SUPPLEMENTAL MATERIAL

miRNA contents of cerebrospinal fluid extracellular vesicles in glioblastoma patients

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Supplementary Methods

Clinical specimen collection

All research performed were approved by IRB boards at University of California, San Diego Human Research Protections Program and were in accordance with the principles expressed at the declaration at Helsinki. Each patient was consented by a dedicated clinical research specialist prior to collection. Written consent was obtained for each patient. The consent process was approved by the ethics committee, and all records were documented in our electronic record system. The written consent from patients was also scanned into our electronic filing system. The plasma and CSF specimens were collected at the University of California San Diego Medical Center under IRB 120345X. Blood was collected using an 18 Gauge-needle venipuncture into clot-activating blood collection tubes with gel separator (BD vacutainer catalog #366450). Attention was paid to minimize mechanical tube agitation. The samples were processed by spinning at 1,100 x g for 10 minutes within 30 minutes of collection, 0.8µm filtered, snap frozen and store at -80°C. The CSF was collected by ventricular/lumbar drain placement or cisternal aspiration at the time of craniotomy. Collected CSF specimens were 0.8µm filtered, snapped frozen and stored at -80°C.

Nanoparticle tracking analysis

The number of vesicles recovered was determined by Nanoparticle Tracking Analysis (NTA) on a Nanosight LM-10HS equipped with a 405nm laser (Nanosight, Wiltshire, UK) that was calibrated with polystyrene latex microbeads at 100 nm and 200 nm prior to analysis. Resuspended vesicles were diluted 1:40 to 1:200 with PBS to achieve between 20-100 objects

per frame. EVs were manually injected into the sample chamber at ambient temperature. Each samples was measured in triplicate at camera setting 13 with acquisition time of 30 s and detection threshold setting of 8. At least 200 completed tracks were analyzed per video. The NTA analytical software version 2.3 was used for capturing and analyzing the data.

Tunable resistive pulse sensing (TRPS)

TRPS was performed using the qNano system from Izon (Christchurch, New Zealand). Samples were serially diluted in PBS and measured using NP200 and NP1000 nanopores. The current was recorded for a minimum of 60 s and 500 blockade events; three measurements were recorded for each dilution. Blockade events were calibrated against particles of a known size measured under identical settings.

exoRNeasy Serum/Plasma Maxi Kit

A beta version of the exoRNeasy plasma/serum maxi kit (EXO50 kit) kindly provided by Exosome Diagnostics was used to isolate extracellular RNA from CSF. The kit uses a spin column format and specialized buffers to purify EVs eliminating the need for ultracentrifugation step from up to 4 ml of 0.8 ∝m prefiltered biofluid. Captured EVs were lysed directly on column with Qiazol, and total RNA was extracted using a modified Qiagen's miRNeasy protocol using the supplied RNeasy MinElute column.

Droplet digital PCR (ddPCR)

EV RNA and miRNA mimic were reversed transcribed with TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems) using the following conditions: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C.

Each 20 µL ddPCR reaction containing cDNA, 10 µL of ddPCR Supermix for Probe (Bio-Rad), 0.5 µL of Taqman primer/probe mix (Applied Biosystems) and DEPC H_2O was loaded into a DG8 plastic cartridge (Bio-Rad) with 70 µL of QX200 Droplet Generation oil (Bio-Rad) and then placed into the QX2000 Droplet Generator (Bio-Rad). The droplets generated from each sample were transferred to a 96-well PCR plate and PCR amplified using the following parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 min, then 1 cycle of 98°C for 10 min, ending at 4°C. The plate was then loaded into the QX200 Droplet Reader (Bio-Rad) for analysis using the accompanied QuantaSoft Application.

Supplementary Figure Legend

Supplementary Fig. 1. Standard curve for miRNA absolute copy determination. 0.02 pmol of miRNA mimic from Qiagen was used in a 20 ∝l cDNA synthesis reaction with miRCURY LNA™ Universal RT microRNA PCR system (Exiqon). 10-fold serial dilution of cDNA was used for the generation of standard curve ($4 \cdot 10\text{-}^{2}$ to $4 \cdot 10^{-6}$ fmol).

Supplementary Fig. 2. Distribution of miRNAs in glioblastoma cell line derived EVs. (a) Nanoparticle tracking analysis (NTA) of extracellular vesicles isolated from glioblastoma cell line by differential centrifugation. 3 measurements were taken for each cell line. (b) Absolute

copy number of miRNA transcripts recovered in microvesicles and exosomes were determined by qRT-PCR. All reactions were performed in triplicate.

Supplementary Fig. 3. The effect of biological replicate on cell line derived EV population. (a) Contribution of microvesicle and exosome fractions to total EV and total RNA recovered across independent biological replicate (b) Cell line-derived microvesicles contain more RNA per vesicle than the corresponding exosomes. Average RNA yield per vesicles was calculated by normalizing total RNA yield to vesicles number as determined by NTA. (c) The expression levels of miR-21, in microvesicles and exosomes were quantitatively assessed. qRT-PCR was performed in triplicate

Supplementary Fig. 4. Distribution of miRNAs in glioblastoma patient plasma derived EVs. (a) Nanoparticle tracking analysis (NTA) of extracellular vesicles isolated from glioblastoma patient plasma by differential centrifugation. 3 measurements were taken for each sample. (b) Absolute copy number of miRNA transcripts recovered in microvesicles and exosomes were determined by qRT-PCR. All reactions were performed in triplicate

