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Supernatant versus exosomal urinary microRNAs. Two fractions with different outcomes in gynaecological cancers

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MicroRNAs (miRNAs) are key regulatory molecules implicated in fundamental cell processes. Recent investigations have been focused to investigate their diagnostic potential also in various body fluids. Plasma and serum are widely used for these purposes. Urinary miRNAs, as the easily available type of sample, have been explored particularly in urological diseases recently. However, we have shown previously that differential expression of urinary cell-free miRNAs may be observed also in gynaecological cancers, such as ovarian and endometrial cancers. In the present article, we focus on the differences in particular urine cell-free miRNA abundance among different samples including particularly ovarian and endometrial cancers and rare gynaecological diagnoses involved in the study. Using raw abundance miRNA expression data, we confirmed significant up-regulation of miR-92a in ovarian cancer, and significant down-regulation of miR-106b in endometrial cancers. As miR-21 appeared up-regulated in the endometrial cancer similarly as in the verification process, where also miR-106b resulted in significant down-regulation in ovarian cancer, these miRNAs may be good candidates for further evaluation as novel diagnostics.

To find out why supernatant but not exosomal urine miRNAs fraction resulted in significant results in regards to deregulation of expression, we performed a comparison of the same urine samples isolated by these two manners. We show that diagnostic potential of cell-free urinary miRNAs may depend on the urine fraction used for the isolation. While particular urinary miRNAs may be enriched, other may reveal unchanged or diminished expression in the exosomal fraction in comparison with supernatant fraction, giving differences also between cancer and control samples. More research will be needed to further explore which kind of cell-free samples would give better results for diagnostic purposes in various diagnoses using urinary samples and investigating cell-free miRNAs expression. Meanwhile, different urine fractions should be explored for their miRNA expression to establish novel diagnostic urinary miRNA markers.

Key words: ovarian cancer, endometrial cancer, cell-free urine, microRNAs, exosomes, diagnostics

Ovarian cancer is the deadliest gynaecological cancer while endometrial cancer is the most common gynaecological cancer in developed countries [1-2]. Epithelial ovarian cancer accounting for about 90% ovarian cancer cases is typical by a heterogeneous histopathological nature resulting in several subtypes based both on classical histological or molecular classifications [3-4]. Different parts of the gynaecological tract are suspected to play a key role in ovarian carcinogenesis while processes of epithelial to mesenchymal (and vice versa) transition and cancer stem cells may be implicated [5-7]. Two types of endometrial cancer have been recognized, however novel ongoing classifications based on the molecular traits suggest new insights into the endometrial cancer classification [8].

The vast majority of ovarian cancer cases are diagnosed in the late stages resulting in significant decrease in overall survival [1, 9]. Disease recurrence and resistance to chemotherapy are common obstacles in the achievement of the treatment success [10]. In endometrial cancer, the majority of cases are diagnosed based on uterine bleeding, however, there is a proportion of patients which do

not exhibit early disease symptoms. Currently, there is no generally accepted marker that could be used in screening of these two cancers. Similarly, the novel diagnostic markers for the early diagnosis of ovarian and endometrial cancers are still needed [11–15].

With the shift from the tissue and cell lines-based investigations towards the clinically more relevant samples such as body fluids, their diagnostic potential has emerged in many diseases recently. Serum and plasma samples are the most prominent investigated sample types; however urine has attracted several studies seeking novel diagnostic markers or tools for ovarian cancer as well. Different approaches have been applied in search for novel urine diagnostic tools exploring for example proteins such as HE-4 [16] or using novel proteomic methods [17], fluorescence characteristics [18-19], or microRNAs expression (see next).

MicroRNAs (miRNAs) are key regulatory molecules operating in a post-transcriptional regulation of gene expression. Being implicated in the fundamental cell processes, they have been found associated with the pathological states including carcinogenesis [20], and also occurring in various body fluids [21]. However, there is only a limited number of studies focused on body fluids based miRNAs expression available in gynaecological cancers (reviewed in [22]). Only two studies have been published for urinary miRNAs in ovarian cancer recently [23-24], one of these including also the endometrial cancer [23]. In addition to ovarian and endometrial cancers, the cervical cancer represents the third most important gynaecological cancer. As far as we are aware, there has not been any study published for urinary miRNAs in this cancer. The vast majority of urinary miRNAs research has been published in urological cancers such as bladder, prostate and kidney cancers (see [26]). However, it may be assumed that studies will be coming soon also in other cancers, breaking the view that urinary miRNA markers are not suitable for the diagnostic purposes in non-urological diseases. Many obstacles yet prevent introduction of urinary miRNAs as cancer diagnostics regarding particularly methodological aspects and insufficient number of independent studies (see [22]).

As above mentioned, we have explored the novel diagnostic potential using urine miRNAs samples of ovarian cancer and endometrial cancer patients recently [23]. We have found that miRNAs may not only be found in cell-free urine, but may be differentially expressed between the cancer and control samples using supernatant miRNAs, but not the exosomal miRNAs. Using supernatant fractions of urine, we have shown that miR-92a may be up-regulated significantly and miR-106b down-regulated significantly in ovarian cancer. In endometrial cancer, we have found miR-106b down-regulated in endometrial cancer [23].

In the present article, we extended this urinary microRNA research including several other gynaecological diagnoses in addition to ovarian and endometrial cancers. We explored the expression in further analyses showing differences in overall abundance of particular miRNAs across different samples. We compared the same samples isolated both as supernatant and exosomal fractions of urinary miRNAs, showing that these fractions may harbour different diagnostic potential associated with different microRNA content. Elucidating the factors affecting urinary miRNA expression is necessary for further investigations and their interpretations. These factors may have important impact on establishing particular miRNAs as diagnostic markers with respect to the methodology applied in various body fluids and clinical diagnoses associated with the gynaecological tract.

Material and methods

Clinical samples, modes of sampling, and isolation of RNA and qPCR analyses generally follow the description (including detailed clinicopathological assessment) provided previously [23], except for the extended data set with additional diagnoses provided in the present study.

