# Small RNA Sequencing of Circulating Small Extracellular Vesicles MicroRNAs in Patients with Amyotrophic Lateral Sclerosis

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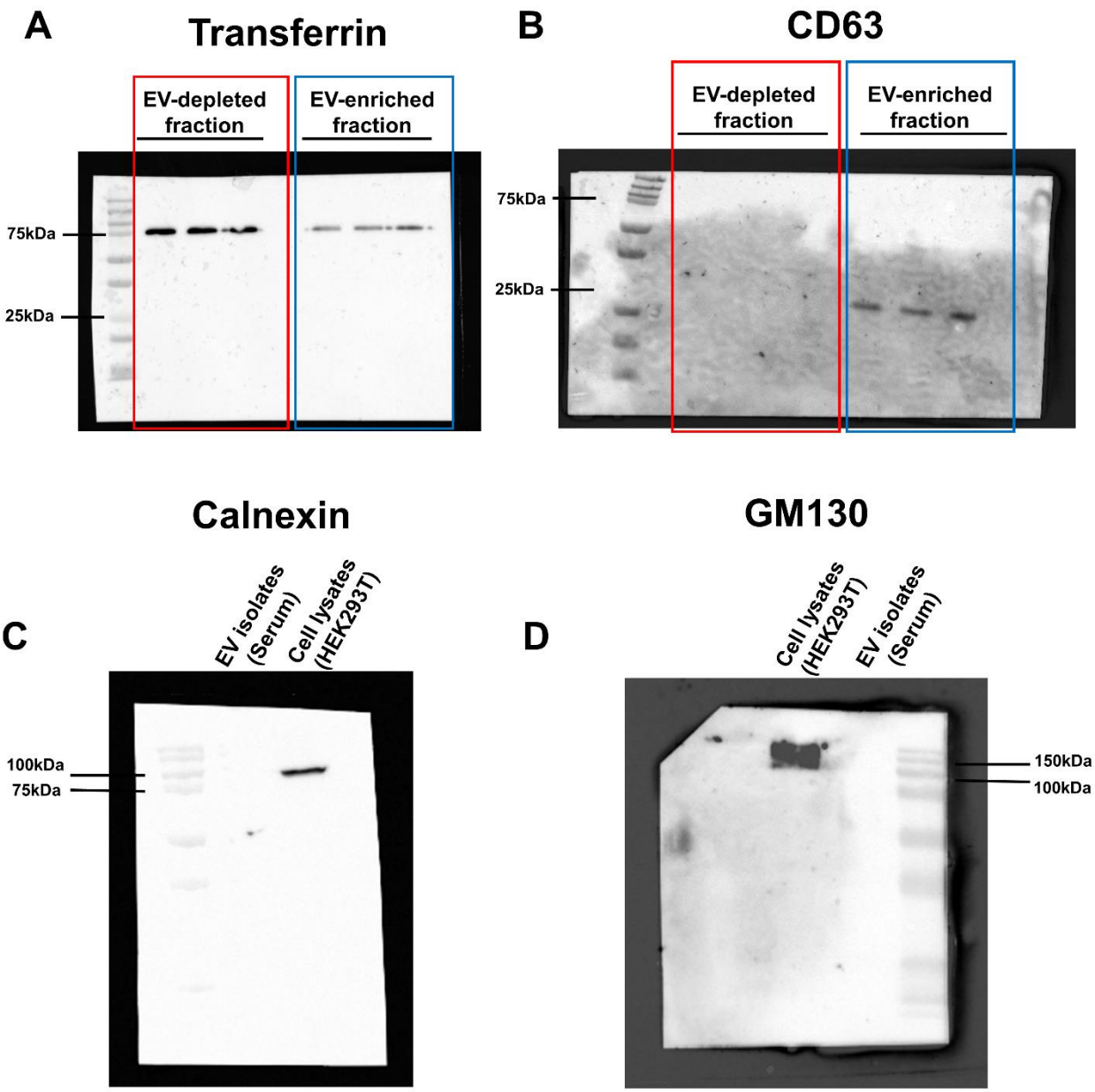
**Supplementary Table 1. Patients' clinical information.**

