EVs is not highly successful and still up to 40% of EV-binding RNA is not removed.
140. Still no clear prptocols for exosome RNA Seq, analysis pipelines missing. The variability between samples is high and solutions to that have not been found
141. Subgroup of EVs from EV population In vivo study
142. technical challenges clinical utility
143. Technical challenges of accurately measuring and profiling low-abundance extracellular/EV RNAs. Especially seeing as the concurrence between studies is still very low.
144. Technical difficulties still remain concerning EV purification and characterization and hence in defining their RNA content. We need further advancements in standardization of EV purification methods.
145. Technical difficulties: normalization issues, detection of small amounts of RNA
146. Techniques to demonstrate selective packaging and delivery of EV cargo.
147. Techniques to overcome bias in RNAseq
148. The basic challenge is to find collaborators and get well trained on exosomes protocols.
149. The field of therapeutic EVs still lacks the other fields of EV research. More funding at the NIH level is required to further advance the field of therapeutic EV research
150. The main challenge is to correlate specific RNAs in EVs with a biological function in in vivo and vitro settings, using EVs in a physiologically sensible amount

(so not in excessive non-natural amounts as usual published). It would be nice to trace single RNAs from EVs into target cell machinery, to adequately show that they are functional. In addition, stoichiometry of RNAs in EVs should be better considered (ie. if there is only 1 given miRNA per 1000 EVs, how can it have an important effect in target cells.

151. The main obstacle continues to be EV isolation and purity of EV isolates, in particular when purified from complex biofluids like blood. This complicates the interpretation of RNA sequencing studies and evaluation of their biological activity in recipient cells/organisms. To ensure success and overcome these obstacles it is imperative to develop standards and identify reliable and specific markers. It is also necessary to continue the efforts to optimize and develop novel methods and technologies to specifically isolate EVs.
152. The main obstacle is that there is not a unique way to isolate and characterise them. Because of that many group claim that they are isolating RNA only from EV but it's not true.
153. The methodology for one-particle analysis of RNA may be needed.
154. The realization that most papers in the field are fake
155. There is a big need need to separate what is EV associated RNA and free RNA associated with carrier proteins only or other ways of which RNA is transported extracellular. Then how EVs and RNA is isolated is really important in order to make comparisons
156. There is a need of defining universal and stringent SOPs when utilizing EV-RNA or other EV contents in point of care diagnosis or therapeutics. While considering EVs as therapeutic modalities there must be a standard or minimal SOP for harvesting GMP grade EVs. Moreover, much has been documented from cell culture EVs, but we need more focus on patient derived samples (e.g. biofluids in case of biomarkers) and finding a way to target organs for therapeutics. Moreover, we need to focus what RNA species are of particular interest and when considering them as biomarkes non-EV circulating RNA stabelized by proteins is equally important for biomarker discovery since it provides mutation based screening or expression profiles from patient body fluids. In which cases we want EV-RNA and in which cases we want non-EV exRNA must also be defined.
157. This initiative has provided a great deal of important information available to the field of researchers in terms of deep sequencing data and needs to be increased to include more researchers and new perspectives.
158. To understand if the RNA is going inside the EV or couple to the EV
159. To understand the rules of RNA loading into EVs and subsequent role of the fragmented and non-fragmented RNAs in the recipient cell.
160. translating RNA into proteins into recipient cells effect of exo miRNA on target gene expression
161. ultimate definition of EV associated markers and function in various diseases.
162. Understanding how a mixture of RNA species mediate a total biological response, and which components dominate the effect
163. Understanding how EV assist in homeostasis, how disease alters EV biology, and how to best interrogate EV cargo in biofluids, and how to exploit EV for therapy. Challenges include improved benchmarking technology by industry-academic partnerships, greater funding for basic EV biological research independent of industry, streamline use of biorepositories, translational studies.
164. Understanding how rna cohorts with in Ev act together to modify cell function