Clinical samples. Briefly, second morning urine of patients with epithelial ovarian cancer and fallopian tube cancer,

Table 1. Summary of clinicopathological data for patients and samples used in the S1 experiment

Type of sample	FIGO stage	Grade	Age at surgery	Metastases status at surgery	Recurrence [1]	Death [1]
Epithelial ovarian cancer						
Serous pre-surgery						
UCB310A	1c	III	53	M0	no	no
UCB318A	3a	III	55	yes, in omentum	yes (10)	yes (12)
UCB417A	3c	III	64	yes, in omentum and peritoneum	no	no
Mucinous (pre-surgery)						
UCB315A	1c	II	72	M0	no	no
Serous post-chemotherapy (disease recurrence)						
UCB425C [2]	3c	III	56	M0	yes (16)	no
Fallopian tube cancer *(pre-surgery)*						
UCB902A	3c	II	77	yes, extensively including omentum	yes (15)	no

[1] month after surgery, [2] five months after surgery, 2 months after chemotherapy (used in [23] in extended data set)

Table 2. Summary of clinicopathological data for patients and samples used in the S2 experiment

Type of sample	FIGO stage	Grade	Age at surgery	Metastases status at surgery	Recurrence [1]	Death [1]
Epithelial ovarian cancer						
Serous pre-surgery						
UCP12	3c	III	68	yes, in peritoneum	n/a	n/a
Serous post-surgery						
UCB318C (7 months after surgery, 8 days after chemotherapy)	3a	III	55	yes, in omentum	yes (10)	yes (12)
Mucinous (pre-surgery)						
UCB315A	1c	II	72	M0	no	no
Mixed endometrioid ovarian cancer/endometrioid endometrial cancer (pre-surgery)						
UCP5	3c	III	57	n/a	n/a	n/a
Endometrial cancer						
Type 1 endometrioid endometrial cancer						
UCP8	1a	II	77	n/a	n/a	n/a
UCP9 (mixed with undifferentiated carcinoma)	n/a	n/a	86	n/a	n/a	n/a
UCP11	3b	n/a	64	n/a	n/a	n/a
UCP13	n/a	n/a	91	n/a	n/a	n/a
UCP15	n/a	II-III	71	n/a	n/a	n/a
Benign samples – ovarian type	-	-		-	-	-
UFB01 (ovarian fibroma)	-	-	66	-	-	-
UCP14 (ovarian thecofibroma)	-	-	74	-	-	-
Benign samples – endometrial type	-	-		-	-	-
UCP7 (endometrial polyp)	-	-	65	-	-	-
UCP10 (endometrial leiomyomas)	-	-	68	-	-	-

[1] month after surgery

endometrial cancer and eventually other gynaecological diagnoses* was collected before the surgery, and partially collected as post-surgery, and post-chemotherapy ovarian cancer samples at the University Hospital Brno (FN Brno, samples designated as UCB) and the Institute of the Care of Mother and Child Prague (ÚPMD Praha – Podolí, samples designated as UCP). Control urine samples came from the Transfusion Department, General University Hospital Prague (VFN Praha) and were provided by healthy blood donor volunteers (post-menopausal women). These samples were used as the reference for post-menopausal patients. Additionally, pre-menopausal women using and not-using hormonal contraception were isolated as pooled urine to observe potential differences. All patients enrolled in the study were Caucasians of the Czech nationality and provided written informed consent. This research was approved by a multicentre Ethical Committee of the General University Hospital Prague. Urine Collection and Preservation Tubes (Norgen Biotek Corp., Canada) ensuring stabilization of urine samples were used for the urine collection. A brief summary of samples and clinical diagnoses used in three experiments (see later) is given in table 1 (S1 experiment), table 2 (S2 experiment) and tables 3a,b (Exosome experiment), Supplementary Table 1 (additional diagnoses in the Exosome experiment), and Supplementary Table 2 (control samples).

Urine processing. The protocol for processing the whole urine (Urine Total RNA Purification Maxi Kit, Slurry Format, Norgen Biotek Corp., Canada) was followed according to manufacturer instructions with some modifications ensuring isolation of cell-free urine fractions. Methods are described in detail in [23]. Briefly, in the Supernatant-1 experiment (further S1 experiment), urine samples were centrifuged for 10 minutes at 100 x g at RT, and then supernatant was centrifuged at 500 x g for 10 minutes at RT. In the Supernatant-2 (further S2 experiment), urine samples were centrifuged for 10 minutes at 300 x g at 4°C, and then supernatant was

**Note 1: Fallopian tube is very closely related to ovarian cancer (e.g., in the annual SEER Cancer Statistics Review reports of the National Cancer Institute, Bethesda, USA, it is treated with ovarian cancer), therefore in some analyses this diagnosis was processed within ovarian cancer set. Note 2: Krukenberg carcinoma of ovary is a secondary cancer coming primarily from other body tissues, particularly of gastrointestinal tract. In our case, the origin could not be evaluated. Note 3: Malignant diffuse large B-Cell lymphoma infiltrating extensively ovaries and fallopian tubes was included in our clinical sample set. In these analyses, we included it as a reference pathological material and for illustration of different miRNAs pattern in urine of this patient.*

Table 3a. Summary of clinicopathological data for patients and samples used in the Exosome experiment (ovarian and fallopian tube cancers)

Type of sample	FIGO stage	Grade	Age at surgery	Metastases status at surgery	Recurrence [1]	Death [1]
Epithelial ovarian cancer						
Serous (pre-surgery)						
UCB318A	3a	III	55	yes, in omentum	yes (10)	yes (12)
UCB417A	3c	III	64	yes, in omentum and peritoneum	no	no
UCP12	3c	III	68	yes, in peritoneum	n/a	n/a
UCB322A (13 days after neoadjuvant chemotherapy)	3a	II	68	yes, in peritoneum	n/a	yes (9)
Serous (post-surgery)						
UCB417B	3c	III	Ibid.	Ibid.	Ibid.	Ibid.
UCB322B (23 days after neoadjuvant chemotherapy)	3a	II	Ibid.	Ibid.	Ibid.	Ibid.
Serous post-chemotherapy (disease recurrence)						
UCB318B [2]	3a	III	Ibid.	Ibid.	Ibid.	Ibid.
UCB318C [3]	3a	III	Ibid.	Ibid.	Ibid.	Ibid.
Mucinous (pre-surgery)						
UCB315A	1c	II	72	M0	no	no
Mixed endometrioid ovarian cancer/endometrioid endometrial cancer (pre-surgery)						
UCP5	3c	III	57	n/a	n/a	n/a
Fallopian tube (pre-surgery)						
UCP1	3c	II	62	n/a	n/a	n/a
Fallopian tube (post-surgery)						
UCB902B	3c	II	77	yes, extensively including omentum	yes (15)	no

[1] month after surgery, [2] chemotherapy 7th day after surgery, sampling B here 10th day after surgery, [3] 7 months after surgery, 8 days after chemotherapy

Table 3b. Summary of clinicopathological data for patients and samples used in the Exosome experiment (endometrial cancer and benign samples)

Endometrial cancer	FIGO stage	Grade	Age at surgery
Type 1 endometrioid endometrial cancer			
UCP8	1a	II	77
UCP9 (mixed with undifferentiated carcinoma)	n/a	n/a	86
UCP11	3b	n/a	64
UCP13	n/a	n/a	91
UCP15	n/a	II-III	71
UCP16	3a	I	61
UCP17	n/a	II	55
UCP19	n/a	I	57
UCP21	n/a	I	64
Type 2 endometrial cancer			
UCP22	n/a	III	51
Benign samples – ovarian type			
UFB01 (ovarian fibroma)	-	-	66
UCP2 (ovarian thecofibroma)	-	-	68
UCP14 (ovarian thecofibroma)	-	-	74
Benign samples – endometrial type			
UCP7 (endometrial polyp)	-	-	65
UCP10 (endometrial leiomyomas)	-	-	68
UCP18 (intramural tumor)	-	-	53

centrifuged at 2,000 x g for 20 minutes at 4°C. Initial input urine volume was 2 ml in S1 and S2 experiments. *Note: this supernatant is not exosomes-free. We use the term supernatant, but this should not be confused with the exosome-depleted supernatant.*

In the Exosome experiment, urine samples were processed following manufacturer protocol for Urine Exosome RNA Isolation Kit (Norgen Biotek Corp., Canada). Initial mean input urine volume was 3 ml. Prior to isolation, urine samples were centrifuged at 1,000 x g, for 10 minutes at RT and stored at 2°C – 8°C until further processing. Two additional centrifugations were then applied, the first at 300 x g, for 10 min., at 15°C, and the second at 2,000 x g, for 10 minutes, at 15°C. Next, supernatant was filtered through a sterile, 0.2 µm PVDF filter (Whatman Puradisc 13mm, GE Healthcare Life Sciences) to 15-ml tube to ensure isolation of vesicles up to 200 nm.