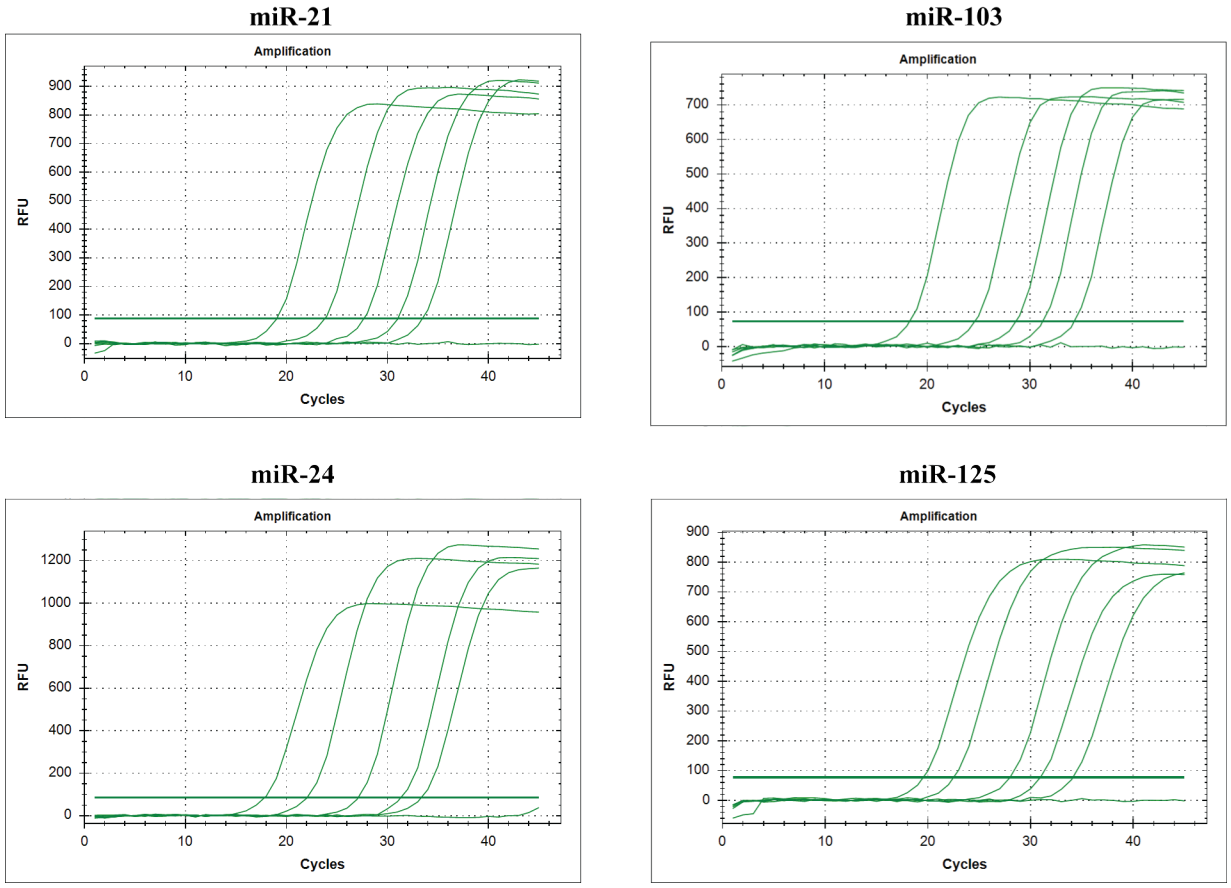
Supplementary Fig. 5. Distribution of miRNAs in glioblastoma patient CSF derived EVs. (a) Nanoparticle tracking analysis (NTA) of extracellular vesicles isolated from glioblastoma patient CSF by differential centrifugation. 3 measurements were taken for each sample. (b) Absolute copy number of miRNA transcripts recovered in microvesicles and exosomes were determined by qRT-PCR. All reactions were performed in triplicate.

Supplementary Fig. 6. Comparison of CSF EV RNA yield by ultracentrifugation vs. exoRNeasy. EV-associated RNAs were isolated from 4ml of CSF either by ultracentrifugation coupled with miRCURY RNA isolation kit or Qiagen exoRNeasy Maxi Kit.

Supplementary Fig. 7. Enrichment of EVs >200nm in microvesicle fractions. Nanoparticle tracking analysis (NTA) of extracellular vesicles isolated from glioblastoma patient CSF by differential centrifugation. (a) Percentage of EVs >200nm in microvesicle and exosome fraction. (b) Percentage of EVs <200nm in microvesicle and exosome fractions.