165. Uniformity and reporting of isolation methods. A major issue is how samples treated with different procedures are compared against one another.
166. Verify at the very first place wheather these exRNA being analyzed are not A artifact.
167. Visualisation of RNA in the vesicles and once targetted in the recipient cell
168. We need artificial nanoparticles to spike EVs-containing biological fluids
169. We need more international collaboration in EV research
170. We need to find a reliable way to label vesicular RNA (other than Syto RNAselec) directly in EV and fluorescently in order to be able to observe transfert and activity of those RNA. It would be also important to consider all RNA species as a whole when looking into biological activity because a single RNA is unlikely to be the reason of the whole phenotype. We should not exclude other components in vesicular RNA study. For those we need to determine a flow chart and techniques validated in all aspects to ensure reproducible results and develop RNA monitoring techniques, specialy at a single cell level.
171. we need to understand better who mediates RNA loading, protection and the real role of ExRNAs. In some part they are specifically loaded with some purpose. Also, RNA binding proteins are not all the same, they have a role in loading and in mediating the RNA effect on their targets
172. We still need to determine what physiological levels of EVs are and whether the RNA numbers in those EVs are capable of producing the effects observed in vitro.
173. What is needed is a robust proof of function in vivo. This is still to some extent inferential due to the complexity of EV content and effects.
174. Wide range of noncoding RNA and unknown function for many of these RNAs indicate that more research for functonal analysis is required.
175. Widely adopted, easy and reproducible protocols for RNA isolation from clinical samples.

Open Response 2 (required). What fundamental aspects/mechanisms/characteristics of extracellular RNA or EV biology remain to be determined in order for the field as a whole to flourish?

1. Cell surface markers, distinction between waste vesicles and signaling vesicle if there is one. Identification of specific receptor entry mechanism. Receptor for RNA beyond TLR-let7. Understanding how transfered RNA can have an effect, how is it taken up and how does it meet target? Does it act at nuclear level?
2. Biogenesis of EVs. How to sort RNA into EVs.

3. Better understanding the links between the cells that release EVs, the content of these EV subtypes, and the cells that take up these EVs. Basically: sender, message, receiver. How do these influence eachother? This will lead to better understanding of EVs as signaling entities.
4. Identification/characterization of different sub-populations of EVs.
5. characteristics of EV is important.
6. 1. function of unidentified EV RNA 2. loading of RNA during EV/microvesicle biogenesis 3. delineation of EV-associated and EV-containing (lumen) RNA and its stability, functional significance for drug delivery
7. A guideline of sample preparation and bioinformatic analysis of NGS data designed for EV RNAs

8. EV donor tissues in body. Mechanism of release and uptake of exRNA or EV.
9. 1) EV release 2) EV uptake 3) cross kingdom communication
10. The ability to determine when an EV is functioning in signaling, and when it is functioning to release contents from overloaded cells (the original junk theory).
11. Not quite sure
12. Detailed knowledge on selective packaging of I.e. RNA upon cellular stress or injury..
13. Factors underlying EV induction.
14. Specificity of the EVs: origin and target.
15. How can EVs transfer their content into the cytoplasm when taken up by the target cell? How are proteins and RNAs specifically loaded into EVs? How can we specifically stop production and uptake of EVs?
16. Transfer of nucleic acids and their function in recipient cells Excluding miRNA
17. Rna subtypes and loading into evs
18. Applications of EVs in drug delivery
19. identifying the roles of these molecules

20. There are too many inconsistencies between claims related to exRNA and the scientific literature. These inconsistencies seem to be overlooked or ignored due to the desire to publish and/or make the field relevant, especially in regards to dietary uptake. The black box needs to be opened and assessed mechanistically and quantitatively and reconciled with the literature.
21. EV subset isolation fundamental EV isolation technique
22. Processing mechanisms
23. Sorting and Loading
24. move towards standardized/agreed methods for these sample types, starting at the isolation process,
25. RNA content specific to the cell and how the cargo can be changed. Can we program ev production.
26. isolation strategy of a cell/tissue specific EVs from the plasma/sera
27. why is it there? what is its target? what does it do to its target?
28. - delineate how RNA is transferred to target cells and how it can affect the behavior of these cells
29. Function of the RNA cargo, integrity analysis of RNA, understanding (specific) loading mechanisms
30. Intraluminal or surface associated RNA ? Types of nucleic acids associated with EVs ? Is there any specificity in the association ?
31. ?
32. There is so much more that needs to be investigated and so many directions to take. The fundamentals need to be determined and agreed the field will naturally expand and flourish.
33. Understanding of microRNA interactions
34. What is an exosome and how we are going to define it.
35. Many...one focus could be to better define cellular identity and conditions under which EVs are obtained to increase reproducibility and allow for mechanistic determinations of how cell phenomena are causative or reflective of EV cargo and bioactivity.
36. The degrees of heterogeneity
37. That the cargoes of EVs survive in sufficient quantities to be absorbed and that these cargoes can be used by recipient cells.