Quality and quantity control of RNA. Nanodrop 1000 spectrophotometer (Thermo Scientific), and Agilent 2100 Bioanalyzer with Agilent RNA 6000 Pico Kit (alternatively Agilent RNA 6000 Nano Kit, Agilent Small RNA kit) (Agilent Technologies), and Qubit fluorometer (Life Technologies) were employed for assessing quality and concentration of samples.

Detection of expression of microRNAs. Individual TaqMan microRNA assays (Life Technologies) were applied in real-time PCR analyses. Single-stranded cDNA was generated

from total RNA using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) following manufacturer´s protocol and scaled-down (1/2, total volume 7.5 μl) reaction volumes. For each sample, the same input of total RNA volume was used. List of microRNA assays used in the experiments is provided in Supplementary Table 3. In the Exosome experiment, RNA input volume in the reverse transcription was increased to 4.6 μl (in relation to manufacturer´s protocol), and cDNA input increased to 1.85 μl in the amplification reactions (in relation to manufacturer´s protocol). PCR amplifications were performed in scaled-down reactions (1/2, total volume 10 μl) in triplicates (S1 and S2 experiments), or duplicates (Exosome experiment) on the Applied Biosystems 7900HT thermocycler (Life Technologies). No-template controls, no-real-time PCR controls, and inter-plate controls were included in the analyses.

Data processing and statistical analyses. Expression data were captured using SDS 2.4 and RQ 1.2.1 software and processed using primarily Real-time PCR Miner to obtain efficiency-corrected expression data. Relative expressions of microRNAs were calculated as follows. First, adjusted efficiency was calculated. It was 1 + mean efficiency of genes (Real-time PCR Miner), or 2 in Cy0 and delta Ct methods. Next, this adjusted efficiency was powered to Ct (i.e., (1+E)^Ct in Real-time PCR Miner data), or using Cy0, or Ct value.

Geometric mean was calculated for each sample and all miRNAs investigated, and used as the normalization factor in parts calculating logistic regression in preliminary ROC analyses in ovarian and endometrial cancers in S1 and S2 experiments, and in part 3.2 comparing S2 experiment data with Exosome experiment data. Cy0 and delta Ct methods were applied within the verification process (see [23] and Supplementary File Verification). Expression data were log-transformed prior to statistical analyses.

Expression was also calculated as the percentage abundance across the miRNAs investigated within the experiments as the alternative normalization used in the present study. Here, the total miRNA expression was calculated as the sum of the normalized individual miRNAs (i.e., 100%), and a proportion for each miRNA was obtained by dividing its expression by the total expression of selected miRNAs (raw data are presented and applied in statistical processing). Abundance data were not log-transformed in the statistical processing.

Statistical analyses (Mann-Whitney test, Wilcoxon test or paired samples t-test, multivariate regression model) were performed using MedCalc Statistical Software (p-value was set as 0.05).

Results

Urine microRNAs expression in the S1 experiment. As we have shown previously [23], two miRNAs were found to be up-regulated significantly (miR-92a, miR-200b), and two miRNAs down-regulated significantly (miR-106b, miR-100) in comparison of ovarian cancer samples (UCB310A, UCB315A, UCB318A, and UCB417A) plus fallopian tube cancer (UCB902A) and controls in S1 experiment. The investigated miRNas were miR-21, miR-223, miR-92a, miR-200b, miR-16, miR-29a, miR-367, miR-106b, miR-100, miR-20a and miR-1228. Up-regulation of miR-92a, miR-200b and down-regulation of miR-106b was found in sample sets involving only serous ovarian cancers and serous ovarian cancers along with fallopian tube cancer, and extended serous ovarian cancer set including post-chemotherapy sample UCB425C (as this patient suffered from recurrence). In all these mentioned combinations, miR-100 could not be confirmed as down-regulated significantly. Similarly, further extending data set with UCP 5 and UCP 12 ovarian cancer samples resulted in a confirmation of results for miR-106b and miR-200b (miR-92a was not assessed in S2 experiment), but not for miR-100 (see [23]). As regards miR-200b, this miRNA could not be confirmed as up-regulated significantly in the verification process (alternative processing algorithms and normalization procedures, see Supplementary File Verification).

In the present study, we performed several other analyses to further explore the data of the S1 experiment. First, we tested combined expression of two selected miRNAs pairs (ratio miR-92a/106b, miR-92a/miR-100) in comparison of ovarian cancer samples (UCB310A, UCB315A, UCB318A, and UCB417A) plus fallopian tube cancer (UCB902A) and controls. In these miRNAs ratio-based combinations, there was a significant difference between pre-surgery cancer samples and controls (P = 0.0027). *Note: This ratio-based combination of miRNAs expression differs from the combined expression calculated in the MedCalc module (see later).*

We next sought, whether diagnostic accuracy in terms of correct classification within ROC analysis of the obtained data indicates the promising diagnostic potential of the miRNAs investigated. We performed preliminary tests (more samples should be included to be more conclusive) on correctness of classification and ROC analysis using logistic regression module (MedCalc). These results confirmed the exceptional position of down-regulated miR-106b (AUC 0.969, 95% CI 0.764 to 1.000) and miR-100 (AUC 0.846, 95% CI 0.601 to 0.970), and up-regulated miR-92a (AUC 1.000, 95% CI 0.815 to 1.000) and miR-200b (AUC 1.000, 95% CI 0.782 to 1.000) as it was discovered in previous analyses, also as good classifiers for cancer samples (Supplementary Table 4). We applied a multivariate regression model and combined expression data for miR-106b/miR-100, and miR-100/miR-92a. This approach improved correct classification to 100% and AUC to 1.000 (Supplementary table 4).

Further, we analysed the microRNAs abundance and differences in proportions between the pre-surgery cancer and control samples within the applied miRNAs set (expression of all the miRNAs investigated was set to 100%). This is to illustrate the different relative proportions of particular miRNAs within urine samples, which cannot be observed in otherwise

presented data (e.g. fold differences, or normalized expression). We also used this approach to test the performance when analysing raw abundance data (%) in comparison with previously applied standard processing procedures (normalized data) and verifications.