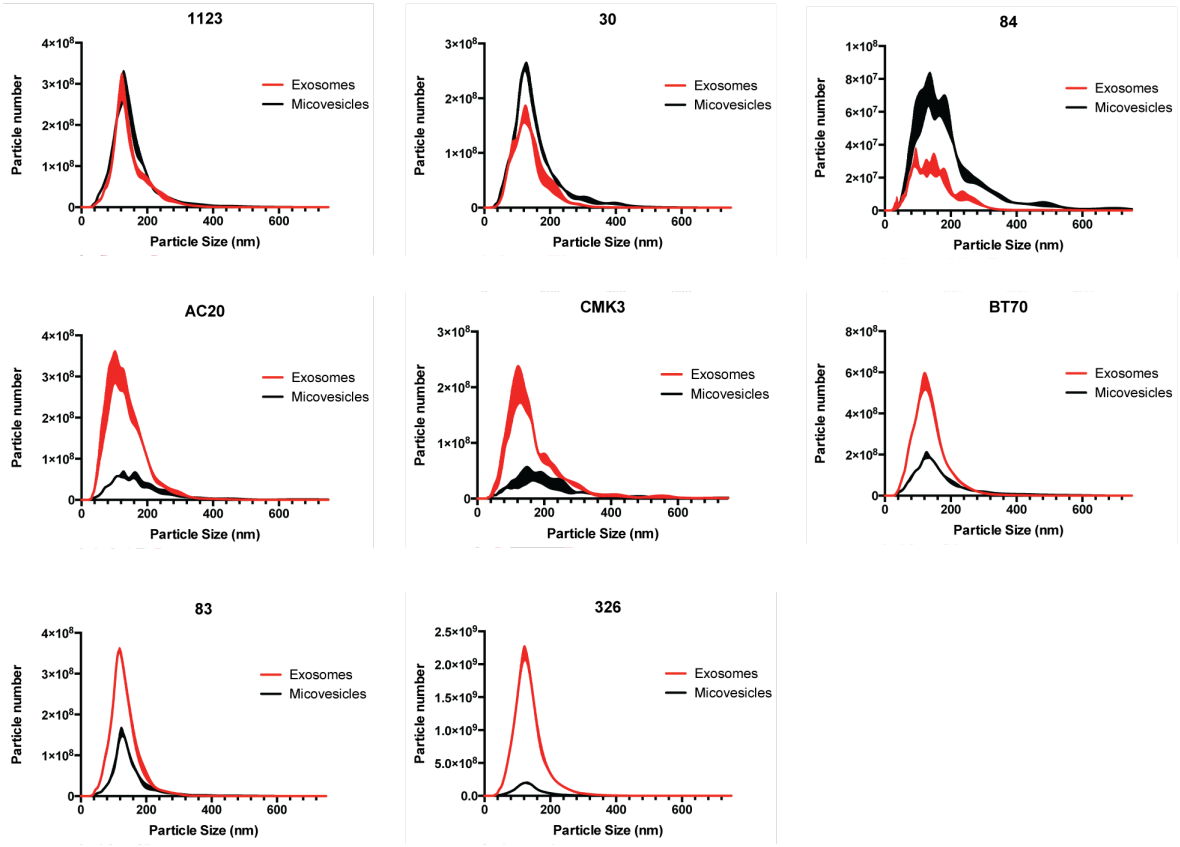
Supplementary Fig. 8. Effect of freeze/thaw cycle on CSF derived EVs. EVs isolated from CSF were analyzed using NTA before and after storage at -80°C. Neither total EV yield (a) nor EV size profile (b) was significantly altered as a result of a freeze/thaw cycle.

Supplementary Fig. 1



Supplementary Fig. 2

a



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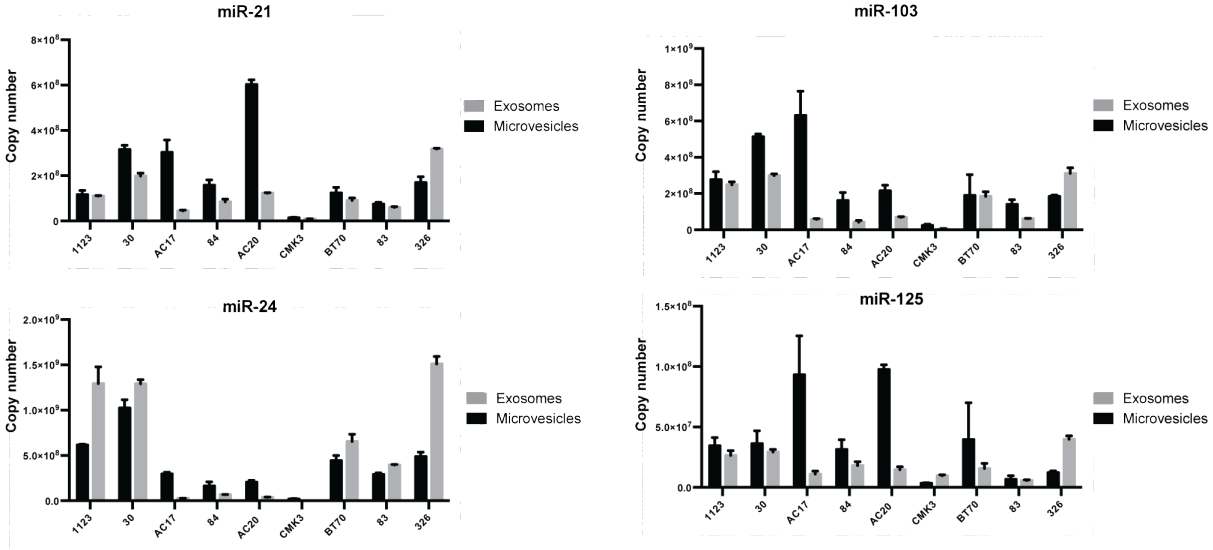
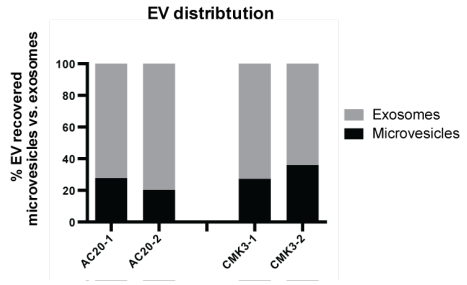
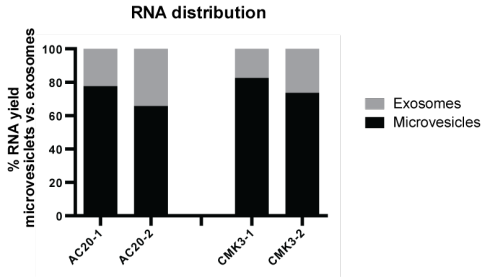


Supplementary Fig. 3

a





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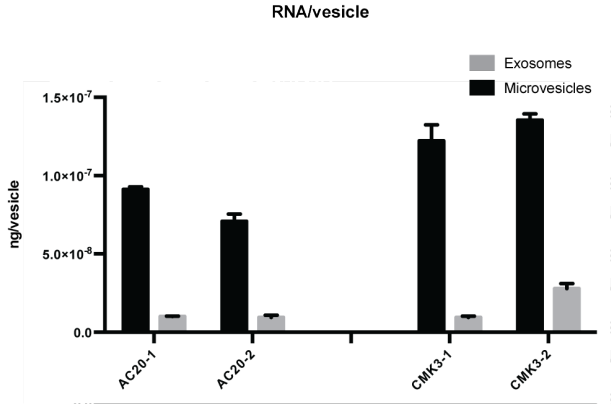