38. relevance in clinical applications that can be translated to actual applications instead of "potential"
39. in vitro effects of RNAs
40. Despite the technological advances in identification, counting and detection, the distinction of the different vesicle types remains a major hurdle. These hurdles depend also on the difficulty in direct visualization techniques of EVs. RNA labeling in single vesicles and single vesicle studies. When this will be possible for the average researcher, we will be a big step forward.
41. Mechanisms of cellular uptake of exosomes and cytosolic release of exosomal contents
42. Do not work in the RNA fields of EVs.
43. Understanding the complexity of different rna species within vesicles
44. Quantity of RNA per EV
45. Transfer of EV cargos
46. How to deliver into cell with no damage
47. Lots of questions wide open (biogenesis/cargo/uptake/whether transfer is functional)
48. Rapid, accurate, and truly useful bioinformatics
49. Being able to separate and identify cell of origin would hugely advance studies of EVs in immunology
50. Heterogeneity and how this is reflected in biological effects. Also understanding the range of ways in which EV RNA has its effects (not just miRNA repressing expression and mRNA being expressed).
51. The biggest hurdle in my opinion is our inability to inhibit exosome biogenesis in a targeted manner so as to define their function in vivo using mouse models.
52. Whether EVs deliver RNAs while extracellular, after fusion, or from inside phagosomes
53. Selection/No selection of RNA found in EVs? All circulating RNA are in EVs?
54. therapeutic applications
55. EV use in diagnostics within a standardised approach/frame work.
56. as noted in my reply to #5 above
57. Whether or not the cargo is arbitrary or programmed.
58. Rna cargo into the Ev and its specificity
59. The shedding mechanism should be specific for different types of cells. A clear and precise differentiation between exosomes and EVs in terms of biomarkers. Determination of EVs contents and affects on cell content post shedding.
60. to determine their potentia role in pathogenesis of different infactious and non-infections diseases
61. Specific markers, inhibitors and stimulators of biogenesis, cargo loading, uptake and release of exosomes
62. I don't know
63. I don't know
64. I got No sufficient expertise on this research field to answer
65. The targeting of exosomes as a whole and considered as a bio-marker.
66. What controls the formation and export of EVs and is cargo uptake specific or passive. Easy methods for isolation of purified populations for study and stopping the proliferation of these kits that are NOT isolating purified populations of EV.
67. How exosome RNA profiling affects target cell
68. More data on relations to clinical aspects

69. Mechanisms of EVs adressing.
70. Identification is key, then function will follow.
71. No idea
72. In my opinion it is very important to elucidate the mechanisms and signals that govern the selective sorting of material to be loaded in EVs and have an holistic view concerning the strategies used by organisms to mediate EVs-mediated inter-organ communication. Also more advances are required in the field of Microvesicles, that have been negleted, when compared to "exosomes".
73. 1. loading of different forms of Microparticles with defined structures and RNA species like mRNA, rRNA, miRNA 2. loading of RNA onto MPs outside the cells? 3. uptake and functions of RNA inside the target cells 4. analysis of functions of RNA inside the target 5. preparation of different forms of synthetic micropartices
74. Novel technological platforms are required to shift analyses from the bulk to the single EV level. With this we should become able to dissect the heterogeneitiy of given EV samples.
75. same as above
76. Relationship with disease
77. specific loading of DNA and RNAs into exosomes
78. Mains questions remaining are which mechanisms are underlying the selective selection of RNA, to be incorporate, and which mechanisms of uptake promote an effective RNA incorporation in target cells
79. many unanswered questions
80. Standardise techniques
81. To what extent does the state of the parent cell influence EV cargo
82. Selectivity
83. How cargo is loaded into EVs, and how EVs are transported/regulated to carry out their biological function.
84. the acquisition of EV and routing in the cell... referred to as "cross-dressing" by immunologists, but needs a more general term
85. How molecules are targeted into EVs
86. Mechanisms of action of RNA component, particularly on influencing the other cells and tissues
87. Mechanisms of ev release
88. to determine the mechanism(s) by which cargoes are loaded during the exosomes biogenesis
89. What are the carriers of exRNAs. how do different signals regulate RNA transfer, how do such small amounts of RNA have such large regulatory function. What about non-mIR RNA function, not much is known.
90. Finding ways to address biological relevance to bolster interesting findings in vitro is I believe critical to the field.
91. Therapeutic or clinical use
92. Exosome receptor Epithelial vs immune cells Exosome constitution
93. No one knows how exosomes deliver cargo.
94. Classification and the biogenesis.
95. We still do not have a good picture of the extracellular RNA profiles of non-diseased individuals. There is no consensus for normalization or nomenclature.
96. Non-standardized EV isolation protocols (for exosomes and sMVs), resulting in often heterogeneous EV mixtures and variation between studies, thereby making

comparative conclusions between studies challenging Complementing experimental developments and purification strategies with refinements in data-sharing resources to ensure that the availability and quality of data for such experimental approaches is accurate Extend understanding of distinct mechanisms that underpin cell-type specific EV recognition and entry into cells Precisely assign EV-mediated phenotypic change to one and/or multiple EV constituents