Using raw abundance data (%), two miRNAs (miR-106b and miR-1228) appeared to be the most abundant miRNAs, both with a decrease observed in cancer samples. Other investigated miRNAs had remarkably decreased relative proportion in samples. Relative miRNA abundances in S1 experiment are depicted in Figure 1. Fold-differences of cancer samples and control samples based on percentage abundance are given in Supplementary Figure 1.

Interestingly, when the raw abundance data (not log-transformed) were analysed statistically between ovarian cancer (additionally also with fallopian tube) and control samples, three miRNAs, i.e. miR-92a (P = 0.0013 incl. tube, P = 0.0032 excl. tube), miR-200b (P = 0.0013 incl. tube, P = 0.0032 excl. tube) and miR-16 (P = 0.0433 incl. tube, P = 0.0894 excl. tube, here non-significantly) appeared significantly up-regulated, however miR-106b was not found significantly down-regulated (even after the log-transformation of data). Expression of miR-92a was found up-regulated in concordance with normal data processing and verifications. However, it should be noted that miR-106b was found down-regulated both in standard data processing and confirmed in data verifications (see Supplementary File Verification). In case of miR-200b, this was found up-regulated partially in the verification, while miR-16 was not found de-regulated in the verification.

In the S2 Experiment, the results based on relative (percentage) abundance appeared in congruence with the other results (see later). It may indicate that using percentage abundance may be congruent with other results in some cases, but it may be possibly affected by normalization procedures (e.g., in our case of miR-106b geo-mean normalization in standard procedure in contrast to raw abundance data). Combination of various procedures should be preferred to obtain more comprehensive and reliable picture on expression de-regulations. Nevertheless, we intended to show these data both for the overall abundance overview and their potential to exhibit clear differences in expression when a relative small set of miRNAs is being investigated.

Urine microRNAs expression in the S2 experiment. Differences in microRNA expression (miR-21, miR-223, miR-200b, miR-16, miR-29a, miR-367, miR-106b, miR-100, miR-20a and miR-1228) between ovarian cancer, endometrial cancer, and control samples have been studied previously [23]. In both cancer groups, only the down-regulated expression of miR-106b was statistically significant. In various combinations of ovarian cancer samples, miR-106b retained capacity to discriminate cancer samples from control samples: a) UCB315A, UCB318C (post-surgery, post-chemotherapy), UCP12, and mixed ovarian and endometrial cancer UCP5 (P = 0.0036), b) UCB315A, UCB318C, UCP12 (P = 0.0094) and c) UCB315A and UCP12 (P = 0.0285) [23].

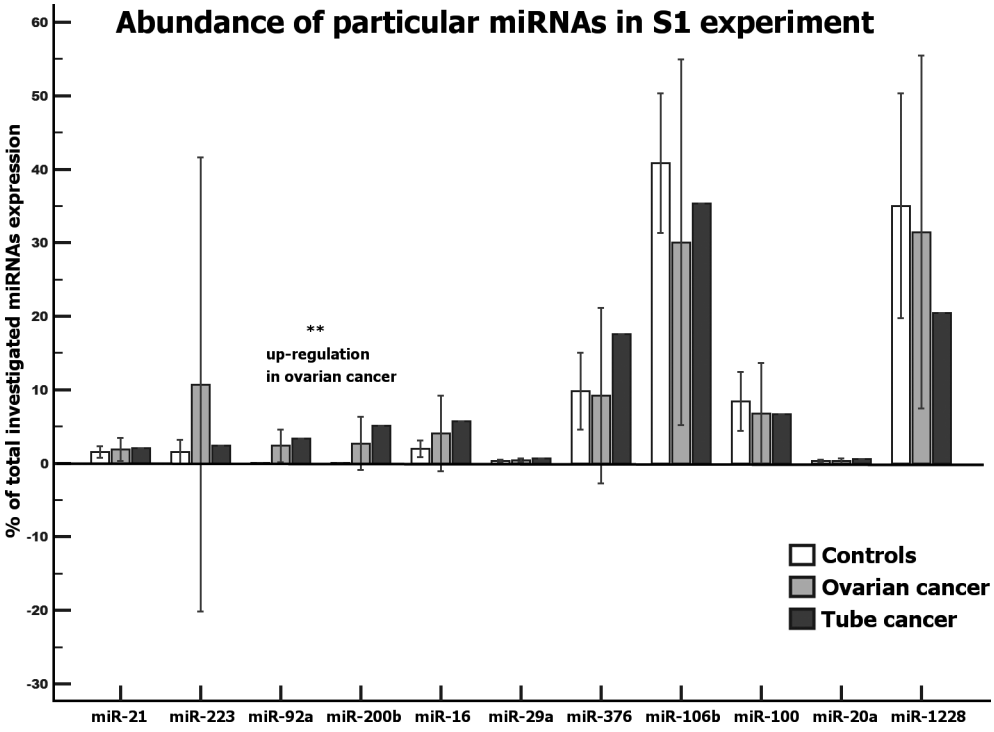

Figure 1. Percentage abundance of particular miRNAs within the S1 experiment including ovarian cancer, fallopian tube cancer and controls. Error bars indicate 95% CI for the mean.

In a preliminary logistic regression model applied in the present study in ovarian cancer (samples UCB315A, DCUCB318C, DCUCP5, UCP12), miR-106b was able to classify correctly 100% cases, with good performance found in preliminary ROC analysis (AUC 1.000, s.e. 0.000, 95% C.I. 0.794 to 1.000). Multiple logistic regression model (MedCalc) was then applied to identify combinations of miRNAs to improve the percentage of a correct classification. Here, a combination of miR-100 and miR-367 resulted in 86.67% correct classification (AUC 0.944, s.e. 0.0648, 95% C.I. 0.696 to 0.999).

In endometrial cancer (samples UCP8, UCP9, UCP11, UCP13, UCP15) and controls, a preliminary logistic regression model confirmed the good classification status for miR-106b (88.24% correct classification, AUC 0.983, s.e. 0.0261, 95% C.I. 0.777 to 1.000). Multiple logistic regression model (MedCalc) revealed a combination of miR-106b either with miR-21, miR-200b, or miR-29a to improve correct classification to 100% (AUC 1.000, s.e. 0.000, 95% C.I. 0.805 to 1.000 in all the combinations). Another combination, miR-21 with miR-29a was also acceptable (correct classification 88.24%, AUC 0.967, s.e. 0.0415, 95% C.I. 0.750 to 1.000). It should be emphasized that more samples should be included to provide ROC analyses more reasonable and conclusive. Thus, these data should be considered particularly as illustrative.

Fold-differences identified in pathological samples in contrast to controls based on percentage abundance are illustrated in Supplementary Figure 2.

Next, we explored the microRNAs abundance and differences in proportions between the pre-surgery cancer and control samples within the applied miRNAs set (expression of all the miRNAs investigated was set to 100%). In control samples, miR-106b and miR-1228 appeared as the most abundant (Figure 2). In pathological samples, the abundance of particular miRNAs varied extensively, but miR-1228 was expressed most abundantly (see Figure 2).