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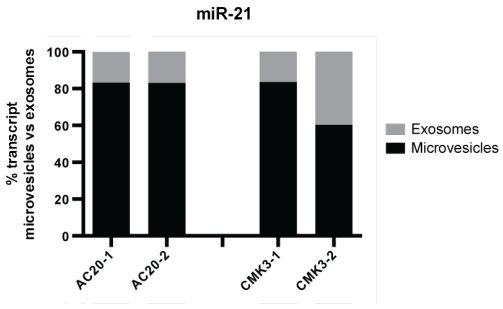
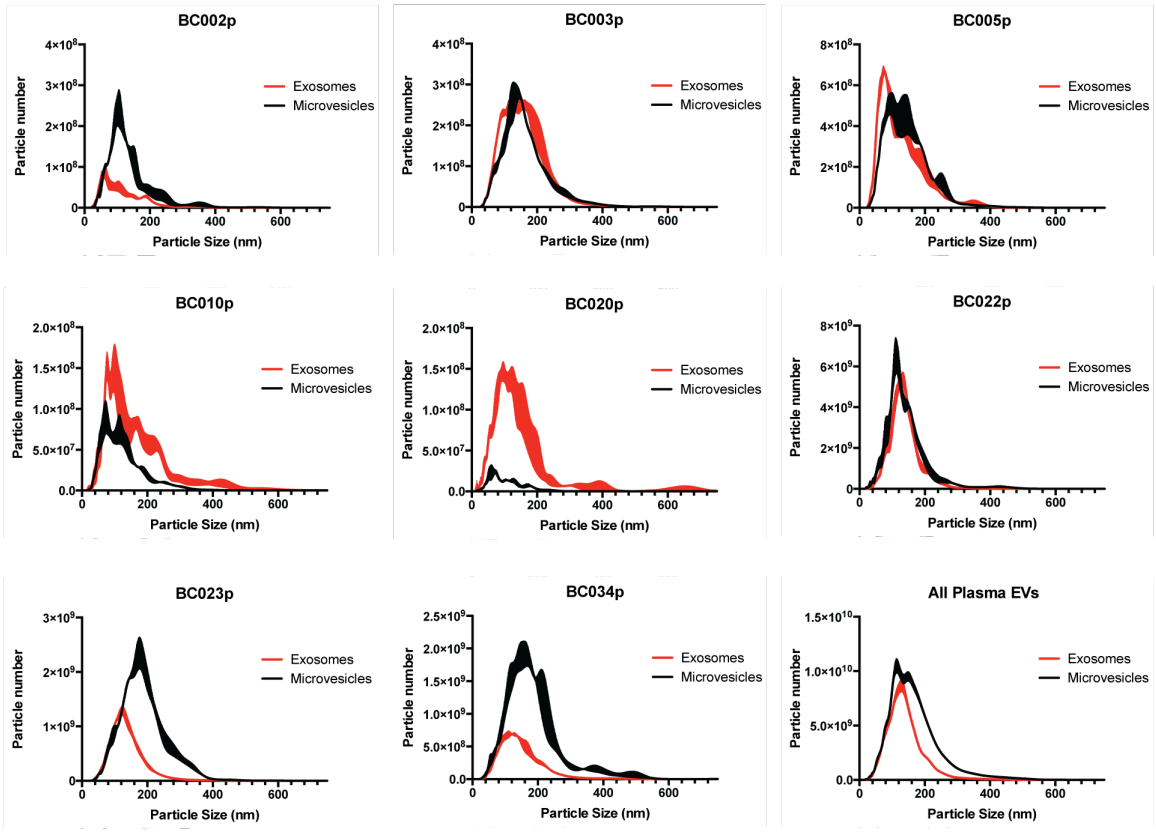