97. not competent enough to give ad educated answer
98. -how cargo is loaded and or changed (different cargo in different types of vesicles) - what specific cargo components do
99. cargo in EV
100. In my opinion the field still needs broadly defined standarization. Comparison of the experimental data from different laboratories should be made simpler. The way of obtaining vesicles should be precisely described in each article published in the field.
101. Translating in vitro to in vivo
102. Is cargo really transferred?
103. If there is any protein responsible to transfer microRNAs into exosomes
104. no field is in depth answered
105. Are particular RNAs loaded onto EVs, how that changes with stressors/disease, does the uptake into recipient cells affect phenotype?
106. EV bound RNA molecules is a complicated issue. They require to be studied in detailed. EV biology is an important area to be extensively perused.
107. Sorting of RNA into The exosomes
108. Not sure
109. cargo unloading in recipient cells
110. Which RNAs are selected in the cargo and how can they being processed?
111. Functional assays, quantitative measurements of Contents and Transfer of extracellular molecules
112. It must be determined whether physiologically-relevant quantities of EV RNAs are transferred between cells in vivo, and the nature of the biologically-relevant EV producer and EV target cells need to be rigorously identified.
113. Diagnostic and prognostic clinical tests
114. Reliable quantitations of the whole population of different size RNAs in EV context.
115. RNA sorting into EVs
116. Do not know
117. Many, for example ESCRT should be recycled and not be part of the nascent vesicles but many of the ESCRT proteins are costantly found in the secreted vesicles. How many copies of each molecules are carried? How many and waht type of protein are actually present in the same single structure?
118. RNA molecules sorting and packing
119. 1. The same issue from previous question. First, We must character what is EVs? What is real lipoprotein (LDL and so on)? What is real Ago2 complex? Whether lipoprotein bind EVs? Ago2 complex only expressed by itself or can express in EVs? Next, Once make the clear for these populations. Next questions will be which types of EVs do we have? exosome? Microvesicle? Apoptotic body Or XXXvesicle?
120. It is clear that subpopulations of exosomes (endosome-derived small EVs) exist. We need better ways to isolate these subpopulations so we can begin to

understand their biogenesis, composition and function.

121. The functions of RNAs and/or other particles (protein, lipid, anything else carried by EVs, or the EVs themselves) in the recipient cells need to be determined. There should be some significance in the specific set of proteins/RNA etc that are secreted, what is their message to other cells?
122. RNA loading
123. the mechanisms by which certain RNAs are loaded into EVs compared to others in a more comprehensive way, screening different cell lines and not only focusing on one or two. For this a consortium of lab would be useful, so that different cell lines-derived EVs are tested.
124. rapid, low sample-requiring measurement methods to quantify and evaluate exosomes
125. Normal ranges. Half-lives.
126. EV biology in non-model organisms and in microbiology
127. Fundamental purpose of EVs in general
128. Amount in a single EV Specificity of RNA cargo in EV population from a particular cell type (is my RNA or miRNA of interest present in every EV produced by one particular cell type or is it present only in 20% or 70% of my EV population?)
129. genetic tools to knock out production of a relevant EV subset
130. What s the role of human Y RNA found in EVs
131. a type of EVs, like the ratio of lipid of EVs
132. transcriptomic of EVs
133. single particle analysis
134. Degree of vesicle heterogeneity in different cell types/systems. Collaborations aimed at determining reproducibility. Packaging and loading of EVs with RNA.
135. mechanisms of export and selection stability
136. reference standards, reproducibility of any EV assay
137. We need to know much more about the specifics of cargo loading and secretion (active enrichment of specific RNA vs. non-selective loading)
138. The spatiotemporal relevance of EV formation of subpopulations related to the packaging of relevant cargo
139. Standardization
140. targeting of EV-RNA in vivo tracing of EV-RNA in vivo However, the Cre lox system is quiet promising
141. mechanisms underlying specific splicing of (pre-) microRNAs and tRNAs into smaller functional molecules and their loading into EVS
142. How different RNA species (e.g. mRNA and lncRNA) are sorted into EVs/exosomes?
143. Types of nucleic acid, size and quality of information encoded
144. Subgroup of EVs according to their cargo Exosome donor cells in vivo
145. generation biological functions
146. Understanding of the 'purpose' for EV release and altered EV cargo. That is, understanding the biological mechanisms and controllers which determine EV release and what is loaded into them. Also, not enough work is done to characterise the cumulative action of all EV constituents in a given EV 'subset'/type in different conditions. As all are theorised to be taken up into cells concurrently, they will exert their effects together.
147. We still need: 1. to shed more light on the processes which allow RNA sorting

