In the research field of extracellular vesicles (EVs), the use of fetal bovine serum (FBS) depleted of EVs for in vitro studies is advocated to eliminate the confounding effects of media derived EVs. EV-depleted FBS may either be prepared by ultracentrifugation or purchased commercially. Nevertheless, these preparations do not guarantee an RNA-free FBS for in vitro use. In this study we address the RNA contamination issue, of small non-coding (nc)RNA in vesicular or non-vesicular fractions of FBS, ultracentrifugation EV-depleted FBS, commercial EV-depleted FBS, and in our recently developed filtration based EV-depleted FBS. Commercially available serum- and xeno-free defined media were also screened for small ncRNA contamination. Our small ncRNA sequencing data showed that all EV-depleted media and commercially available defined media contained small ncRNA contaminants. Out of the different FBS preparations studied, our ultrafiltration-based method for EV depletion performed the best in depleting miRNAs. Certain miRNAs such miR-122 and miR-203a proved difficult to remove completely and were found in all media. Compared to miRNAs, other small ncRNA (snRNA, Y RNA, snoRNA, and piRNA) were difficult to eliminate from all the studied media. Additionally, our tested defined media contained miRNAs and other small ncRNAs, albeit at a much lower level than in serum preparations. Our study showed that no media is free of small ncRNA contaminants. Therefore, in order to screen for baseline RNA contamination in culturing media, RNA sequencing data should be carefully controlled by adding a media sample as a control. This should be a mandatory step before performing cell culture experiments in order to eliminate the confounding effects of media.

Fetal bovine serum (FBS) contains essential factors required for cell growth, metabolism, attachment, and stimulation of proliferation. Thus, it is the most widely used supplement for culturing human and animal cells. One of the major concerns that has become evident recently is the presence of large amounts of extracellular vesicles (EVs) in FBS. Secreted by most cell types, EVs are mediators of cell-to-cell communication and immune regulation. They carry the genetic material (DNA and RNA) as well as proteins, lipids and other molecules, the transfer of which can alter the functions of the recipient cells. EVs present in FBS are co-isolated with cell-derived EVs and therefore act as contaminants that affect the reliability of the read-out from cell culture experiments, as FBS-derived EVs are structurally and functionally similar to cell-derived EVs. In addition, FBS-EVs taken up by cultured cells cause substantial physiological effects. To overcome this problem, different methods are used to deplete EVs from FBS. Ultracentrifugation (UC) is commonly used, but this method removes EVs only partially. Commercially available depleted FBS are produced using proprietary protocols to reach higher purity levels. Also, commercially available defined, serum-free and animal protein free (xeno-free) media developed for clinical cell therapy purposes are an alternative to the largely undefined FBS in culture media. However, we and others have shown that EV-depleted FBS produced by UC or even commercial EV-depleted FBS are not completely free of FBS-derived EVs. Recently we have developed a simple, standardized, cost- and time-effective protocol to produce EV-depleted FBS by means of ultrafiltration (UF) for cell culture purposes that support cell viability and proliferation.

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### Table 1. Suppliers and pricing information of culture media used for small ncRNA sequencing. Pricing information include consumables and supplementation needed for preparation of 50 ml of complete media. **culture vessels need to be coated with CELLstart**<sup>™</sup> Substrate (Thermo Fisher cat# A1014201) 1:50 dilution 3.6 eur/ml.