Supplementary Fig. 4

a



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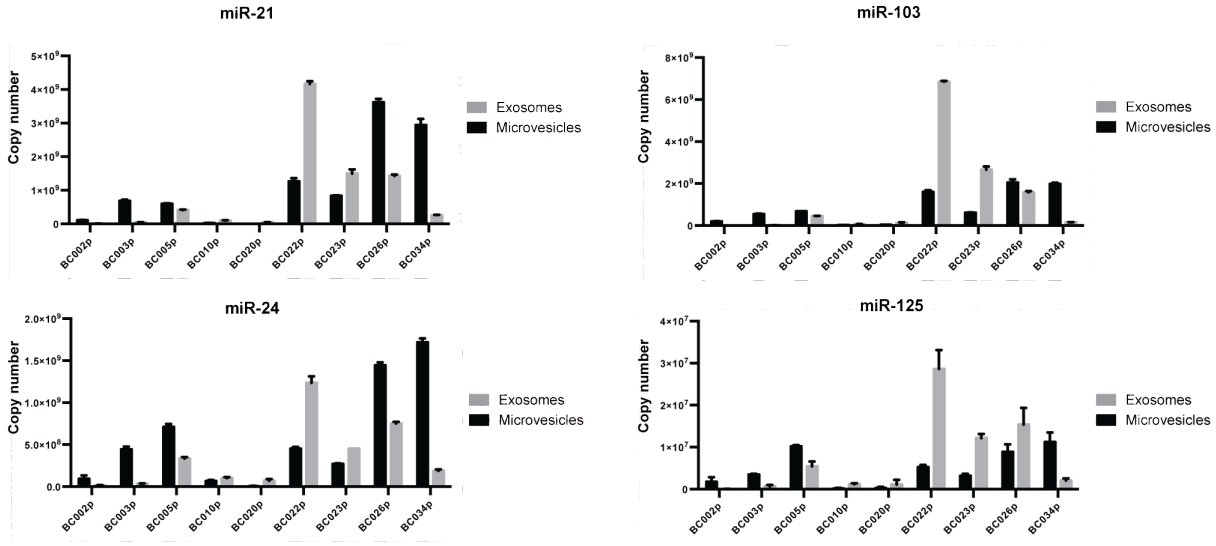
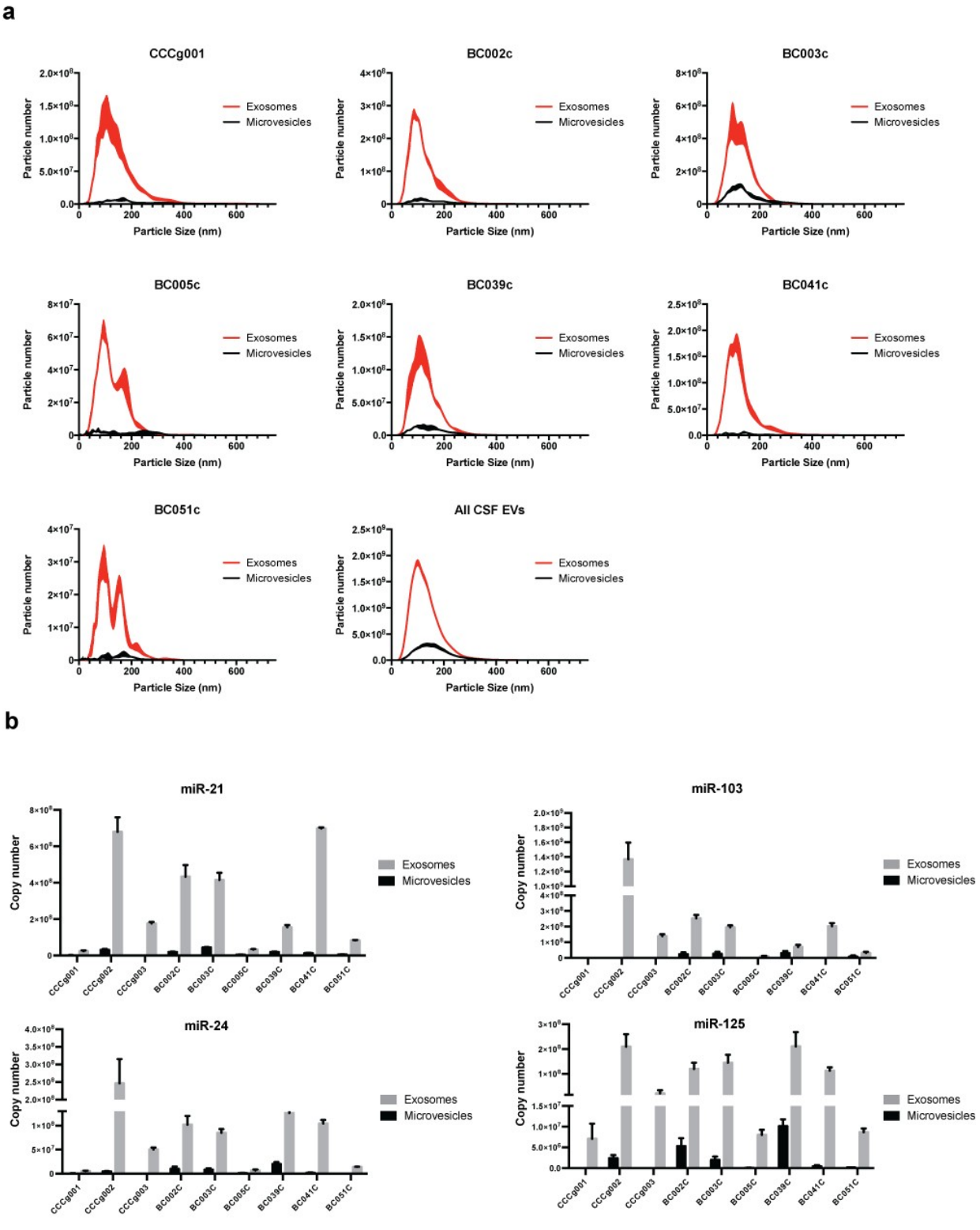
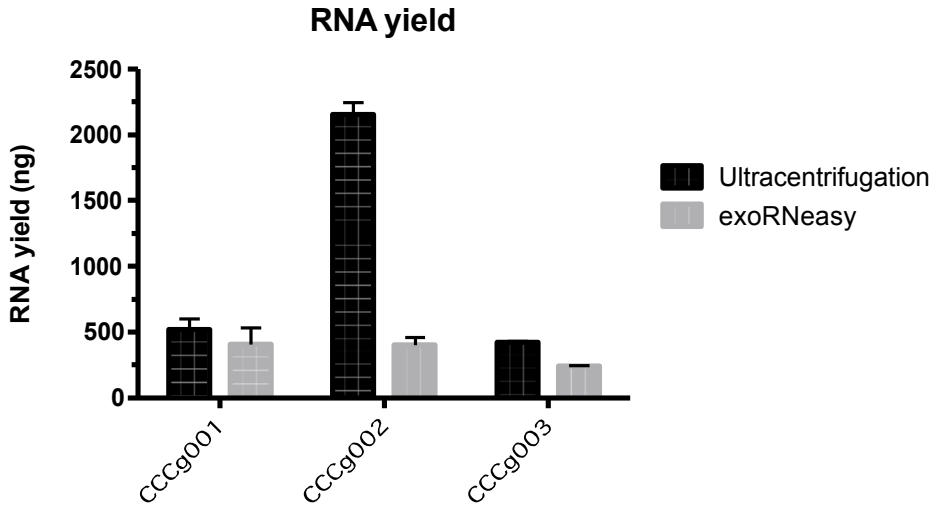