to vesicles; 2. to clarify whether different RNAs are present in different EV populations released from the same cells; 3. To clarify the role of RNA-binding proteins in RNA sorting and, on the other hand, the role of RNA in protein sorting to vesicles.

148. in vivo functional transfer of RNA via EV
149. Clear demonstration of EV cargo delivery to recipient cells.
150. Show how specific organs or compartments within organs use EV to communicate and coordinate action
151. Th role of specific EV cargo in each cancer type and its relatedness to metastasis.
152. Better ways for scale up and production of therapeutic EVs
153. EV functional RNA cargo delivery to target cells.
154. Mechanisms of RNA loading into EVs
155. We need to improve isolation methods in order to have the only EV content and then do a better characterisation in term of absolute amounts.
156. We may have to know if the low copy number of RNA in EV could modulate gene expression in target cells.
157. The field should sober up and realize that RNA transfer does not occur to evoke biologically relevant responses
158. How is the protein and RNA content sorted into EVs what are the mechanisms of this sorting/loading. More EM studies on biogenesis when and where is the RNA loaded in exosomes for example. Is this an alternative route from the regular endosomal pathway or just a part of it?
159. We stil lack a recomendation on whethet we should focus on a standard method of purity issue or regardless of which method was used we should focus on biological effects? when using EVs as theraputics. Additionally, one of the basic question in EV-RNA is how RNA species are precisely loaded/packaged into EVs (particularly those of exosomes). This is relevant when using EVs as gene delivery vector. Therefore, finding best answers with well elaborated mechanisms will help us using exosomes as gene delivery vectors. Next, we lack theethods to load therapeutic RNA or drugs into EVs, dose, routes of administration and uptake by targeted organs. we need more focus on in-vivo models for therapeutic testing and functional readouts of RNA delivery.
160. It appears that each exRNA may have a different stability and function based on its associations extracellularly. Thus, no single study will explain the function of all exRNA similarly no single research group will have all the answers.
161. Its function in a normal system
162. What is the role(s) of EV's RNA (not only miRs) in the cell following EV's cargo transfer?T
163. Put less focus on defining the difference between exosomes, microvesicles etc and focus more on specific functions/biomarkers that can be used in particular disease settings
164. Role of NTA-modification for RNA sorting into EV and resolving loading machinery
165. all
166. How cargo (especially proteins, nucleic acids) is naturally or how it can be artificially loaded. We need to understand how exRNA species (miRNA, lncRNA, piwiRNA, mRNA, etc) are biologically active. We need to understand how EV can be

readily purified from biofluids and low copy number or low protein content can be analyzed at the 'omics level.

167. How we can determine which components if EV are critical for entry into, delivery of cargo and functional changes to acceptor cells
168. How much RNA is inside EVs, and whether it is biologically significant.
169. We urgently need to verify wheather these exRNA being analyzed are not A artifact rather than anything else in this field.
170. proximity of exosomes and viral particles, capacity of exosomes to participate to the infectious process
171. Stability of extracellular RNA outside EVs
172. distinguish between normal and diseased exosomes
173. There is more than one type of RNA and they act as a whole. We need to work on RNA-Protein networks and global impact.
174. the mechanism of loading and the role of specific proteins on EV targets
175. A conclusive understanding of the mechanism of EV production would enable it to be manipulated in other experiments to determine EVs contribution to disease states
176. One needs to understand fundamental pathways of EV regulation and assign contribution of EVs (and extracellular RNA) to specific processes and disease states. One also has to prepare mentally to the possibility that while in some cases EVs/exoRNA may play important roles in other this may be minor or nil, and pushing the notion to the contrary will only produce a backlash.
177. Function of noncoding RNA.
178. Understanding packaging mechanisms of RNA into EVs and determining which EVs (or sup-populations of EVs) contain RNA (or which RNAs).

Open Response 3 (optional). What standardized protocols or resources, if any, would you like to see developed for the EV community?

