1st EV Meeting 2024: Minutes on Discussion Points

WG Leads

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Key Take Aways

- Checklists: there are checklists in existance for all three biofluids we discussed (blood, CSF, urine). We can contribute to distributing them to increase awareness among different societies (ELBS, different biobanks, etc.) and to develop them further. While we cannot control all sample parameters, reporting them can increase reproducibility of the experiments.
- We still don't understand all the impact of the different pre-analyticals on our downstream liquid biopsy targets. We should look into which studies we could collectively set up to evaluate the impact of such parameters. For example the impact of blood collecting tubes or the removal of platelets, etc
  --> if we really identify the most important pre-analytics that we need to evaluate in a survey, we can maybe help and support interactions between different partners that can help to determine t the impact of this parameters
- Universal SOP: it would be fantastic, if we have one universal bio banking protocol that covers not only EV, but also other liquid biopsy targets
- Follow-up meeting on the 1st October: Topic will be the analysis of multiple liquid biopsy analytes (EV, CTC, ctDNA) from the same bio fluid
- Survey on what the current EV biobanking issues are. What is hampering successful biobanking (e.g. lacking information on sample collection), important pre-analytics, check-lists for biobanks – Indication by participants on willingness to contribute to experimental work in establishing and validating a joint SOP

MEETING MINUTES / QUESTIONS TO THE SPEAKERS & DISCUSSION

1st Presentation by Ursula Sandau, Oregon Health and Science University, Portland, USA:

An Hendrix: You mentioned the composition of CSF, if you compare it for example to blood. The abundance of proteins is of course much lower and probably also other types of particles. What does this mean in terms of stability of the EVs as contaminants can alter the storage stability?
Ursula Sandau:
We haven't done any really controlled studies to address that, but based on our experience: In its original biofluids the EV seems to be pretty good in regards to stability. For a lot of our studies we track how long the fluid has been biobanked and whether or not we have more variance in our assays or outliers with the older ones and yes in cases where it gets to be a decade or so it become a little bit more of a problem, but that a pretty long time. So a decade versus a more immediate time. But we do feel based on what we can see is, that once you take EVs out and you put them in to like a PBS and then try to store them, we feel that there is a bit more issues with stability. And I do think that it is because a lot of the enrichment and separation protocols further dilute and you have more loss. So I think that is definitely a challenge. We regularly especially for trying to do like a vesical flow or something like that, we try to aliquot to keep them as concentrated as possible. And we only do one freeze/thaw cycle on EVs that have been separated.

Franz Ricklefs: Looking at biobanking from these sources: I always wondered, how you guarantee that you have the same quality if you store something in a biobank, let's say for more than 12/24 months, that it is still similar to something that you actually froze just a few weeks back. Because I mean from tissue samples we know, if you have it stored multiple years then the quality goes down quite a bit.
Ursula Sandau:
Right, yes I think we definitely need to do some studies to look more longitudinally what's the impact of storage. But most of what we've gotten, the closest that we've gotten to looking at that, is when we do a statistical analysis: we've look to see like in some of our analysis, like over time do we see a drift in our marker that we are measuring. Because as you know since you're working with this, sometimes we work a lot with the US-Alzheimers disease biobanks and bio repositories, which is a collection of NRH funded bio repositories around the US that are collected the same manner but then they going to a biobank and some of these sample have been banked for a very long time, they come with a lot of very valuable information, like MRI, so you want to make use of them, but you haven't that definite caveat of how long is too long. Yes, I think we do definitely need studies to look at the impact of fresh but also shorter term (e.g. one year) storage.

Basant Kumar Thakur: We also working on gliomas and medulloblastoma especially in pediatrics. And what we are realizing: we are getting mostly lumbar puncture CSF and we are seeing that when we do proteomics we have tons of immunoglobin coming up, which normally I expect that it should be mostly in the blood. Sometimes it's very difficult to say which finding has functional biomarker potential and which is because of blood brain barrier. Do you have experience on whether hemoglobin leveld differ depending on from where it is captured in the brain?