| Acronym | Culture media | Basal media | Serum manufacturer | Serum content | Supplementation | Price/50 ml (excl. VAT) |
|---------|---------------|-------------|--------------------|---------------|----------------|------------------------|
| FBS-1   | FBS-1 media   | DMEM/F12 + Glutamax cat# 31331093 | Sigma, ref. 10270106, lot. 42F8554K | 10% FBS | 1% pen-strep | 4 eur |
| FBS-2   | FBS-2 media   | DMEM/F12 + Glutamax cat# 31331093 | Gibco, ThermoFisher cat# 10270106 lot. 42G8468K | 10% FBS | 1% pen-strep | 4 eur |
| UC-dFBS-1 | Ultracentrifugation EV-depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | Sigma, ref. 10270106, lot. 42F8554K | 10% UC-dFBS | 1% pen-strep | 36 eur |
| UC-dFBS-2 | Ultracentrifugation EV-depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | Gibco, ThermoFisher cat# 10270106 lot. 42G8468K | 10% UC-dFBS | 1% pen-strep | 35 eur |
| UF-dFBS-1 | Ultrafiltration EV-depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | Sigma, ref. 10270106, lot. 42F8554K | 10% UF-dFBS | 1% pen-strep | 53 eur |
| UF-dFBS-2 | Ultrafiltration EV-depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | Gibco, ThermoFisher cat# 10270106 lot. 42G8468K | 10% UF-dFBS | 1% pen-strep | 52 eur |
| SBI-dFBS-1 | Commercial Exosome depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | System Biosciences, ref. EXO-FBS-50A-1 lot.170501-001 | 10% SBI-dFBS | 1% pen-strep | 39 eur |
| SBI-dFBS-2 | Commercial Exosome depleted FBS media | DMEM/F12 + Glutamax cat# 31331093 | System Biosciences, ref. EXO-FBS-50A-1 lot.170718-001 | 10% SBI-dFBS | 1% pen-strep | 39 eur |
| StemPRO | Commercial StemPro MSC Serum free medium | StemPro<sup>®</sup> MSC SFM Basal Medium | Gibco, Thermo Fisher, A1067501 | — | 0.3% pen-strep, 1% StemPro MSC CFM Xenofree supplement, GlutaMAX<sup>®</sup> Supplement | 37eur* |

In the EV research field, identifying the ‘RNA contaminants’ derived from media itself is of high importance when developing RNA biomarkers. To address this question, we undertook this study to characterize the extracellular small non-coding (nc)RNA contaminants present in FBS, EV-depleted FBS, commercially available EV-depleted FBS as well as serum- and xeno-free defined media to assess their small ncRNA content and diversity.

### Results

#### EV characterization.  
EVs were isolated from FBS, ultracentrifugation EV-depleted FBS (UC-dFBS), ultrafiltration EV-depleted FBS (UF-dFBS), commercial depleted FBS (SBI-dFBS) and commercial serum- and xeno-free media (StemPRO) by ultracentrifugation (Table 1). EVs were further characterized by nano tracking analysis (NTA), transmission electron microscope (TEM) and western blotting (WB). Based on TEM analysis, while no vesicles could be detected in UF-dFBS, vesicles structures and protein aggregates were detected in regular FBS, UC-dFBS and SBI-dFBS. StemPRO displayed scarce vesicle-like structures and large protein aggregates (Fig. 1A). For western blotting (WB) analysis, bovine specific EV markers are lacking and species cross reactivity for bovine is rarely reported. Anti-tetraspanin antibodies were not used as it is difficult to estimate whether these antibodies specific for mouse or human samples recognize the bovine form. We therefore selected anti-transferrin receptor/CD71 H68.4 monoclonal antibody, which is abundant in serum-derived material, suitable for both mouse and bovine proteins and also recognizes a transmembrane protein, therefore its detection confirms that EVs are analyzed<sup>10,11</sup>. WB results indicated a strong CD71 band in FBS and SBI-dFBS, whereas no bands were detected in StemPRO and UC-dFBS. Faint band of CD71 protein was detected in StemPRO (Fig. 1B). To address the contamination issue, we also performed an additional WB analysis (Supplementary Fig. 1) with HDL (ApoA1) to assess the presence of non-EV bound miRNAs in our samples. Since the antibody was specific for human proteins, species cross-reactivity was analyzed running human plasma samples in parallel with FBS samples. No primary antibody control was used to assess if any non-specific binding or false positives may be due to non-specific binding of the secondary antibody. Pure human HDL was added as a positive control. Thereby confirming the reliability of the results.