Similarly as in S1 Experiment, we explored the performance of raw abundance data (%) in statistical analyses of S2 Experiment. In ovarian cancer (samples 315A, 318C, UCP5, UCP12), we found miR-106b significantly down-regulated (P = 0.0153), along with miR-367 significantly up-regulated in ovarian cancer samples (P = 0.0203). In case of miR-106b, this result is congruent with standard normalized and log-transformed data and verifications. However, in miR-367 this could not be confirmed in either alternative data processing previously (see Supplementary File Verification).

In endometrial cancer (samples UCP 8, 9, 11, 13, 15), we found miR-106b significantly down-regulated (P = 0.0061), while miR-21 was shown up-regulated significantly (P = 0.0350). In case of miR-106b, the results are corresponding with standard normalized and log-transformed data and verifications. In miR-21, this result is in line with different verification procedures (see Supplementary File Verification).

Differences between cancer groups and controls using exosomal miRNAs. We could not find any significant dif-

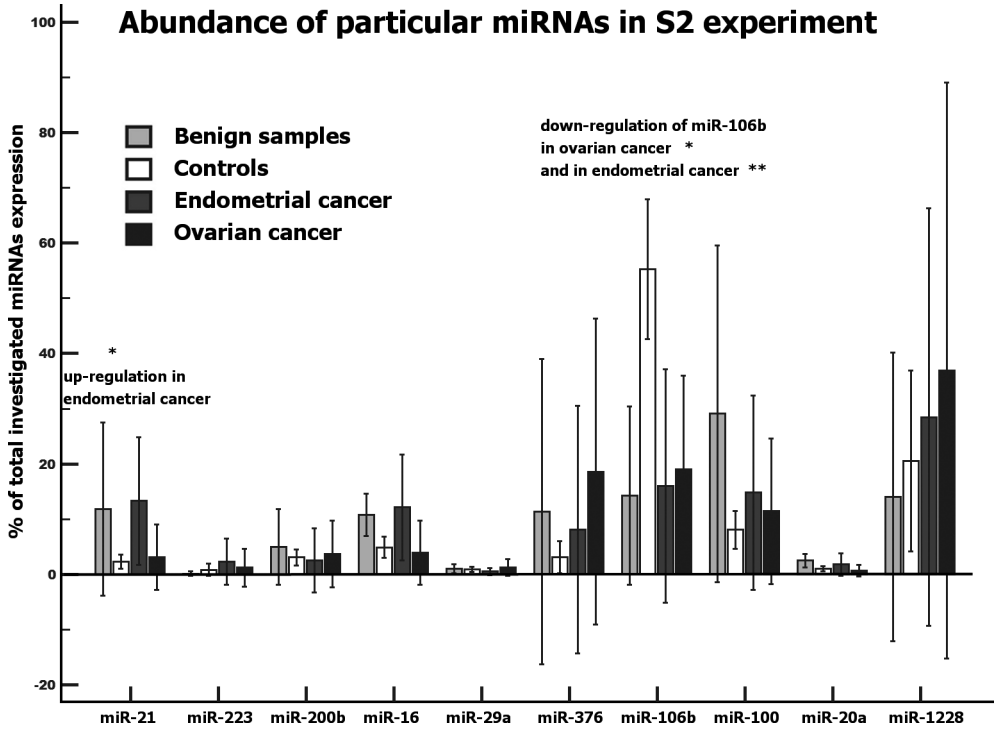

Figure 2. Percentage abundance of particular miRNAs within the S2 experiment including ovarian cancer, endometrial cancer, benign and control samples. Error bars indicate 95% CI for the mean.

ferences between pathological samples (particularly ovarian and endometrial cancers) and control samples using exosomal miRNAs, while using supernatant miRNAs the results were more promising (see above and in [23]). This may implicate that different isolation procedures and/or the differences in the miRNA content themselves may affect the results so that one methodology (supernatant) may be superior to another methodology (exosomes) in particular miRNAs. In our case, these effects resulted in the impossibility to find significant deregulation in miRNA expression in any combination of pathological samples and controls using exosomal miRNAs. However, with respect to a limited number of samples and a selection of several candidate miRNAs, we cannot conclude that exosomal miRNAs are generally less suitable for diagnostic purposes. In the expression analyses, exosomal miRNAs were expressed; however, many missing values affected the analyses negatively. However, we did observe differences in miRNAs abundance and fold differences in their expression between the pathological samples and controls (Figure 3), and Supplementary Table 5 (ovarian cancer), Supplementary Table 6 (endometrial cancer), Supplementary Table 7 (other diagnoses), Supplementary Table 8 (controls), and Supplementary Table 9 (fold differences to control samples). In additional diagnoses (Supplementary Table 1), insufficient number of cases for one particular diagnosis disabled to perform reasonable statistics (pathological samples versus controls), in contrast to ovarian cancer, endometrial cancer, and benign samples. Therefore, these data are treated here only as differences in the percentage abundance and fold differences.

In our pilot study, we could not find statistically significant differences between pre- and post-surgery ovarian cancer samples using supernatant fractions [23]. However, this does not mean that the differences do not exist at all. As regards these effects in exosomal miRNAs, we were limited by a low number of available samples. However, we observed significant increases and decreases between the pre- and post-surgery samples of malignant granulosa tumor of ovary (UCB331, see Supplementary Table 7). We observed also the changes in expression within the course of therapy in UCB322 (see Supplementary Table 7).

With respect to generally limited information on urinary miRNAs regarding the menopausal status of patients, we included in the Exosome experiment also two pre-menopausal groups of patients (using and not using the hormonal contraception, pooled urine samples isolated), in addition to post-menopausal control patients used as controls for post-menopausal pathological samples in all experiments. Interestingly, the preliminary data showed differences involving decreases and increases in relative expression and percentage abundance (see Supplementary Table 8). The limited number of samples does not allow making definite conclusions. However, it is probable that hormonal status of patients may affect the results and should be considered while involving patients as controls in the miRNAs studies based