| Patient number | Gender | Age | Disease duration (Month) | Onset region | ALSFRS-R | Level of diagnostic certainty |
|---|---|---|---|---|---|---|
| Discovery set | | | | | | |
| P1 | M | 55 | 11 | Bulbar | 26 | Definite |
| P2 | F | 61 | 10 | Limb | 35 | Probable |
| P3 | F | 65 | 31 | Bulbar | 31 | Definite |
| P4 | F | 76 | 23 | Bulbar | 26 | Probable |
| P5 | M | 55 | 18 | Limb | 18 | Probable |
| P6 | F | 37 | 17 | Limb | 30 | Definite |
| P7 | M | 59 | 10 | Limb | 37 | Definite |
| P8 | M | 66 | 12 | Limb | 28 | Probable |
| P9 | F | 50 | 9 | Bulbar | 46 | Probable-laboratory supported |
| P10 | F | 64 | 10 | Bulbar | 44 | Probable-laboratory supported |
| P11 | F | 58 | 20 | Bulbar | 31 | Definite |
| P12 | M | 59 | 18 | Limb | 30 | Probable-laboratory supported |
| Validation set | | | | | | |
| V1 | M | 50 | 5.5 | Limb | 39 | Definite |
| V2 | M | 56 | 5.6 | Limb | 39 | Probable-laboratory supported |
| V3 | F | 72 | 3 | Bulbar | 40 | Probable |
| V4 | M | 56 | 5.4 | Bulbar | 42 | Definite |
| V5 | M | 64 | 5.8 | Limb | 44 | Probable |
| V6 | M | 61 | 6 | Limb | 45 | Definite |
| V7 | M | 56 | 5 | Bulbar | 45 | Probable |
| V8 | M | 55 | 5 | Bulbar | 29 | Definite |
| V9 | M | 50 | 10 | Limb | 19 | Definite |
| V10 | M | 56 | 9 | Limb | 38 | Definite |
| V11 | F | 66 | 5 | Limb | 43 | Probable-laboratory supported |
| V12 | F | 60 | 7 | Limb | 36 | Definite |
| V13 | F | 81 | 5.3 | Limb | 33 | Probable |
| V14 | F | 67 | 5.8 | Bulbar | 45 | Definite |
| V15 | M | 56 | 5.5 | Bulbar | 41 | Definite |
| V16 | M | 55 | 7 | Bulbar | 34 | Definite |
| V17 | F | 65 | 8 | Bulbar | 23 | Definite |
| V18 | F | 66 | 5 | Limb | 36 | Definite |

M, male; F, female; ALSFRS-R, ALS functional rating scale-revised

**Supplementary Figure 1. Uncropped images of Western blots.** Uncropped Western blot images of transferrin (77kDa) (A), CD63 (37-50kDa) (B), Calnexin (98kDa) (C), and GM130 (130kDa) (D)