Supplementary Fig. 5

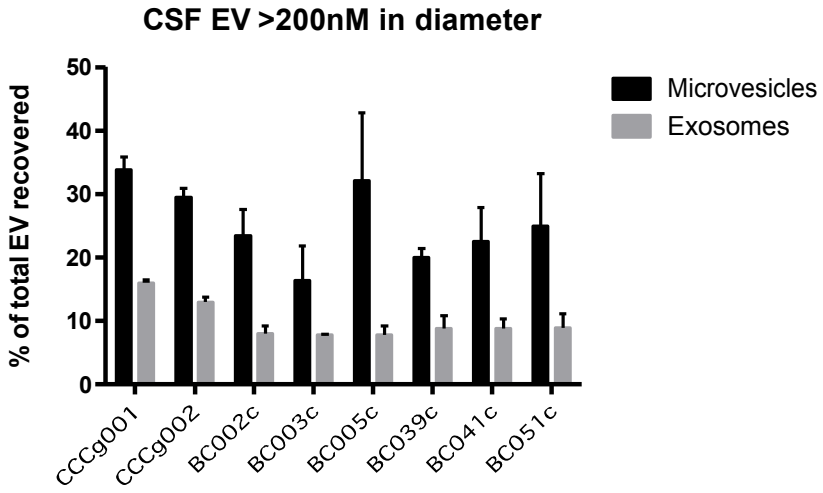


Supplementary Fig. 6



Supplementary Fig. 7

a



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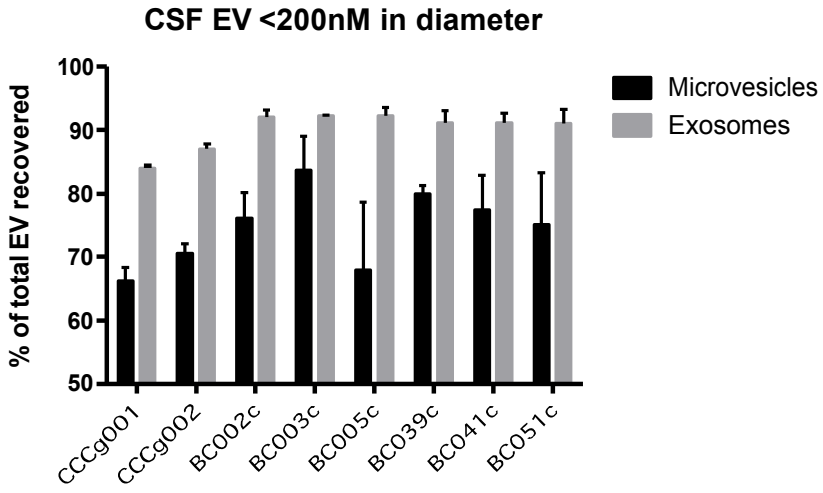
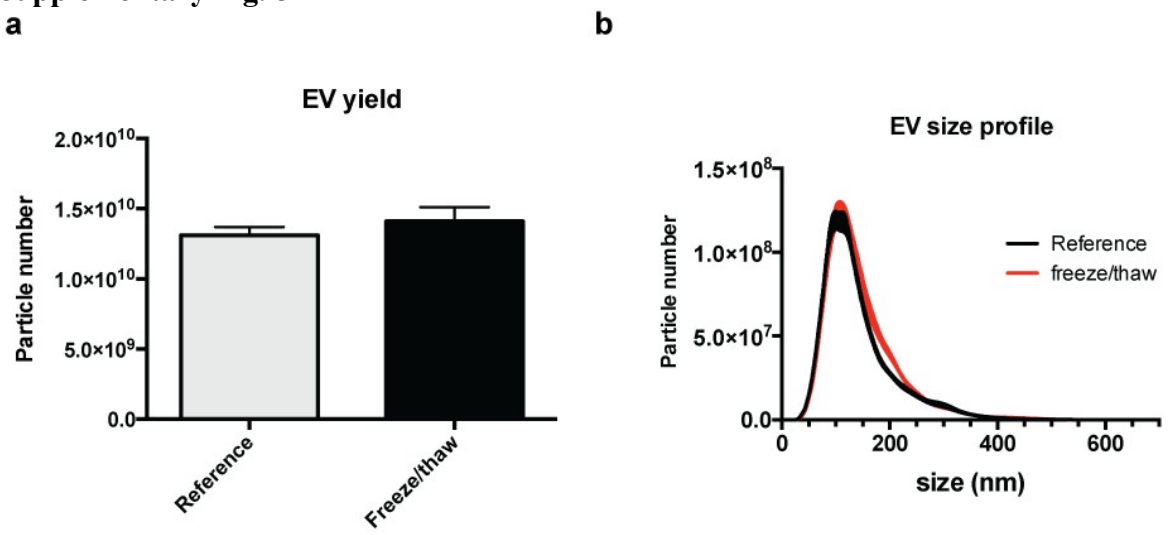


Supplementary Fig. 8



Supplementary Table 1: Recovery of cell line derived EVs

Cell line	Microvesicles (10,000×g)		Exosomes (120,000×g)	
	EV number*	RNA yield (ng)	EV number*	RNA yield (ng)
1123	$2.81\pm0.31 \cdot 10^{10}$	700	$5.71\pm0.65 \cdot 10^{10}$	442
30	$2.66\pm0.09 \cdot 10^{10}$	1465	$4.22\pm0.41 \cdot 10^{10}$	373
AC17	$1.64\pm0.24 \cdot 10^{10}$	2611	$2.50\pm0.11 \cdot 10^{10}$	332
84	$1.23\pm0.11 \cdot 10^{10}$	1828	$1.07\pm0.02 \cdot 10^{10}$	327
AC20	$9.83\pm0.68 \cdot 10^{9}$	690	$3.87\pm0.47 \cdot 10^{10}$	358
CMK3	$8.43\pm1.74 \cdot 10^{9}$	618	$2.54\pm0.20 \cdot 10^{10}$	221
BT70	$2.41\pm0.08 \cdot 10^{10}$	2654	$5.54\pm0.30 \cdot 10^{10}$	676
83	$1.21\pm0.09 \cdot 10^{10}$	491	$2.80\pm0.20 \cdot 10^{10}$	338
326	$1.78\pm0.08 \cdot 10^{10}$	902	$1.83\pm0.13 \cdot 10^{11}$	798

* EV number as determined by NTA. Results are presented as mean ± SEM from three measurements.