Characterization of the EV samples by NTA supported the WB results (Fig. 1C–F). The NTA results showed that as compared to other FBS and SBI-dFBS (Fig. 1D), the number of particles in StemPRO and UC-dFBS was lower, while UF-dFBS had a very low particle count (Fig. 1E). Regular FBS and SBI-dFBS had similar particle number (e<sup>11</sup>) and particle distribution (Fig. 1C). Together, these results indicate that UF-dFBS has a low particle and protein content, potentially being nearly EV-free. Next, we wanted to address the small ncRNA content in these preparations.

#### General mapping results of small ncRNA sequencing.  
Small ncRNA sequencing was performed on FBS containing media (regular FBS and EV-depleted FBS) and serum- and xeno-free media (Table 1). RNA sequencing was not feasible on basal media and supplements due to low RNA yield. Mapping of our sequencing data to human reference genome showed that 0.8 to 9.6 million reads per sample were obtained in the sequencing experiments (Fig. 2). No clear pattern in raw read counts was observed with the different depletion treatments.
but samples based on FBS-2 (dFBS-UC-2 and dFBS-UF-2) showed 3- to 10-fold lower raw reads than their FBS-1-based counterparts.

Mapping of small ncRNA sequencing reads to bovine and human genome displayed indistinguishable miRNA reads and non-overlapping small ncRNA reads between the two species. Small ncRNA sequencing reads were mapped to both human and bovine genome to detect the extent of species-specificity and overlap between the species. Our analysis showed (Fig. 3) that human miRNAs in the tested culture media mapped almost completely (90–100%) to bovine miRNAs, which is also consistent with previous report4. The similarity between bovine and human miRNAs makes distinguishing bovine miRNAs from human miRNA difficult, which may be a confounding factor when assessing miRNA derived from cell culture experiments. In StemPRO medium the overlap was only 73%, which suggests that a much more significant
proportion of these reads may not have originated from miRNA at all, but may rather be experimental noise, suggesting that the actual miRNA content of the defined media was very low. In contrast to miRNAs, other human small ncRNAs were also mapped to a high degree (90–100%) to the bovine genome. The reason for this is that the database for bovine small ncRNAs did not contain any tRNA fragments, which made up most of the human small ncRNA alignments. Therefore, almost all human small ncRNA reads were classified as mapping to the bovine genome instead of mapping to small ncRNAs.

**Vesicular and non-vesicular derived small ncRNA contaminants in FBS, dFBS and commercial media.**

We further characterized the small ncRNA composition of FBS media preparations. As a quality control, serum and xeno-free media (StemPRO) was included in the study and evaluated for RNA contamination, as this medium should not contain any animal derived components. To facilitate comparison between different media types, total miRNA and other small ncRNA levels were normalized according to the external spike-in levels. As shown in the Fig. 4, StemPRO contained small amounts of small ncRNA, source of which is not clearly known. As shown in Fig. 4, considerable variation was observed in RNA content between different media types and manufacturers. In untreated FBS, most (93%) of all small ncRNA counts were mapped to tRNA, whereas 6.5% were mapped to miRNA. Other types of small ncRNA (snRNA, Y RNA, snoRNA, piRNA) were detected in only small amounts (<0.2%). As expected, highest levels of miRNA were detected in untreated FBS, whereas commercial SBI-dFBS had 60±20% lower total miRNA counts. Ultracentrifugation and ultrafiltration lowered the total miRNA content by 80±9% and 99.8±0.1%, respectively. Commercial serum-free medium StemPRO had 99.95±0.01% lower total miRNA content than FBS. For tRNA and snRNA, a similar clear trend was not observed, and depleted media had similar levels of tRNA and snRNA as untreated FBS, although large variation between individual samples was observed (Fig. 4). For Y RNAs and snoRNAs, approximately 10-fold reduction was observed in UF-dFBS compared to other FBS-based media. StemPRO had very low levels of any kind of small ncRNAs. Full list of spike-in normalized miRNA and other small ncRNA counts are listed in Supplementary 1. It should be noted that since the spike-ins used were miRNA-sized (20–24 nt), the normalization is likely to be more accurate in the case of miRNAs than other small ncRNA, which were mostly longer (~30 nt).