1. - purification of EV away from other molecular entities such as ribonucleoprotein complexes and lipoparticles that can contain RNA - nanosized materials for calibration of equipment: for interlaboratory comparison of data it is essential to see the sensitivity of detection of the equipment + chosen settings - standardization in reporting all relevant experimental details
2. 1. Finding an appropriate endogenous control (housekeeping gene) for study of gene expression analysis with qPCR. 2. Establishing a good protocol for removing EV-binding exogenous RNA to be sure about specificity of RNA inside EVs. The current effort using RNase and proteinase K treatment of EVs is not highly successful and still up to 40% of EV-binding RNA is not removed. 3. Standardization of EV/exosome isolation protocols since there are different isolation methods.
3. 1. Isolation and characterization of small RNAs especially from body fluids 2. quantification and isolation of tissue-specific EVs
4. 1. Statistical evaluation of exRNA or EV proteins as a biomarker 2. Detection of impact of EV/exRNA uptake on recipient cells or tissues
5. A common standard of EV use in diagnostics...see Miroslaw Kornek et al...
6. A set of standards for EV cargo research, similar to those established for EV research.
7. all of the basic methods need to be standardized

8. All protocols
9. An updated position paper on the "Minimal experimental requirements for definition of extracellular vesicles and their functions (Lotvall et al JEV 2014) should be published taking into account findings over recent years which help further define exosomes relative to other E Vs such as microvesicles.
10. Bank of standardised cell lines; isolation protocols; EV RNA protocols; pipelines for expression analysis
11. better and cheaper tools to enrich exosomes. How to separate and characterize the different populations of exosomes or microvesicules
12. better more direct functional assays
13. Bioinformatic resources for analysis
14. Bioinformatics for DNA and all RNA, integrative bioinformatics including in modules all available data for one sample and which can add more modules as the filed advances
15. Biologic activities
16. calls for international collaboration
17. Cargo delivery
18. clinical studies
19. Community accepted standards for protein and RNA work or a standard set of requirements for presenting the data
20. counting and mapping subpopulations
21. Dedicated isolation protocols for various biofluids, subpopulations of EVs ("to get genuine exosomes from biofluid x do this") and questions of interest ("Want really pure EVs? Do this! Want maximal enrichment of marker x? Do that!") Stringent, standardized protocols for analysis of EV cargo that emphasize quality control, reproducibility and validation
22. Define more clearly what an exosome is
23. Devices that can rapidly and easily analyze sizes and numbers of EV with wide dynamic range.
24. Different types of EVs isolation and RNA extraction.
25. Easy and reproducible protocols for EV and/or RNA isolation from clinical samples.
26. Efficient cargo loading
27. EV characterisation EV production from different cells
28. Ev isolation
29. Ev isolation Ev characterization Rna normalization
30. EV isolation sample prep for NGS (DNA, small RNA, large RNA) bioinformatic analysis of NGS data
31. EV isolation and definition
32. EV isolation from clinical samples to improve the yield and avoid contamination with proteins or other molecules. Quantification of EVs.
33. EV isolation using commercially available kits EV isolation from biological fluids
34. EV isolation, EV analysis by flow cytometry
35. EV isolation/purification from biofluids. Isolation of miRNA from EV NGS protocols for low-copy number samples (esp blood, CSF)
36. Ev levels measurements in human plasma/serum.
37. EV markers, cargo sorting, EV transport
38. EV purification
39. EV purification and characterization