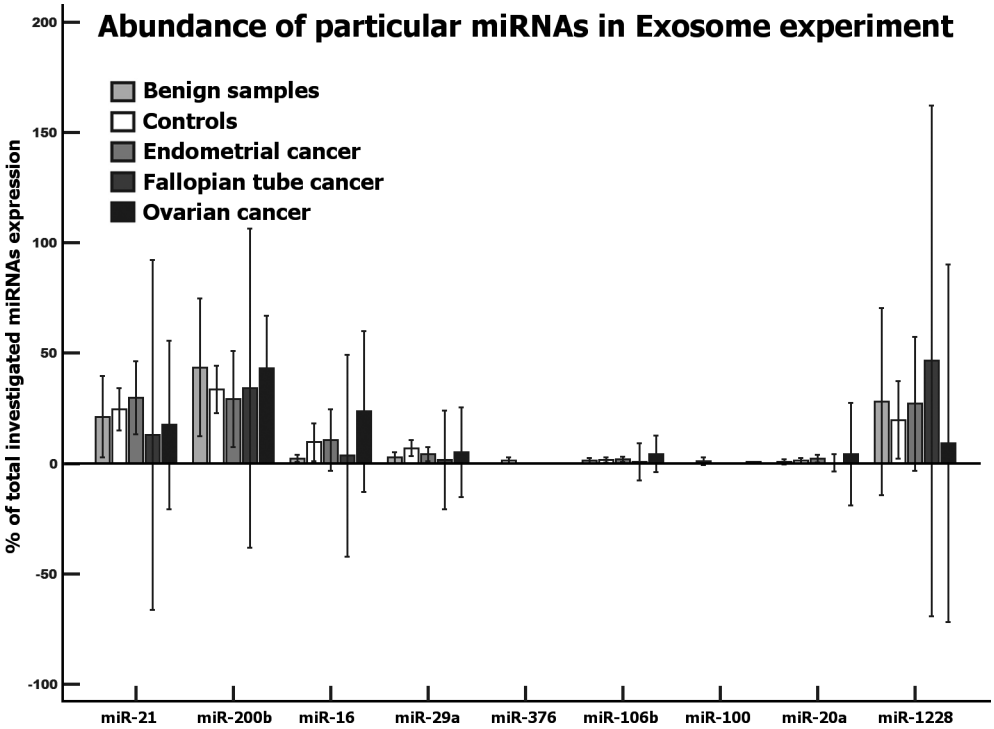

Figure 3. Percentage abundance of particular miRNAs within the Exosome experiment including ovarian cancer, endometrial cancer, benign and control samples. Error bars indicate 95% CI for the mean.

on body fluids. Further research is warranted to elucidate these factors in detail.

Despite interesting expression pattern of exosomal urinary miRNAs, the differences could not be evaluated as the significant changes between cancer and control samples. Therefore, we decided to further inspect the differences between the same samples isolated either as supernatant, or exosomal fraction of urine. As supernatant fraction yielded promising and significant diagnostic differences in contrast to exosomal fraction, we expected that the differences between the fractions may be attributed to a different composition of fractions rather than to be a technical issue. The results of this approach are presented in the next section.

Impact of isolation procedure on results: S2 experiment versus Exosome experiment. We compared results from two experiments using the supernatant (S2 experiment) and exosomal RNA (Exosome experiment) coming from the same urine samples and geometric mean-normalized data. When analysing pathological samples (UCP5, UCP7 to UCP15, n = 10) isolated in S2 and Exosome experiments, there was miR-200b up-regulated in the exosomal fraction ($P = 0.0078$, Supplementary Figure 3), and miR-16 ($P = 0.0020$, Supplementary Figure 4) along with miR-106b ($P = 0.0078$, Supplementary Figure 5) down-regulated in the exosomal fraction, other four tested miRNAs (miR-21, miR-29a, miR-20a, and miR-1228) remained unchanged statistically.

We analysed also the performance of control samples (UN9, 10, 11, 13, 32, 33, 41, 42, 44, n = 9) in a comparison of S2 and Exosome experiments. Here, we found higher expression of miR-21 ($P = 0.0006$, Supplementary Figure 6), miR-200b ($P = 0.0005$, Supplementary Figure 7), and miR-29a ($P = 0.0032$, Supplementary Figure 8) isolated as exosomal miRNAs in comparison with S2-isolated samples. miR-106b appeared as down-regulated in exosomal fraction ($P < 0.0001$, Supplementary Figure 9) while miR-20a, miR-16 and miR-1228 did not differ significantly between S2 and Exosome experiments.

Discussion

Understanding the mechanisms affecting the microRNAs expression and detection belongs to crucial factors necessary for an appropriate interpretation of results of the expression studies. The source of RNA samples may be one of the most important factors. Circulating miRNAs in blood and in other body fluids such as urine may reflect the impact of many cells and body compartments contributing to a global miRNA expression (reviewed in [22]). Therefore, it is very difficult to ascertain the particular source of hundreds of miRNAs and their clear relationship to a pathological state such as cancer.

Many miRNA expression studies have been focused on the expression of exosomal miRNAs, particularly in plasma. However, it should be emphasized that there is a limited number of studies that identify differences between cell-free fraction and exosomes with respect to the differences in miRNA content and expression. Turchinovich et al. [27] have suggested that only a very limited proportion of plasma miRNAs is distributed in vesicles such as exosomes. On the contrary, they consider that circulating extracellular miRNAs are mostly (90% to 95%) microvesicle-free and associated with the RNA-binding Argonaute proteins [28].

On the other hand, Cheng et al. [29] indicated both the impact of isolation procedure on exosomal miRNA fraction amount estimates, and also on their expression, in a preliminary study on three patients and their plasma/serum. Comparison of cell-free plasma/serum samples and exosomes isolated by ultracentrifugation or by the commercial kit revealed an enrichment of exosomes with miRNA. The authors also observed high variation in proportions of particular miRNAs in exosomal fractions isolated by the two methods.

As regards urinary miRNAs, there is very limited knowledge on their exact origin as relevant *in vivo* studies are still lacking. Transrenal passage of the miRNAs from the blood could be suspected along with other sources involved in cell-cell communication or passive leakage from the injured or dead cells [30]. Within the vast majority of papers on urine miRNA expression, the focus has been put on the diseases with a direct association with the urological system affected either by cancer or organ dysfunctions and injuries. The cells within the tract and associated with the tumor tissues have been mostly considered as the basis for the assessment of deregulated expression or considered as the basic source of differentially expressed cell-free miRNAs [31-34].

However, urinary miRNAs have been explored in several other diagnoses recently. For example, urinary miR-1 has been shown as the marker for acute myocardial infarction [35], or along with serum as the marker suitable for monitoring open-heart surgeries with cardiopulmonary bypass [36]. Down-regulated expression of miR-203 in urine has been identified as the biomarker for the severity of inflammation in children with atopic dermatitis [37]. The study focused on forty-eight dyspnea STAMPEDE subjects and analyzing bronchoalveolar lavage (BAL), urine and serum identified several candidate miRNAs. For example, miR-371a-5p was found to be similarly overexpressed in BAL, urine and serum [38]. Very recently, Erbes et al. [39] investigated expression of several candidate urinary miRNAs in breast cancer. They sustained the potential role of urinary miRNAs as non-invasive innovative biomarkers for breast cancer detection.

Similarly as a handful of above-mentioned studies, we have suggested a theory that the pathological states may be reflected not only in the blood but eventually in cell-free urine also in other types of diagnoses than those related to urogenital tract. In our case, we focused on gynaecological cancers. As a proof of principle, we have performed a pilot study based on urine of ovarian and endometrial cancers, identifying several candidate miRNAs with a novel diagnostic potential [23]. However, there exist several constraints that should be taken into consideration, e.g. a limited number of patients and candidate miRNAs investigated.