For depicting the overlap of small ncRNAs in the different media, Euler diagrams were created (Fig. 5). Our data showed that almost all of the detected miRNAs were found in FBS (109 miRNA detected). Only one miRNA, miR-133a-3p, was not detected in FBS or FBS-derived media, but only in commercial SBI-dFBS media. As expected, all the miRNAs in depleted media were also found in untreated FBS. EV depletion techniques resulted in lower number of miRNAs detected above threshold: 68 for SBI-dFBS, 36 for UC-dFBS and only 1 for UF-dFBS. Similar pattern was observed for other small ncRNA, with all the RNAs detected in FBS-based depleted media also present in untreated FBS and 40 unique tRNA fragments and one Y RNA found in SBI-dFBS media. Less effective depletion was observed with tRNA, snRNA, Y RNA and snoRNA, with UF-dFBS still containing 87 out of the 178 tRNA, 10 out of 18 snRNA and 2 out of 8 Y RNA detected in untreated FBS.

**Comparative analysis of RNA depletion efficiency.**

Subsequently, we evaluated the RNA depletion efficiency of different EV depletion methods. Unsupervised clustering analysis (Fig. 6A) showed that in the case of miRNAs, sample types clustered together, and a uniform decrease in miRNA levels across almost all miRNAs was
Figure 4. Spike-in normalized concentration of different small ncRNA in studied media. Total miRNA, tRNA, snRNA, Y RNA, snoRNA, and piRNA concentration is shown for each media sample. Error bars depict standard deviation of the spike-in fit.

Figure 5. Euler diagrams showing the relationships of the small ncRNA sets detected in different media types. RNAs detected at above 1 spike-in normalized count level (moderate to high abundance) were included in the diagrams. Total number of different RNAs per media type are shown in parenthesis. No small ncRNAs were detected above this level in the StemPRO sample.
observed with the depletion treatments. hsa-miR-122–5p was clearly the most abundant miRNA in FBS-based media. Furthermore, we also investigated how many of the top 20 most commonly reported EV miRNAs in the literature are present in different media preparations (Supplementary 2, table B). Several of the commonly reported miRNA in EVs like hsa-miR-21-5p, hsa-miR-451a, hsa-miR-16-5p, hsa-let-7a, hsa-let-7b and hsa-miR-93-5p has-miR-93-5p were found in high levels even in some of the depleted media preparations. In contrast, clustering based on other small ncRNAs (Fig. 6B) occurred based on FBS supplier (1 and 2; Table 1) rather than sample type. No clear reduction in tRNAs was observed by the depletion treatments. This indicated that tRNA,
which were the most abundant type of small ncRNA detected in the media samples, was not effectively removed with the depletion treatments. However, a large difference in tRNA levels was observed between the two FBS suppliers tested.

**Enrichment of mir-203a in all media.** The sequencing data clearly indicated ineffective depletion of RNA in dFBS, commercially available EV-depleted FBS and defined media, and the efficiency of miRNA depletion in these depleted media is unclear. To address this, we focused on specific miRNAs and traced their levels in the different media preparations. Based on the clustering analysis, two groups of miRNAs were selected for further analysis: miRNAs strongly affected by depletion methods and miRNAs weakly affected by depletion methods (Supplementary 2, figure). As can be seen, there is little difference between the groups, indicating that almost all the miRNAs are similarly affected by the depletion methods. A notable exception is hsa-miR-203a, which was present in StemPRO and appeared to be unaffected by all of the depletion methods.

**Discussion**

Interference by FBS derived RNA (vesicular or non-vesicular) in the *in vitro* studies has recently become evident. Therefore, reliability of the results depends on the complete elimination of serum derived RNA. Until now, little attention has been paid to characterizing these RNA contaminants. Ultracentrifugation, the standard method used for EV depletion, is unable to provide EV-free FBS. Moreover, commercially available EV-depleted FBS are also not completely EV/RNA-free. Therefore, we recently developed an ultrafiltration-based protocol to generate EV-free serum. Consequently, we undertook this study to define the small ncRNA contamination present in different depleted FBS preparations, including our ultrafiltration-based EV-depleted FBS, commercially available EV-depleted serum and xenogeneic media.