Ursula Sandau:
I think trying to establish the baseline levels in a controlled population is really a good way to determine whether this is sampling or normal variation. This is critical in establishing what are good criteria for cut off in your studies, because you want to make sure that you are not accidently getting a slightly reduced quality LP in some patients and that's impacting the biomarker read out. We've done studies were we've had a look at mirco RNAs and it appears a very small amount of contaminant really changes the biomarker composition of the mirco RNA.

Basant Kumar Thakur: Exactly, this brings me to the second question and that is also the idea of our society, to bring some standardization: asyou said, controls. For blood we can take healthy blood as a control and make plasma out of that, but we are struggling with what will be the control for the CSF? You cannot obtain CSF by lumbar puncture from a healthy individual. Is there a possibility that in this consortium to develop some kind of controls, via a standardized protocol to then collect the samples of CSF from something that is really close to healthy and then make proteomics, micro RNA analysis or something like that, that would be a standard reference and you can then cross-check your data with?
Ursula Sandau:
I think that is really important. The control aspect is really a hard bit. Especially when you start to look at pediatric or ventricular shunt. We've done EV isolation from CSF and have done RNA-seq on them for mRNA and we compared this to mRNA from plasma. The mRNA that is present in the CSF is very neurologically aligned and the most reliable transcripts are brain origin related. It's a really important biofluid, but there are huge challenges coming up with what is base-line to measure against.
I think improving reporting can help this tremendously. If more and more people start to report numbers of what they've seen when they submit their samples to a clinical lab, we can start to try get a grasp of what is "normal" and what is different under X,Y,Z conditions.

2nd Presentation by Fabrice Lucien PhD, Mayo Clinic, Rochester, USA

Klaus Pantel: I just want to say that from the ELBS side, I mean we can really help to promote whatever is possible and I think you gave a really nice conclusion-slide. And of course I mean to develop a "one fits all" liquid biopsy procedure (SOP), that would be a very challenging but good goal and it would also help the people who are biobanking. They always ask us, as ELBS 'Can you promote or describe some biobanking recommendations'. I guess we can really work on that. Let's try to put that on our list, absolutely. Of course ISEV is already doing a wonderful job, but if we can help to promote and develop things and also combine it with the other biomarkers that we analyze. I just want to say the door is wide open!
Fabrice Lucien: Thank you! I really appreciate that and we definitely going to reach out, for sure.

An Hendrix: I think that was exactly one of the aims we also had within the ELBS, to come to a common ground when looking to the different biopsy targets.
Fabrice I was also wondering when you were questioning the different biobanks for their protocols. Did you also ask about the origin of their protocol or how they came up with their protocol that they are

currently use to process the blood?

Fabrice Lucien:
That's an excellent question. So in some of the biobanks it's really tailored to specific studies. Some of the big biobanks, we found one in Australia I think, they have a really specific purpose you know, in terms of biobanking for specific studies, so they kind of tailored their biobanking. But a lot of them are using the recommendation of the International Society for Biological and Environmental Repositories (ISBER), which established some best practices. But when you look at the different details there is no specific guidelines, it's kept more general. Even when you look at the SPREC coding system, they don't indicate any specific steps e.g. centrifugation, here they give rough suggestions such as 'between 1000-5000' or 'between 5000-10.000'. I would say that there are the three different sources. Either really tailored to the study, ISBER, or the circulation guidelines.

Ursula Sandau: I think about that some of the really big biobanks, that have been banked samples for years and years and have a lot of very valuable samples, that are maybe not with the ideal protocol and a lot of clinical data that is associated with which could be helpful. How do you get your recommendations of when you may have not the ideal sample, based on how its collected- how do you best process it?
Fabrice Lucien:
Yes, this is a common question and we have to be aware of the resource access for different groups. So some people have the chance to have access to a really robust biobank in their institutions and others have trouble trying access samples at all. The position right now is that we don't have a way to say 'this sample is bad and this sample is good' because we don't have the consensus. But what we want is that people report the quality of the samples. For example, having done a study in samples that contain residual platelets doesn't mean that it's a bad study. Reporting this, we know that you have controlled the material you have. That way, if you have data that is different from another group who don't have residual platelets, this directs towards a potential reason for these differences. We don't want to prevent anyone to use biobanked samples because like you said, they are extremely valuable. But we just have to keep in mind that we need to assess rigorously what work with. And that would help with enhance reducibility.
And maybe down the road, in a couple of years we would come with some more expert based guidelines, like evidence based guidelines to really kind of shift and have some samples that are more certified for EV research versus other types of analysis.