To investigate the differences between supernatant and exosomal fractions, we performed the above-mentioned experiments. There exist, however, several limitations, such as the fact that the supernatant still contained the exosomes and was not exosome-depleted, limited number of samples, one methodology to isolate exosomes. Based on our results, we may conclude that the alternations in expression rather reflect the real differences between the two fractions. Different miRNAs may reveal different expression levels in comparison of the supernatant fraction (S2 experiment) with exosomal fraction (Exosome experiment). The support for this assumption may be found in the distinct expression pattern in miRNAs investigated, including all the changes possible, i.e. increased, decreased and unchanged expression, differing also between the pathological and control samples. This may suggest that particular miRNAs may be enriched or diminished in exosomal fraction of miRNA. It is congruent at least partially with another observation in the preliminary study of Cheng et al. [40] suggesting the enrichment of miRNAs in urinary exosomes in contrast to cell-free urine. The authors also observed differences in the representation of the most up-regulated miRNAs between different methods of exosomal RNA isolation and in contrast to cell-free miRNAs [40].

Conclusions

We have demonstrated that miRNAs may be found deregulated in cell-free urine of ovarian cancer and endometrial cancer patients. We further explored the differences between the supernatant fraction and exosomal fraction of urine. The aim was to elucidate why supernatant fraction but not the exosomal fraction has exhibited a diagnostic potential. The differences between the fractions regarding the miRNA content may be suspected as one of the main reasons for these observations. There is currently no consensus whether the exosomes or circulating supernatant-based miRNAs should be preferred in investigations searching for novel diagnostic miRNAs. Which portion of the circulating miRNome (supernatant versus exosomal RNA) represents more truly the pathological states and performs better in diagnostic applications thus should be further elucidated.

Our results might indicate that the source of miRNAs should be carefully considered as it may result in the differences in diagnostic performance of particular miRNAs. Moreover, the differences we have observed between three groups of female patients (pre-menopausal using and not using contraceptives, post-menopausal) may indicate the impact of patient´s hormonal status on the miRNAs expression in urine. Similarly, other factors such as the isolation procedures, normalization, biological variation, along with the impact of external factors such as food and smoking suggested recently to affect miRNA expression (see [22] for a review) should be further taken into consideration when exploring the diagnostic potential of cell-free miRNAs, either coming from supernatant and/or exosomal fractions of body fluids. We propose that different isolation techniques using supernatant, exosomes-depleted supernatant, and exosomal fractions of urine should be now explored to establish novel diagnostic urinary miRNA markers in terms of the best diagnostic accuracy for gynaecological cancers. Many obstacles thus remain to be solved prior to introducing cell-free urinary miRNAs as novel diagnostic tools.

Supplementary information is available in the online version of the paper.

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Supplementary Information

Supernatant versus exosomal urinary microRNAs. Two fractions with different outcomes in gynaecological cancers

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Verification of the S1, S2 and Exosome experiments

In order to eliminate potential bias resulting from a normalization procedure, alternative approaches were applied in addition to geometric mean normalization used previously. Using BestKeeper and NormFinder Excel-based applications, miRNAs were selected for normalization. As the second approach applied in the verification, alternative real-time processing algorithms (Cy0, delta Ct) were used. Here, detailed results of alternative processing applied in the miRNAs expression data verification are provided.

Verification of S1 experiment

- *Verification of S1 experiment by miR-29a normalization (Real-time PCR Miner data included)*

Using above mentioned algorithms, miR-29a was selected as the normalization gene for S1 experiment. We could confirm exceptional position of miR-92a ($P < 0.0001$), miR-200b ($P = 0.0051$), and miR-100 ($P = 0.0114$) the three miRNAs which differed between ovarian cancer and controls significantly. miR-106b gave tightly insignificant results ($P = 0.0731$).

- *Verification of S1 experiment by Cy0 method*

Expression data as the Cy0 values (ovarian cancer samples included, here without fallopian tube) normalized to geometric mean confirmed significant up-regulation of miR-92a ($P = 0.0042$), and down-regulation of both miR-106b ($P = 0.0114$) and miR-100 ($P = 0.0452$). Including fallopian tube into ovarian cancer samples, only miR-92a ($P = 0.0042$) and miR-106b ($P = 0.0069$) were significantly de-regulated.

- *Verification of microRNAs expression in S1 experiment by delta Ct method*

First, geometric mean normalized data were used and ovarian cancer samples excluding fallopian tube sample were analyzed. Up-regulated expression of miR-92a ($P = 0.0120$), and down-regulated expression of both miR-106b ($P = 0.0341$) and miR-100 ($P = 0.0341$) were confirmed. De-regulated expression of miR-200b could not be confirmed similarly as in Cy0 verification. In addition to above mentioned de-regulated miRNAs, miR-223 was found significantly up-regulated ($P = 0.0036$). Next, normalization by miR-29a selected according to NormFinder was applied. This normalization affected the results. Even though confirming up-regulation of miR-92a ($P = 0.0014$) and miR-223 ($P = 0.005$), and down-regulation of miR-100 ($P = 0.0433$), miR-106b was not de-regulated significantly.

Verification of S2 experiment

- *Verification of S2 experiment by miR-16 normalization (Real-time PCR Miner data included)*

Data normalized to miR-16 identified as the best choice for the S2E experiment normalization confirmed significant down-regulation of miR-106b in endometrial cancer. In addition to this finding, up-regulated expression of miR-21 ($P = 0.0204$), and down-regulated expression of miR-29a ($P = 0.0153$) were found for this cancer. In ovarian cancer (samples UCB318C and UCP12 included), the down-regulation of miR-106b was confirmed ($P = 0.0285$) similarly as in endometrial cancer. In contrast to endometrial cancer, miR-29a was found to be significantly up-regulated ($P = 0.0285$) in ovarian cancer.

- *Verification of S2 experiment by Cy0 method*

Cy0 analyses resulted in many missing expressions complicating the statistical analyses. Using geometric mean normalized data including zero values, no significant de-regulation could be found in ovarian cancer. In endometrial cancer no miRNA was found significantly deregulated using geometric mean normalized data. Alternatively, all missing values were omitted and data were normalized to miR-1228 selected according to NormFinder. No significant deregulation was found in ovarian cancer. In endometrial cancer, there was only miR-21 found de-regulated significantly (up-regulated in cancer samples, P = 0.0304).