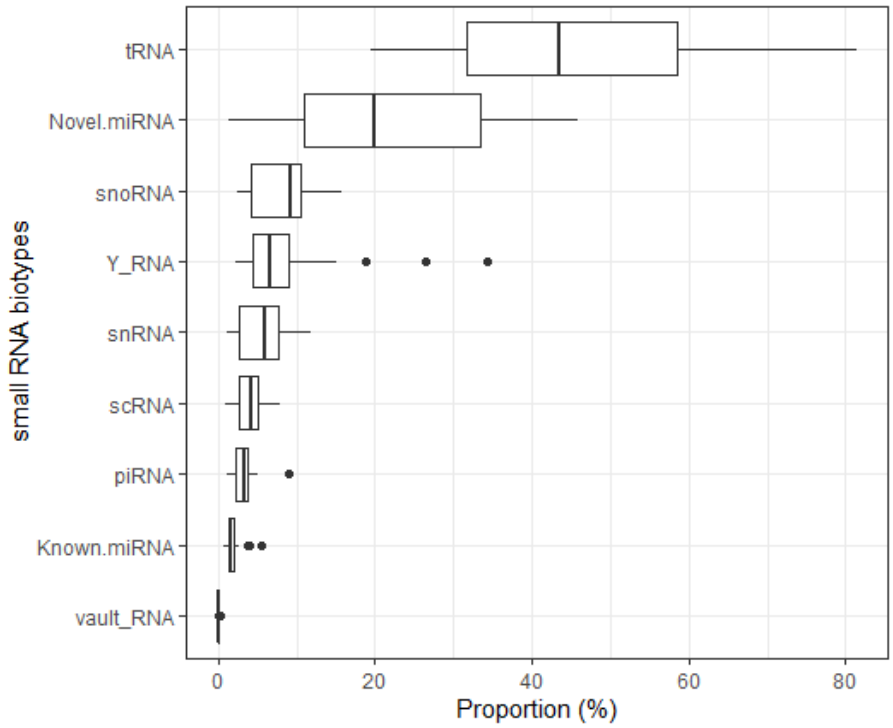


**Supplementary Table 2. Summary of read processing.** Numbers represent raw counts of the corresponding reads. The reads start at the first base after the 5' sequencing adapter and typically end after 51 bp. As mature miRNAs are normally up to 25 bp in length, the reads will contain part of 3' adapter sequence that has to be removed. If a read matches at least first 5 bp of 3' adapter sequence, it is regarded as an adapter sequence, and then trimmed from the read. Trimmed reads should be at the minimum of 18 bp in order to be considered reliable for analysis. In addition, trimmed or non-adapter read with 'N' base was regarded as low quality read and filtered. Sequenced reads are classified as trimmed reads, non-adapter reads, short reads and low-quality reads according to the following definition: Trimmed Read, Read that is removed adapter sequences; Non-adapter Read, Read that has not adapter sequences; Short Read: Read with below 17 bp in read length after adapter trimming, Low-quality Read: Read with one or more 'N' base in trimmed or non-adapter read. Both trimmed and non-adapter reads were analyzed to see comprehensive small RNA profiles.

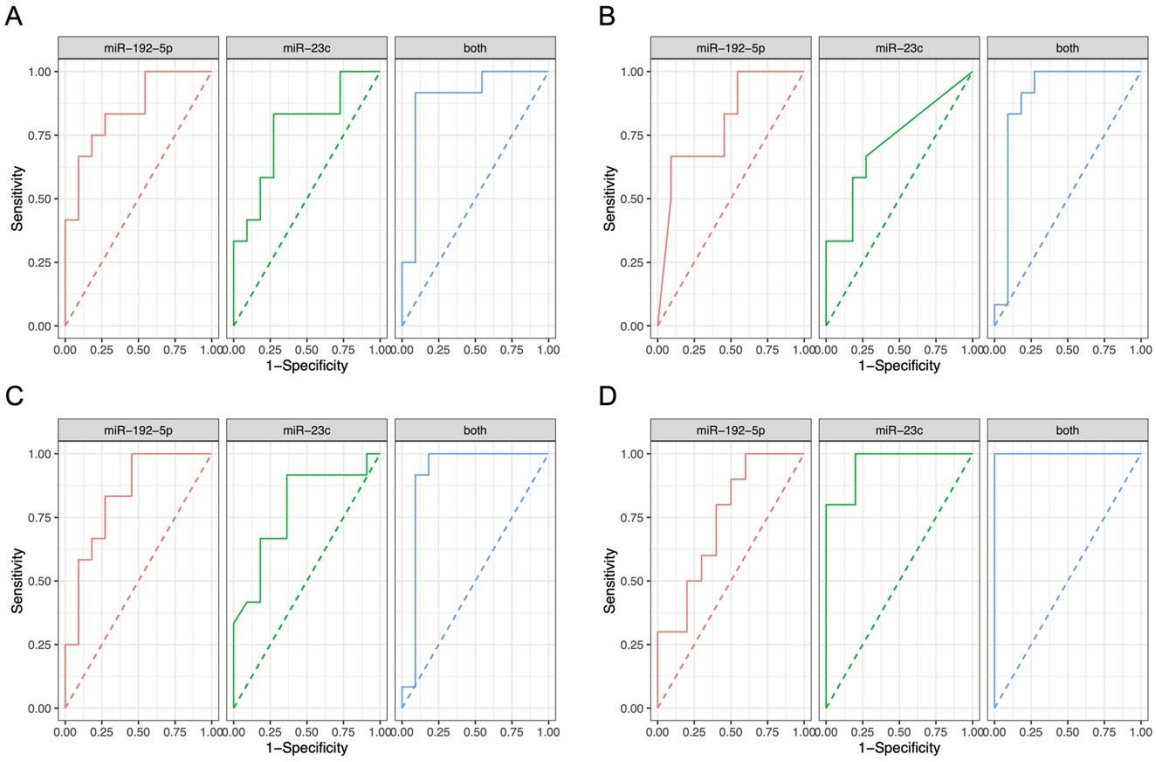
| Sample | Total Read | Trimmed Read | Non-adapter Read | Short Read | Low-quality Read |
|---|---|---|---|---|---|
| C1 | 34,054,152 | 16,302,132 (47.87%) | 348,469 (1.02%) | 17,393,694 (51.08%) | 9,857 (0.03%) |
| C2 | 29,153,184 | 9,669,621 (33.17%) | 82,691 (0.28%) | 19,395,904 (66.53%) | 4,968 (0.02%) |
| C3 | 43,875,832 | 16,116,888 (36.73%) | 1,744,532 (3.98%) | 26,003,705 (59.27%) | 10,707 (0.02%) |
| C4 | 23,895,479 | 11,394,718 (47.69%) | 171,416 (0.72%) | 12,303,429 (51.49%) | 25,916 (0.11%) |
| C5 | 19,666,679 | 10,730,775 (54.56%) | 392,092 (1.99%) | 8,531,159 (43.38%) | 12,653 (0.06%) |
| C6 | 20,420,623 | 8,591,291 (42.07%) | 185,783 (0.91%) | 11,635,318 (56.98%) | 8,231 (0.04%) |
| C7 | 23,682,022 | 9,291,036 (39.23%) | 151,221 (0.64%) | 14,222,829 (60.06%) | 16,936 (0.07%) |
| C8 | 24,178,483 | 11,319,389 (46.82%) | 219,435 (0.91%) | 12,613,443 (52.17%) | 26,216 (0.11%) |
| C9 | 24,183,218 | 13,174,220 (54.48%) | 556,219 (2.3%) | 10,365,374 (42.86%) | 87,405 (0.36%) |
| C10 | 26,210,781 | 9,255,895 (35.31%) | 217,576 (0.83%) | 16,710,072 (63.75%) | 27,238 (0.1%) |
| C11 | 28,984,881 | 14,790,149 (51.03%) | 463,393 (1.6%) | 13,684,704 (47.21%) | 46,635 (0.16%) |
| C12 | 24,732,679 | 5,007,423 (20.25%) | 115,179 (0.47%) | 19,598,446 (79.24%) | 11,631 (0.05%) |
| P1 | 14,886,671 | 4,157,080 (27.92%) | 15,152 (0.1%) | 10,712,328 (71.96%) | 2,111 (0.01%) |