40. EV quantitation, characterization and sorting.
41. Evaluation of methods
42. EVs purification and characterization, including markers
43. Excess Production of Exosomes Cargo Loading
44. Exosome preparation for different purpose Exosome detection for protein, RNA, lipids etc
45. FFF
46. Generally accepted markers for EV subtypes should be developed. Monoclonal antibodies to EV markers should be standardized by and for the community.
47. Good mouse models to look at EV function.
48. how to present methods and data
49. I am trying to develop standardized nanoparticles and / or protocols allowing to use deep-frozen breast milk samples to study Evs. Current trends is to process fresh milk.
50. I don't even feel like there's a gold standard protocol for EV isolation
51. I have no answer
52. I think it needs a big study with a standard protocol to determine this. For scalability qiagen exorneasy (or equivalently quick and easy) would fit.
53. I'd like to see any standardized protocols or resources.
54. I'm not sure if standardised protocols are the way forward. Recommendations perhaps. I think clarity of methodologies used in publications is more important. This allows researchers to form there own opinions and fosters new ideas and methodologies.
55. Improved techniques for single vesicle phenotyping are essential. Labelling techniques have not kept pace with improvements in cytometry and other single particle analysis methods
56. ISEV has already too strong emphasis on standardization. This risks development of novel methodologies, if e.g. certain isolation methods are formed as standards. However, relevant isolation method depends totally on the downstream application. Also novel methods should be tested more actively. EV field is too young for labeling certain techniques as "golden standards". Rather emphasis should be in thorough reporting of the used techniques, to allow reproducibility.
57. Isolation Storage
58. Isolation methods and ev analyses
59. Isolation of all the components. A real consensus of how we have to do it so everyone makes sure we are working in the same.
60. Isolation of EVs from plasma and other body fluids
61. Isolation of EVs without compromising their biological constitution and activity
62. Isolation of EVs. Which kit should use or should not use for EV research.
63. Isolation of exosomes and microvesicles
64. isolation of pure Microparticles and exosomes and identification of different content also from plasma or blood protocols to study the uptake of MP by different methods
65. Isolation of purified EVs (ie without lipoproteins) that doesn't compromise too much yield
66. Isolation protocol
67. Isolation protocols based on specific biological properties/complexity of the bodily fluid.
68. Isolation techniques
69. Isolation techniques, normalization of RNA analysis from EVs
70. Isolation, identification, and characterization to distinguish each type of vesicles

71. Isolation, Quality Control,
72. it is too early to provide standardized protocols for isolation. It may be time now to develop lists of protein markers of subtypes of EVs, but it requires a joint effort to compile all the recently published and ongoing comparative proteomic studies, ie further develop/use the EVpedia and Vesiclepedia resources
73. It might vary from group to group. For example those working in EV purity feild they would like to see standardized protocols for EV purity. But those working in EV-based therapeutics might have different intersts such as protocols on in vivo administration, doses, time intervals, routes of administration and related handeling. However, those working in liquid biopsies might be interested in knowing/developing suitable protocols for mining EVs from body fluids especially blood considering related complexities. I would like to see standardized protocols developed for therapeutics and liquid biopsies. The later one needs more attension.
74. Loading of EVs
75. Many (e.g. RNA extraction etc), but i'm still wary of establishing 'standardized' protocols before they have been fully explored by the community. If we standardize to early then we just fix in place methods that are not optimal.
76. mikroRNA quantitation in biofluids
77. Mostly those related to extraction and purification of EVs and to explore better ways to detect them using flow cytometry
78. Na
79. Nanoparticle tracking analyzer
80. New stringent definition of Exosomes and other classes of EVs
81. nomenclature, isolation.
82. Non-biased platforms for the quantification of nucleic acids in EVs.
83. not sure
84. Not sure we know enough for 'standardization' - already too many in the field seem ready to establish themselves as having answers
85. optimize and standardize the isolation procedures for different matrices
86. Preparation of pure (?) EVs remains a challenge. Different strategies should be used and guide line for acceptance of the preparation should be formulated.
87. Protein study for complicated samples such as blood.
88. Proteomics
89. Protocol for the isolation of EVs from cell culture supernatant; protocol for quantification of EVs (to easily determine the dose of EVs in experiments); The list of controls that should be used during various experiments involving the use of EVs
90. Protocols that can separate various EVs and various lipoproteins all together
91. Protocols to confirm that the RNA observed associated with EVs are indeed INSIDE those EVs. Proteinase k - RNAse protocols and functional activity of EVs RNA based on more than just one molecule.
92. protocols to produce clinical batches
93. Purification
94. Purification mRNA normalization
95. Purification of EVs from various body fluids.
96. purification/extraction protocols
97. Purified EV isolation methods that every lab (including clinical labs) can use and know they are getting the right material.
98. Question too broad