It is well known that the specificity of isolating EVs is low for both ultracentrifugation and other EV isolation methods. Various studies have demonstrated that EV preparations derived using these methods contain small amounts of non-EV soluble components, which might carry RNA as well. EV isolation and RNA sequencing was done according to the stringent workflow of the Qiagen services. Furthermore, qPCR was performed on our media samples with endogenous miRNAs (mir-451a, miR-103a-3p, miR-191-5p, miR-23a-3p, miR-30c-5p, mir-23a-miR451a and 3 spike in controls; UniSp6, UniSp-101, and UniSp-100); miR-451a, used as an EV marker, was clearly expressed in our samples, indicating that miRNAs analyzed are EV related. Despite using a stringent protocol for isolating EV-related RNA for RNA sequencing, we still could not rule out the possibility of presence of non-vesicular RNA in our data. As the genuine association of sequenced RNA with EVs was not demonstrated (by using differential nuclease/protease and detergent treatments, for instance) in our study due to technical difficulties, it is more likely that our samples contained both "cell-free RNA" (non-vesicular RNA) and "EV-bound RNA" (vesicular RNA). Therefore we prefer to use the term "vesicular or non-vesicular fractions" in this study.

A vast majority of miRNAs (90–95%) are estimated to be non-vesicular. Based on information retrieved from the literature, similar trend was observed for our miRNA data (Supplementary 2, table A). A possible source of miRNA contamination could be the circulating miRNA bound to various carriers, including Argonaute2 complexes, albumin and lipoproteins. Since high density lipoprotein (HDL) HDL-miRNA transport appears to be more robust than low density lipoprotein (LDL), we focused on analyzing HDL (ApoA1) in our samples. As expected, all the samples except UF-dFBS showed positive expression of HDL, indicating that none of the EV-depleted media are EV or lipoprotein free. These HDL-associated miRNAs, could be considered as one of the source of miRNA contamination.

Our data was in agreement with Wei et al., where 100% overlap of RNA sequencing between bovine and human genome was observed. These results indicate, that media selected for cell culture experiments should be chosen carefully, prioritizing EV/RNA-free media. Screening of contaminants from the culture media should be a routine procedure in order to establish the baseline level of RNAs as a control to minimize the confounding effect of media. To highlight the importance of these findings, we redacted the most commonly reported miRNAs in cell-culture-based EV studies (Supplemental 2, table B) and observed their levels in different media types. Majority (15 out of 20) of the most commonly reported miRNAs were found in moderate to high levels, while 5 out of 20 were found in very high levels.

UF-dFBS showed an exceptional performance in depleting miRNAs compared to UC-dFBS and SBI-dFBS. Surprisingly, miRNA levels in commercially available SBI-dFBS were slightly higher than UC-dFBS, and some miRNA, such as miR-122-5p and miR-203a were present in at least moderate levels in all dFBS. miR-122-5p was also recently reported to be present in abundant amounts in FBS and dFBS, which indicates that certain miRNAs are difficult to remove completely. In the case of miR-122-5p, this is due to its high abundance in FBS. Even though more than 99% is removed, moderate levels are still left in UF-dFBS. Liver specific miR-122 is exclusively non-vesicular, with key roles in hepatocyte growth, metabolism, and homeostasis, and miR-122 is also associated with human liver cancer metastasis and lung cancer.

It is difficult to explain the inefficiency of removing miR-203a. It was detected mainly as short, 16-nt inserts, and could potentially be an experimental artifact, which would explain its presence in all the samples, even in StemPRO. It has been reported that miR-203 has an effect on many properties of cells in culture, e.g. as an inhibitor of ‘stemness’ of mammalian stem cells and pluripotent stem cells and it may also have anti-tumorigenic activity in cancer stem cell populations.