Siegfried Hauch: I'm referring back to this slide you showed with the study from Dhont, B. et. al., JEV, 2023 where they showed the influence of different blood stabilization or anticoagulating systems. Do we also have information what that means concretely? So if there is a platelet contamination for example, what kind of mircoRNA does appear suddenly that have nothing to do with EVs, but with platelets more or less and data like that. Is that available or is that still something that has to be done?
Fabrice Lucien:
I mean, to answer your question I just give the mic to An Hendrix, because she has led that study. So she will know better than me. The only thing I want to add first, is that what you mentioned is very interesting: you could do two types of research downstream. One is more single-vesicle-analysis,

for example via cytometry. There is always the question: do your residual platelets really matter, when you measure for example pancreatic EVs by flow cytometry? Maybe not. We don't know. But when you start doing transcriptomics, proteomics on bulk EV, you have to consider those limitations. But An, maybe you want to add something on that.

An Hendrix: Yes, indeed. So for the bulk analysis it's extremely important to control for these parameters, because depending on every single pre-analytical, but also downstream processing step that you implement or the blood collection tube, it does impact the proteomic and the RNA profile. Both the microRNA profile and the total RNA profile. Now in terms of RNA and blood collection tub, Fabrice also mentioned that the processing time, so the time between collecting the sample, processing and storing the sample is really crucial in this, because I think for the 'classical' blood collection tubes, if you really process within in a short timeframe the impact on the downstream -omix profiles is smaller than if you wait for a very long time period. And this is what we actually observed for example with blood collection tubes that enable preservation, so they are typically used for example for DNA or RNA studies, these definitely do not preserve the EV -omix profiles over a longer time period. So this is really important to consider.

3rd Presentation by Elena Martens-Uzunova PhD, GUSEV (Group on Genitourinary System EVs)

Basant Kumar Thakur: Mof the time we work in very collaborative settings and there you have one place collecting the samples, but sometimes it has to be frozen before it can be sent and in one other location you have direct access to the samples that someone can bring directly to your lab. Now for blood we have optimized to do the initial spins and then you can freeze it at a certain stage and then later you can isolate EVs, but what about urine? As far as I know this albumin and also this urine globulin is very high, you can get such a thick band of that. So how to avoid that for the initial clearing?

Elena Martens-Uzunova:

It is very important and people often tent to ignore it and then try to go and use archived urine biobanks. What we recommend is to process urine within 4 hours of collection. And processing means to actually remove any cellular component that is present in there. So have a low speed centrifugation step within 4 hours of collection to remove the cells that are present in urine and then aliquot and freeze immediately. But what you have to realize is that urine is not a sterile liquid. There is bacteria in there, there is virus in there, sometimes even yeast in there, so keeping the urine cool and processing it, pre-clearing to remove the cellular component early, is a very important step. And then to particularly regarding the uromodelin removal: there is still a lot of controversies. Some people think you have to do it, some people say you don't. The issue there is a little bit that if you have a large amount of uromodelin present in your sample and you try to remove it, you probably also remove a lot of vesicles that get captured within that network there. So it really depends on what you want to look at afterwards. If you want to do a whole proteomic study, well there uromodelin doesn't really interfere with what you want to do.

Additional considerations and content:

Questions from the chat:

Markus Sprenger-Haussels an Alle

Hi Fabrice, nice talk! Do you know about or are even involved int generating the ISO standard: https://www.iso.org/standard/85347.html#lifecycle

Martin Schlumpberger an Alle

Just one more comment, because I think it hasn't been mentioned explicitly: During the time interval between blood Collection and Plasma Generation, blood cells will also keep producing EVs.

Fabrice Lucien an Alle

Hello Markus, I am not aware of anyone involved in this but it would be important to have a representative. Surprisingly, during my discussion with some biobank leaders, it is not always a requirement to align with ISO certification.