| | | | | | |
|---|---|---|---|---|---|
| P2 | 30,338,701 | 17,683,705 (58.29%) | 619,382 (2.04%) | 12,024,624 (39.63%) | 10,990 (0.04%) |
| P3 | 33,815,917 | 17,647,256 (52.19%) | 188,727 (0.56%) | 15,970,849 (47.23%) | 9,085 (0.03%) |
| P4 | 32,055,313 | 16,407,543 (51.19%) | 810,531 (2.53%) | 14,827,373 (46.26%) | 9,866 (0.03%) |
| P5 | 23,990,215 | 5,662,082 (23.6%) | 105,844 (0.44%) | 18,208,857 (75.9%) | 13,432 (0.06%) |
| P6 | 23,555,073 | 8,925,460 (37.89%) | 59,118 (0.25%) | 14,565,980 (61.84%) | 4,515 (0.02%) |
| P7 | 23,287,639 | 10,831,014 (46.51%) | 960,165 (4.12%) | 11,482,849 (49.31%) | 13,611 (0.06%) |
| P8 | 35,037,627 | 15,577,105 (44.46%) | 1,843,470 (5.26%) | 17,588,706 (50.2%) | 28,346 (0.08%) |
| P9 | 28,371,671 | 14,268,622 (50.29%) | 262,260 (0.92%) | 13,805,103 (48.66%) | 35,686 (0.13%) |
| P10 | 30,844,432 | 16,904,759 (54.81%) | 417,731 (1.35%) | 13,458,704 (43.63%) | 63,238 (0.21%) |
| P11 | 30,187,225 | 18,877,929 (62.54%) | 343,758 (1.14%) | 10,939,214 (36.24%) | 26,324 (0.09%) |
| P12 | 28,114,739 | 12,325,273 (43.84%) | 224,844 (0.8%) | 15,533,887 (55.25%) | 30,735 (0.11%) |