One striking observation in our study is that other small ncRNAs (snRNA, Y RNA, snoRNA, and piRNA) contaminants are clearly more difficult to eliminate, even with our UF-dFBS (Figs 4 and 6). Despite being fully defined, StemPRO media was not completely RNA free, small traces of both miRNA and small ncRNA were detected. This is an important point to consider, as these GMP produced media are intended for clinical stem cell therapy.
Regardless of its source, presence of RNA (vesicular/non-vesicular) in all the tested media should still be considered contamination, which needs careful consideration for further applications. While there is no data readily available, we speculate that possible source of the RNA in these media could be from the recombinant proteins such as human serum albumin, essential amino acids, or vitamin constituents. 

It is likely that RNA detected in our study could have originated from the different components of media supplements. In the future additional studies are needed to formulate an RNA free culture media, where all media components should be precisely screened. This will provide the details about the function and origin of the different RNA components in media.

In conclusion, our results highlight the need and importance of screening of EV- and RNA-free media for the in vitro studies in the EV field, specifically related to the investigation of RNA biomarkers. Furthermore, functional impact of these contaminant RNA in recipient cells need to be addressed to understand their biological roles.

**Materials and Methods**

**FBS.** FBS was obtained from two different suppliers, product details are described in Table 1. FBS obtained from Sigma was referred as FBS-1 and from Gibco as FBS-2.

**EV-depleted FBS preparations.** EV-depleted FBS was prepared from both FBS-1 and FBS-2. Ultracentrifugation and ultrafiltration were used to deplete EVs from FBS. The preparation of UC-dFBS and UF-dFBS from regular FBS was done according Kornilov et al. Briefly, UC-dFBS was prepared by 19 hours UC of regular FBS at 26,000 rpm (121 896 gmax) with SW28 rotor (k-factor 284.7, Beckmann-Coulter). Light-colored top layer of supernatant was filtered with a 0.22μm filter (Millipore Stericup-GP, 0.22μm, polyethersulfone filter). UF-dFBS was prepared by centrifuging regular FBS for 55 min at 3,000 g in Amicon ultra-15 centrifugal filters (ref: UFC910024, 100kDa Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) and collecting of flow-through. FBS-1, UC-dFBS-1 and UF-dFBS-1 refer to FBS obtained from Sigma and FBS-2, UC-dFBS-2 and UF-dFBS-2 refer to FBS obtained from Gibco (Table 1). Commercially available EV-depleted FBS (Exo-FBS™, System Biosciences, EXO-FBS, Mountain View, CA, USA), termed as SBI-dFBS, was used as a control (Table 1).

Flowchart of the study design is illustrated in Fig. 7.

**Defined media.** StemPRO® MSC SFM medium, a fully defined media formulation for culturing mesenchymal stem cells, was used as a serum-free, xeno-free culture media control. For media supplementation, see Table 1.

**EV isolation from FBS preparations and defined media.** EV characterization was performed on EVs isolated by UC due to technical issues related to using exoRNeasy Serum/Plasma Maxi Kit (Qiagen) used for the sequencing study. This issue has been described in our previous article. Briefly, EVs were isolated from 3.5 ml of FBS, UC-dFBS, UF-dFBS, SBI-dFBS, and StemPRO by ultracentrifugation for 2 hours at 26,000 rpm (121 896 gmax, SW28 rotor, 4°C) to collect an EV pellet, which was washed with filtered DPBS (0.1μm filter) and stored in Protein LoBind microcentrifuge tubes (Eppendorf) at −80°C. For small ncRNA sequencing, EVs and RNA were isolated from 3.5 ml of FBS, UC-dFBS, UF-dFBS, SBI-dFBS, and StemPRO by exoRNeasy Serum/Plasma Maxi Kit (Qiagen).

**Nanoparticle tracking analysis.** The number and size distribution of particles in EV samples were analysed using NTA (Nanosight LM14, NanoSight Technology, Salisbury, U.K., http://www.malvern.com) as described previously. Briefly, isolated EVs were diluted in filtered (0.1μm) DPBS to obtain the optimal detection concentration of 10⁶–10⁹ particles/ml, and triplicate videos were recorded. The data was analysed using NTA software.

