It was mentioned that all blood samples were left at room temperature for a period of 16-24 hr before processing with a standardised protocol. Why the authors left the samples for so long a time before further processing? Does such a long period at room temperature have any effect on both the bioactive cargoes of exosomes? Does the delay of processing change the component of small extracellular vesicles isolated, for example, more apoptotic bodies will be produced?

Why the authors left the samples for so long a time before further processing?

The bloods that we used in our study were not collected exclusively for extracellular vesicle miRNA biomarker studies. They were collected as part of an Australian consortium effort to incorporate a range of blood biomarkers into an expansive oesophageal cancer research program. The protocol for blood collection and processing was developed in 2010. Our aim was to standardise as many aspects of the collection and analysis protocol as possible, including consistent use of specific blood collection tubes, eliminating haemolysed samples from analysis, etc, so as to minimise variation across collection sites.

One aspect that we had no control over was the time at which a patient would have the clinic appointment during which their blood was taken. Sometimes this would be early in the day, sometimes it would be in the middle of the day, and sometimes it would be late in the day. In the case that it was late in the day, laboratory staff were not available to process the specimen soon after it was collected. Therefore, it made sense to standardise a time frame between collection and processing so as to enable a degree of control over this factor. Hence, we set 16-24 hours as the time frame.

In some clinical scenarios, such as when patients in rural areas have their blood taken for diagnostic purposes, processing delays are unavoidable because of the time taken to transport the sample from the rural collection site to the testing laboratory. In this regard, the protocol that we set for our research studies might provide a more representative translation of what would take place in a diagnostic setting, compared to if we had been able to further process the samples immediately after collection.

To this day there remains no universally agreed upon and set guidelines regarding sample processing for circulating sEV miRNA studies. In regards to circulating miRNA stability in general, our standardised approach of processing the samples no later than 24 hours after collection, and within a fixed consistent and relatively narrow window of time, is supported by the findings reported in Glinge et al, 2017 (PMID: 28151938) and Poel et al, 2018 (PMID: 29520111).

Other biomarkers that have been successfully studied with our protocol, throughout our research consortium, include cell free tumour derived circulating DNA, circulating glycoproteins, and genotyping using DNA extracted from peripheral blood mononuclear cells. The fact that we can extract viable peripheral blood mononuclear cells, from the blood collected in K3-EDTA tubes for processing plasma , indicates that apoptosis is low. Our unpublished studies indicate that there is no detectable background of contaminating cellular DNA in our serum sEV preparations, thus providing evidence that apoptosis is low in the blood collected in the gel+clot activator tubes used for processing serum. Furthermore,

for another study we sent RNA specimens derived from our serum sEV preparations to a commercial provider and their analysis for haemolysis specific markers indicated that there is no haemolysis.

Does such a long period at room temperature have any effect on both the bioactive cargoes of exosomes?

We are unsure what the reviewer means by, 'both the bioactive cargoes of exosomes'. One possibility is that the reviewer is asking us whether leaving the tubes at room temperature for 16-24 hours before processing could influence which blood cell derived miRNAs are sorted into exosomes. While this might be possible, we can't comment from our study about whether such an effect occurs, and it would be a separate and detailed study to determine whether it does. One of the reasons that we kept the tubes at room temperature, rather than at 4 degrees Celcius , before further processing, was so as to avoid the types of physiological shock that might impact upon miRNA expression and sorting of miRNAs into exosomes. Another possibility is that the reviewer is asking whether the miRNAs already packaged into exosomes at the time that blood is collected might decay in the exosomes before processing. We suggest that other studies, including the study by Glinge et al, 2017 (PMID: 28151938), indicate that this is unlikely.

Does the delay of processing change the component of small extracellular vesicles isolated, for example, more apoptotic bodies will be produced?

This is a very interesting question. While our data indicate that apoptosis is low in our samples (see above), we cannot exclude this possibility. This is something that could be explored in future studies. Apoptotic bodies are larger in size (500nm – 2µm) than small extracellular vesicles, and we specifically included a serum centrifugation step at 16,000 x *g* for 30 minutes so as to minimise contribution from these larger vesicles. Hence, we do not expect them to be present in the serum/plasma aliquot that was used for the Exoquick^{TM} sEV isolation procedure.

It has been recommended to use terms for extracellular vesicle subtypes according to their physical characteristics such as size ("small EVs" (sEVs) and "medium/large EVs" (m/lEVs) instead of exosomes and microvesicles, since there is still no accepted specific markers of exosomes and MVs

We agree with the reviewer and have changed the terminology throughout our manuscript, including when referring to other authors' who described 'exosomes' in their studies.

The authors should discuss in the discussion section the possible reasons why there is difference of exosomal profile between serum and plasma exosomes.

We have added a paragraph, on this topic, to the discussion