99. Reference materials
100. Removal of protein contaminants
101. Report cell identity and conditions (media components, passage, substrate, density, etc) when EV isolation performed...we need to start better connecting EVs to the cells that generate them in order to eventually understand and control EV production or inhibition.
102. RNA cargo delivery by EVs. Next Generation Sequencing of EVs RNAs.
103. RNA extraction EV purification EV separation
104. Rna research
105. RNA-seq, RT-qPCR
106. see 10
107. Small RNA sequencing, EV purification
108. Small volume sample collection
109. something along the lines of EU nano standards
110. SOPs for body fluid collection methods of purification RNA extraction (everything basically since now nothing is standardized)
111. Standardisation may lead to better reproducibility of data but also to dogmatism and attempts to dictate unilateral views. I suggest to set up the guidelines of reasonable standardisation, e.g. to validate biomarkers across the field or formulate exosome-based therapeutics. On the other hand standardizing the discovery process is dangerous.
112. Standardised cell culture protocols for EV isolation for clinical applications
113. Standardised protocols are needed for uptake and labelling assays - many protocols still use protein concentration (from surface, not lysed) to determine the amount of EVs used.
114. standardized EV isolation methods dedicated to the downstream profiling
115. Standardized EV isolation protocol, surface markers
116. Standardized Isolation using as final separation step either size exclusion chromatography or density gradients.
117. Standardized methods for qPCR normalization on EV-RNA (how to set it up and validate normalization genes)
118. Standardized naming, in order to allow for more efficient archiving and literature reviews. Standard protocol for EV isolation.
119. Standardized protocols of isolation and characterization (e.g. staining for flow cytometry, extraction of protein, nucleic acid for western blot and sequencing respectively)
120. Standardized protocols to purify EVs from biological fluids such as blood/plasma/serum, saliva or CSF should be of the most importance for both basic and applied science.
121. standardized, quick, cheap and efficient isolation of EVs from blood plasma that can be used in diagnostic setting
122. the real trouble now is that centrifugation is based on old guesses and literature. we should put aside literature and standardize the best and reliable isolation protocol. Also FACS analysis for characterization should be standardized
123. UC, SEC, Filtration, bead isolation, ELISA maybe and so on
124. unbiased isolation protocols / functional quantification protocols
125. Update of exocarta that seems to include a lot of contaminants
126. We need novel strategies to enrich EVs to higher purities and to isolate EV-

subfractions.

127. Whuch endogenous control to use for real time pcr on EVs RNA... as well as an optimal microRNA as endigenous control
128. will Change with progress
129. Would be nice to see an update of Therys protocol in current protocols

Open Response 4 (optional). Please use this space to add any comments about EV or exRNA science that were not captured by the questions above.

1. A further problem concerns the kind of interaction between EVs and RNAs: are RNAs carried inside the vesicles or are they (at least in some cases) attached to the external shell of EVs?
2. analysis of exRNA is one of the most promising strategies for liquid biopsy and certainly needs attention and more fundings from the NIH and other agencies.
3. Development of clinical aprroaches
4. EV surface antigens.... but please involve me ;-)
5. Expand the comparative species approach, utilizing multiple species in discovery (rodents, veterinary species, non-mammalian species, etc).
6. ExRNA is a field of research quite important. In my vision, proteins and lipids bring a acute response to EVs while RNA bring a long term modulation of cell activity. The study and correlation of those activities would be a very interesting subject to determine if they go the same way or block each other.
7. exRNA science is a subspecialty of ISEV and should not be placed on a pedestal. It's by all means valuable but is not one and the same as EV research, it has its own complexities distinct from other sensitive biomolecule transport. Otherwise consider renaming ISEV&RNA
8. For a single sample, does anyone see the same particle count by EM and NTA? My hunch is no.
9. Guide line for accepting the pure preparation of EVs is an important aspects to study their functions. Methods of preparation and characterization are very important for their research.
10. Have the website to share information, such as protocols, videos, key publications
11. Imaging techniques for thd future, we need to be able to use tools to follow natural vesicles in vivo and in organoid in non transparent tissue, two photon microscopy is one but more and more accessible are needed.
12. ISEV needs to continue to partner with ISAC and ISTH on methods
13. It is a great opportunity to push this field of science by such great specialized community.
14. It's a wonderful "new" field of science and cell-to-cell communication, and a game changer. So thank all of you at ISEV for being the first to believe in and support this field of science!

15. Looking forward to novel technology developments in the field of extracellular RNA and the upcoming intense and exciting research.
16. Molecules (including RNA) that are associated outside EV should be more focused.
17. Thank you
18. Thanks for the survey

19. The bio-activity of EVs and their cargoes is still sufficiently plausible, despite the challenges, for it to be worth researching.
20. The definition and "markers" defining samples are rarely quesioned, I think we are basing on ancient and wrong definitions and markers. A new and more stringent set of markers of size and source should be found
21. There is no focus or recomendation on reference material. I beleive that there must be a reference material for EVs that may represent/mimic EV properties both in terms of physical/chemical and biological point of view.
22. This is a fascinating field and there is a great potential in it but it needs to be integrated with other biological processes rather than become insular and self-absorbed. Other forms of cellular interactions such as EV-free extracellular RNA, non-EV cell communication and other processes should (and with time will) be a part of this amazing science.
23. Unfortunately there are to many publications in the field but few of them are well done, in many of them there are clear misunderstanding due to inadequate characterisations. We have to be more strict.