**Transmission electron microscopy.** Particle morphology was examined using TEM as described previously. Briefly, isolated EVs were diluted in filtered (0.1μm) DPBS, loaded into 200 mesh copper grids and negatively stained with neutral uranyl acetate and embedded in methyl cellulose uranyl acetate mixture.

**Western blotting.** WB was performed as described previously using primary antibodies against anti-transferrin receptor/CD71-H68.4 (#13-6800, Thermofisher Scientific) at 1:1000 dilution and ApoA1 29,30 (kind gift from Matti Jauhiainen) at 1:2000 EVs isolated by ultracentrifugation from equal volumes (20 ml) of each sample were loaded to gels. As a control, protein from adipose tissue derived mesenchymal stem cells, was used as a serum-free, xeno-free culture media control. For media supplementation, see Table 1.

**Small non-coding RNA (small ncRNA) sequencing.** Small ncRNA sequencing was performed by Qiagen (Qiagen, Hilden, Germany). EVs and RNA were isolated using exoRNeasy Serum/Plasma Maxi Kit. EVs were isolated from 3.5 ml of media followed by RNA isolation protocol optimized for serum/plasma (no carrier RNA was added). Library preparation was done using the QIAseq miRNA Library Kit. Out of 12μl of isolated RNA, 5μl
was converted into microRNA NGS libraries. Adapters containing 12nt-long unique molecular indices (UMIs) were ligated to the RNA. Then RNA was converted to cDNA. The cDNA was amplified using PCR (22 cycles) and during the PCR indices were added. After PCR the samples were purified. Library preparation quality control was performed using either Bioanalyzer 2100 (Agilent) or TapeStation 4200 (Agilent). Based on the quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pools were quantified using the qPCR ExiSEQ LNA™ Quant kit (Exiqon). The library pools were then sequenced on a NextSeq500 sequencing instrument according to the manufacturer instructions using 1 × 75 bp reads. Raw data was demultiplexed and FASTQ files for each sample were generated using the bcl2fastq software (Illumina Inc.).

**Data analysis.**  **UMI correction and trimming.** To correct PCR bias with UMI information, raw reads were processed as follows: First, cutadapt 1.1131 was used on each raw read with the adapter sequence to acquire information about the presence of adapter. Only reads that fulfilled the following criteria were kept: (1) read contained adapters, (2) read had at least 16nt insert sequence length, and (3) read had at least 10nt-long UMI sequence. Second, insert sequences with incomplete UMI sequence were extracted as partial-UMI reads. Third, all unique insert + UMI combinations were identified from reads with full-length UMI and collapsed into a single read. Fourth, collapsed full-length UMI reads and partial-UMI reads were combined. After UMI-correction, reads were trimmed using cutadapt output and an in-house script.

**Mapping.** Reads were mapped using Bowtie 2 (2.2.2)32 as follows: First, reads mapped to spike-ins or outmapped (mapped to adapter sequences, polyA and polyC homopolymers, or abundant ribosomal or mitochondrial RNA...
The RNA sequencing data has been deposited to the GEO (accession number GSE120594).

**Data Availability**

The RNA sequencing data has been deposited to the GEO (accession number GSE120594).

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Author Contributions
Bettina Mannerström. The author carried out the experiments, data analysis, performing administrative tasks, and participated in writing of manuscript. Riku O. Paananen. The author contributed by analyzing the data (RNAseq), participating in the writing of the manuscript and proofreading of the manuscript. Ahmed G. Abu-Shahba. The author contributed by performing analyses (Western blot) and proof-reading of the manuscript. Jukka Moilanen. Editing the manuscript and administrative tasks. Riitta Seppänen-Kaijansinko. The author contributed by providing the resources, facilities and financial support to the study, proof-reading of the manuscript. Sippy Kaur. The author designed and supervised the project, interpretations of results, and writing of manuscript. All authors critically read and approved the final version of the manuscript.

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