Supplementary Table 2: miRNA content in cell line derived EVs

Sample	miR-21		miR-24		miR-103		miR-125	
	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo
1123	4.10×10^{-3}	1.92×10^{-3}	2.19×10^{-2}	2.26×10^{-2}	9.83×10^{-3}	4.35×10^{-3}	1.23×10^{-3}	4.63×10^{-4}
30	1.18×10^{-2}	4.72×10^{-3}	3.85×10^{-2}	3.06×10^{-2}	1.93×10^{-2}	7.10×10^{-3}	1.37×10^{-3}	6.94×10^{-4}
AC17	1.84×10^{-2}	1.80×10^{-3}	1.81×10^{-2}	1.08×10^{-3}	3.84×10^{-2}	2.34×10^{-3}	5.68×10^{-3}	4.40×10^{-4}
84	1.28×10^{-2}	7.89×10^{-3}	1.34×10^{-2}	6.38×10^{-3}	1.32×10^{-2}	3.87×10^{-3}	2.57×10^{-3}	1.69×10^{-3}
AC20	6.12×10^{-3}	3.18×10^{-3}	2.10×10^{-2}	1.02×10^{-3}	2.20×10^{-2}	1.77×10^{-3}	9.95×10^{-3}	3.80×10^{-4}
CMK3	1.55×10^{-3}	3.39×10^{-4}	2.45×10^{-3}	3.58×10^{-5}	2.95×10^{-3}	1.37×10^{-4}	4.14×10^{-4}	3.83×10^{-4}
BT70	5.12×10^{-3}	1.64×10^{-3}	1.85×10^{-2}	1.18×10^{-2}	7.88×10^{-3}	3.33×10^{-3}	1.65×10^{-3}	2.82×10^{-4}
83	6.12×10^{-3}	2.14×10^{-3}	2.43×10^{-2}	1.41×10^{-2}	1.16×10^{-2}	2.21×10^{-3}	5.56×10^{-4}	2.01×10^{-4}
326	9.53×10^{-3}	1.74×10^{-3}	2.76×10^{-2}	8.27×10^{-3}	1.04×10^{-2}	1.71×10^{-3}	6.88×10^{-4}	2.18×10^{-4}

Supplementary Table 3: Recovery of plasma derived EVs

Plasma	Microvesicles (10,000×g)		Exosomes (120,000×g)	
	EV number*	RNA yield (ng)	EV number*	RNA yield (ng)
BC002p	$2.27\pm0.27 \cdot 10^{10}$	195	$8.11\pm1.11 \cdot 10^{9}$	90
BC003p	$3.51\pm0.29 \cdot 10^{9}$	140	$4.03\pm0.22 \cdot 10^{10}$	80
BC005p	$7.51\pm0.17 \cdot 10^{10}$	135	$7.39\pm0.58 \cdot 10^{10}$	195
BC010p	$1.01\pm0.08 \cdot 10^{10}$	105	$2.03\pm0.17 \cdot 10^{10}$	65
BC020p	$2.18\pm0.16 \cdot 10^{9}$	160	$1.91\pm0.29 \cdot 10^{10}$	155
BC022p	$5.88\pm0.69 \cdot 10^{11}$	170	$4.55\pm0.28 \cdot 10^{11}$	410
BC023p	$3.20\pm0.03 \cdot 10^{11}$	115	$1.26\pm0.06 \cdot 10^{11}$	375
BC026p	$1.34\pm0.07 \cdot 10^{11}$	160	$5.76\pm0.22 \cdot 10^{10}$	180
BC034p	$2.96\pm0.19 \cdot 10^{11}$	180	$9.03\pm0.78 \cdot 10^{10}$	140

* EV number as determined by NTA. Results are presented as mean ± SEM from three measurements.

Supplementary Table 4: miRNA content in plasma derived EVs

Sample	miR-21		miR-24		miR-103		miR-125	
	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo
BC002p	1.38×10^{-2}	3.37×10^{-4}	1.17×10^{-2}	6.70×10^{-4}	2.50×10^{-2}	1.58×10^{-5}	2.18×10^{-4}	1.68×10^{-5}
BC003p	1.95×10^{-2}	1.21×10^{-3}	1.27×10^{-2}	8.42×10^{-4}	1.55×10^{-2}	3.77×10^{-4}	9.91×10^{-5}	1.66×10^{-5}
BC005p	7.98×10^{-3}	5.58×10^{-3}	9.49×10^{-3}	4.55×10^{-3}	9.15×10^{-3}	6.09×10^{-3}	1.36×10^{-4}	7.39×10^{-5}
BC010p	2.73×10^{-3}	4.91×10^{-3}	6.78×10^{-3}	5.09×10^{-3}	2.68×10^{-3}	3.24×10^{-3}	1.66×10^{-5}	5.94×10^{-5}
BC020p	2.57×10^{-3}	2.52×10^{-3}	2.45×10^{-3}	3.73×10^{-3}	1.97×10^{-2}	6.51×10^{-3}	1.30×10^{-4}	5.85×10^{-5}
BC022p	2.16×10^{-3}	9.17×10^{-3}	7.69×10^{-4}	2.72×10^{-3}	2.74×10^{-3}	1.51×10^{-2}	8.94×10^{-5}	6.30×10^{-5}
BC023p	2.61×10^{-3}	1.19×10^{-2}	8.48×10^{-4}	3.58×10^{-3}	1.92×10^{-3}	2.11×10^{-2}	9.95×10^{-5}	9.65×10^{-5}
BC026p	2.71×10^{-2}	2.48×10^{-2}	1.08×10^{-2}	1.31×10^{-2}	1.54×10^{-2}	2.75×10^{-2}	6.65×10^{-5}	2.67×10^{-4}
BC034p	9.95×10^{-3}	2.92×10^{-3}	5.81×10^{-3}	2.07×10^{-3}	6.72×10^{-3}	1.74×10^{-3}	3.80×10^{-5}	2.25×10^{-5}