**Supplementary Figure 2. Small RNA composition.** The distributions of small RNAs are shown as boxplots representing the proportions of each biotype among the final processed reads mapped to the category of small RNA (excluding rRNA). The relatively low proportion of known miRNA (1.52% of the processed reads mapped to the category of small RNA, excluding rRNA) may be explained by size selection and gel extraction of small RNA libraries prior to sequencing. In order to investigate the composition of various small RNA biotypes, we excised a band corresponding to 138-220 bp (18 to 100bp of cDNA plus 120 bp of adaptors). This would have decreased the proportion of small RNAs such as miRNA and piRNA (26-31 bp), while increasing the proportion of larger small RNAs such as tRNA (typically 76-90 bp in length).



**Supplementary Figure 3. Receiver operating characteristic (ROC) curves for the diagnosis of ALS based on the miRNA levels of miR-192-5p, miR-23c and both (fitted by binary logistic regression) measured by small RNA-seq (A, B, C) and ddPCR (D)**. The miRNA levels were normalized by different algorithms (A: DESeq2, B: EdgeR, C: Quantile normalization), and by an endogenous reference miRNA in ddPCR (miR-191). Diagnostic metrics were summarized in a table below.

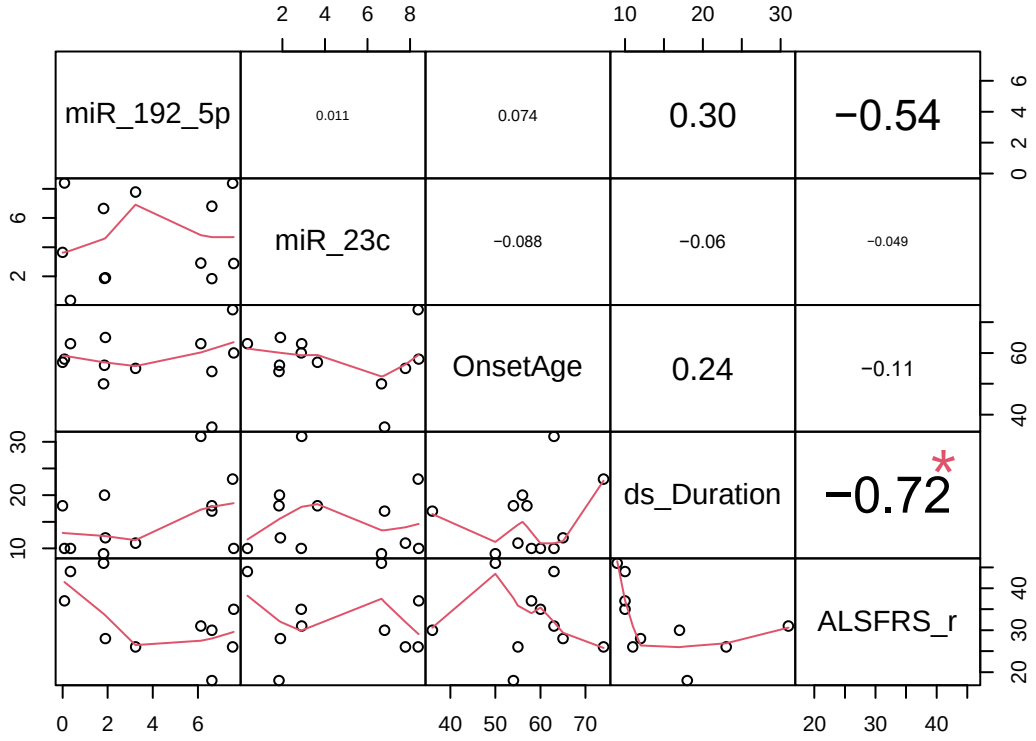



**Supplementary Table 3. The measures of sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve in the ROC curves of Supplementary Figure 3.**

| | Sensitivity | Specificity | PPV | NPV | AUC (95% CI) |
|---|---|---|---|---|---|
| miR-192-5p | | | | | |
| DEseq2 | 0.667 | 0.909 | 0.889 | 0.714 | 0.848 (0.69-1) |
| EdgeR | 0.667 | 0.909 | 0.889 | 0.714 | 0.795 (0.607-0.984) |
| Quantile | 0.833 | 0.727 | 0.769 | 0.800 | 0.833 (0.662-1) |
| ddPCR | 1.00 | 0.400 | 0.625 | 1.00 | 0.74 (0.515-0.965) |
| miR-23C | | | | | |
| DEseq2 | 0.833 | 0.727 | 0.769 | 0.800 | 0.773 (0.573-0.972) |
| EdgeR | 0.583 | 0.818 | 0.778 | 0.643 | 0.72 (0.521-0.918) |
| Quantile | 0.917 | 0.636 | 0.733 | 0.875 | 0.784 (0.586-0.982) |
| ddPCR | 1.00 | 0.800 | 0.833 | 1.00 | 0.96 (0.849-1) |
| Both | | | | | |
| DEseq2 | 0.917 | 0.909 | 0.917 | 0.909 | 0.894 (0.742-1) |
| EdgeR | 0.833 | 0.909 | 0.909 | 0.833 | 0.894 (0.728-1) |
| Quantile | 0.917 | 0.909 | 0.917 | 0.909 | 0.909 (0.745-1) |
| ddPCR | 1.00 | 1.00 | 1.00 | 1.00 | 1 |

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve

**Supplementary Figure 4. A representative figure of multiple correlation plots between the level of miRNA (miR-192-5p and miR-23c) measured by small RNA-seq (quantile normalization) and clinical parameters (onset age, disease duration, and the revised ALSFRS total score).** Each panel on the lower triangle is a scatter plot with smooth curve fitted by Loess (locally estimated scatterplot smoothing) for a pair of variables whose identities are given by the corresponding row and column labels. Spearman correlation coefficient for each pair was presented on the corresponding panel on the upper triangle. *p<0.05. All spearman correlation coefficients (p-values within parentheses) were summarized in a table below.




**Supplementary Table 4. The correlation coefficients of Spearman correlation analysis in Supplementary Figure 4.**

| | Onset age (years) | Disease duration (months) | ALSFRS-R total score |
|---|---|---|---|
| miR-192-5p | | | |
| DEseq2 | 0.207 | 0.620 | -0.492 |
| EdgeR | 0.110 | 0.582 | -0.458 |
| Quantile | 0.055 | 0.517 | -0.543 |
| ddPCR | -0.348 | -0.067 | -0.067 |
| miR-23C | | | |
| DEseq2 | -0.018 | -0.043 | -0.006 |
| EdgeR | -0.092 | -0.019 | -0.043 |
| Quantile | -0.116 | 0.031 | -0.049 |
| ddPCR | -0.273 | -0.104 | 0.313 |

**Supplementary Table 5. Comparison of the methods and cohort between studies on the circulating EV miRNA in ALS.** The discovery and validation cohort are presented separately with the number of patients and controls within parentheses.

| Author (year) | Sample | EV isolation | miRNA profiling | Discovery cohort | Validation cohort |
|---|---|---|---|---|---|
| Xu et al. (2018) | Serum | Precipitation (ExoQuick) | qRT-PCR | 30<br>(10 vs. 20) | NA |
| Katsu et al. (2019) | Plasma | Precipitation (polyethylene glycol) & immuno-affinity (L1CAM) | miRNA Oligo Chip | 10<br>(5 vs. 5) | NA |
| Saucier et al. (2019) | Plasma | Peptide affinity (Venceramin) | NGS (discovery)<br>ddPCR (validation) | 26<br>(14 vs. 12) | 15<br>(12 vs. 3) |
| Banack et al. (2020) | Plasma | Precipitation (exoQuick) & immuno-affinity (L1CAM) | NGS (discovery)<br>qPCR (validation) | 20<br>(10 vs. 10) | 20<br>(10 vs. 10) |
| Pregnolato et al. (2021) | Serum | Precipitation (miRCURY) | qRT-PCR | 10<br>(7 vs. 3) | NA |
| Lo et al. (2021) | Serum | Immuno-affinity (CD63) | NanoString | 21<br>(14 vs. 7) | NA |
| Present study | Serum | Membrane affinity (exoEasy) | NGS<br>ddPCR | 23<br>(12 vs 11) | 33<br>(18 vs 15) |