Supplementary Table 5: Recovery of CSF derived EVs

Sample	Microvesicles (10,000×g)		Exosomes (120,000×g)	
	EV number*	RNA yield (ng)	EV number*	RNA yield (ng)
CCCg001	$1.15\pm0.01 \cdot 10^{9}$	232.5	$1.68\pm0.24 \cdot 10^{10}$	418
CCCg002	$2.57\pm0.23 \cdot 10^{10}$	265	$6.15\pm0.19 \cdot 10^{10}$	2684.5
CCCg003	$4.41\pm0.77 \cdot 10^{8}$	221	$1.01\pm0.07 \cdot 10^{10}$	411
BC002C	$1.95\pm0.31 \cdot 10^{9}$	240	$2.52\pm0.25 \cdot 10^{10}$	555
BC003C	$1.28\pm0.14 \cdot 10^{10}$	190	$5.25\pm0.68 \cdot 10^{10}$	165
BC005C	$4.85\pm2.03 \cdot 10^{8}$	235	$6.36\pm0.32 \cdot 10^{9}$	100
BC039C	$1.97\pm 0.33 \cdot 10^{9}$	210	$1.34\pm0.18 \cdot 10^{10}$	135
BC041C	$5.77\pm0.42 \cdot 10^{8}$	195	$1.67\pm0.20 \cdot 10^{10}$	160
BC051C	$2.26\pm0.48 \cdot 10^{8}$	190	$2.90\pm0.20 \cdot 10^{9}$	165

* EV number as determined by NTA. Results are presented as mean ± SEM from three measurements.

Supplementary Table 6: miRNA content CSF derived EVs

Sample	miR-21		miR-24		miR-103		miR-125	
	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo	Copy/MV	Copy/Exo
CCCg001	7.03×10^{-4}	1.43×10^{-3}	3.03×10^{-4}	3.34×10^{-4}	ND	5.87×10^{-6}	5.38×10^{-6}	4.23×10^{-4}
CCCg002	1.19×10^{-3}	1.11×10^{-2}	1.65×10^{-4}	4.01×10^{-3}	4.49×10^{-5}	2.23×10^{-2}	9.30×10^{-5}	3.42×10^{-3}
CCCg003	1.98×10^{-4}	1.76×10^{-2}	9.99×10^{-5}	5.00×10^{-3}	ND	1.39×10^{-2}	ND	2.18×10^{-3}
BC002C	9.74×10^{-3}	1.72×10^{-2}	4.88×10^{-3}	4.05×10^{-3}	1.16×10^{-2}	1.01×10^{-2}	2.74×10^{-3}	4.78×10^{-3}
BC003C	3.35×10^{-3}	7.92×10^{-3}	6.04×10^{-4}	1.63×10^{-3}	1.96×10^{-3}	3.73×10^{-3}	1.56×10^{-4}	2.78×10^{-3}
BC005C	7.27×10^{-3}	5.16×10^{-3}	9.62×10^{-4}	1.05×10^{-3}	ND	1.41×10^{-3}	1.41×10^{-4}	1.26×10^{-3}
BC039C	9.31×10^{-3}	1.16×10^{-2}	1.00×10^{-2}	9.44×10^{-3}	1.45×10^{-2}	5.21×10^{-3}	5.15×10^{-3}	1.57×10^{-2}
BC041C	2.10×10^{-2}	4.19×10^{-2}	2.01×10^{-3}	6.29×10^{-3}	ND	1.22×10^{-2}	7.54×10^{-4}	6.80×10^{-3}
BC051C	2.31×10^{-2}	2.90×10^{-2}	5.25×10^{-4}	4.79×10^{-3}	3.21×10^{-2}	1.10×10^{-2}	6.33×10^{-4}	3.00×10^{-3}

ND: Not detected

Supplementary Table 7: EV number as determined by NTA vs TRPS

Samples		NTA*	TRPS*
CSF1	Microvesicles	$5.17\pm0.61 \cdot 10^{9}$	$4.39\pm1.75 \cdot 10^{10}$
	Exosomes	$8.43\pm0.68 \cdot 10^{10}$	$7.52\pm1.33 \cdot 10^{10}$
CSF2	Microvesicles	$6.54\pm0.66 \cdot 10^{10}$	$4.95\pm1.19 \cdot 10^{10}$
	Exosomes	$4.51\pm0.54 \cdot 10^{11}$	$1.08\pm0.34 \cdot 10^{11}$
CSF3	Microvesicles	$5.10\pm0.42 \cdot 10^{9}$	$8.35\pm1.77 \cdot 10^{10}$
	Exosomes	$9.38\pm0.75 \cdot 10^{10}$	$1.78\pm0.29 \cdot 10^{11}$

* EV numbers are presented as mean ± SEM from three different dilutions, and three measurements were performed at each dilution

Supplementary Table 8: Comparison of miR-125 quantitation methods (qPCR vs ddPCR)

	Ratio (qPCR:ddPCR)*	
CSF sample	**Microvesicles**	**Exosomes**
BC002C	5.07	6.80
BC003C	2.34	7.13
BC039C	6.18	6.66
BC041C	4.64	9.64
BC051C	2.79	4.78

* miRNA copy number was determined by qPCR and ddPCR. Standard curves generated by serial dilution of known quantities of miRNA mimic were used for calibration

Supplementary Table 9: List of primers

miRNA	**Primer**
miR-21	LNA™ PCR primer set, Product No. 204230
miR-103	LNA™ PCR primer set, Product No. 204063
miR-24	LNA™ PCR primer set, Product No. 204260
miR-125	LNA™ PCR primer set, Product No. 204465