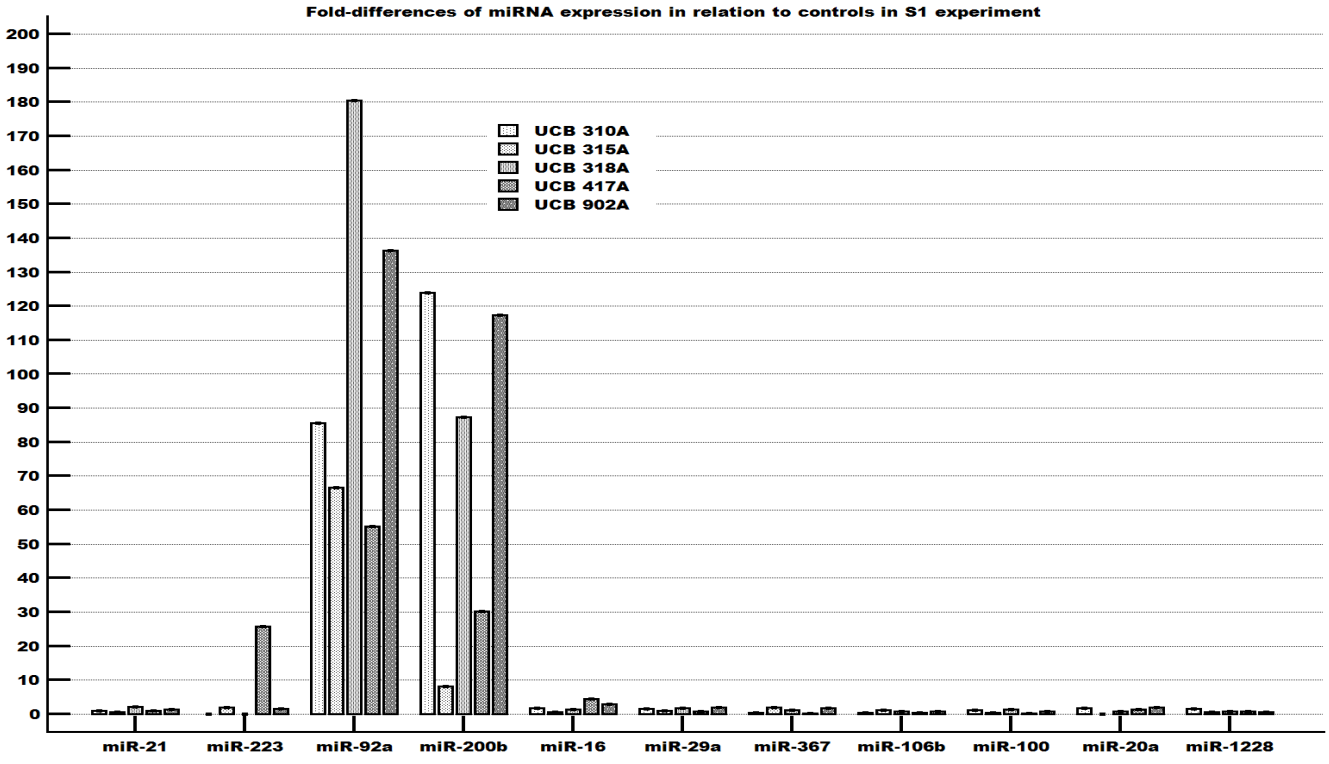
- *Verification of S2 experiment by delta Ct method*

First, geometric mean normalized data were used. No significant de-regulation was found in ovarian cancer. We next confirmed that miR-106b was down-regulated significantly (P = 0.0268) in endometrial cancer samples. Tight results were obtained for miR-21. Here, when applying strict criteria on data normality (one out of four normality tests failed) and non-parametric test, the results were insignificant (P = 0.0578). Applying alternatively the parametric test, the results become significant (P = 0.0450). No other miRNAs appeared de-regulated significantly. Then, based on NormFinder findings, normalization factor as the geometric mean of miR-21 and miR-20a was calculated and used for data normalization. Using this approach the down-regulation of miR-106b in endometrial cancer was confirmed (P = 0.0032). Also significant de-regulations were observed in miR-29a. Here, this miRNA appeared down-regulated in endometrial cancer (P = 0.0001), and up-regulated in ovarian cancer (P = 0.0141). This state was able to distinguish the both cancer groups (endometrial versus ovarian cancers, P = 0.0253). No other miRNAs were shown as to be de-regulated significantly in this verification.

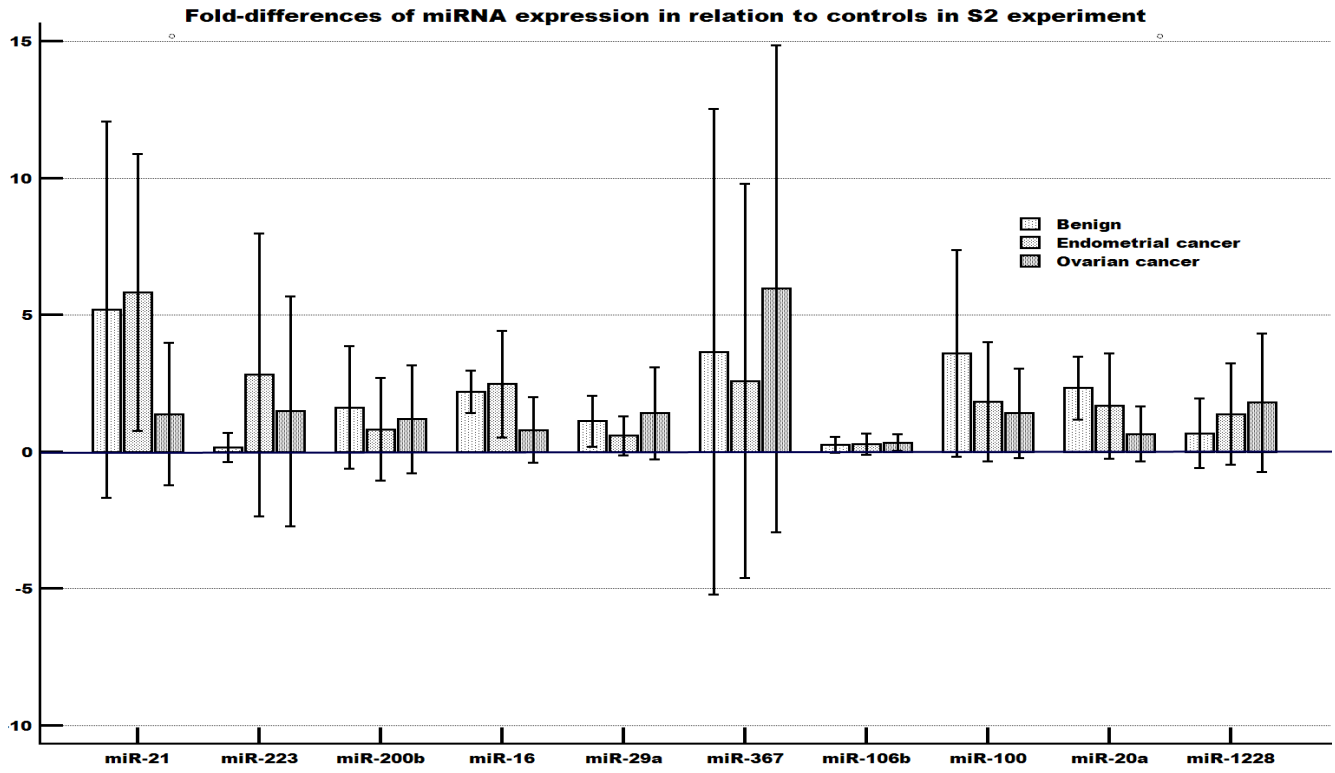
Verification of Exosome experiment

- *Verification of Exosome experiment by miR-16/106b normalization*

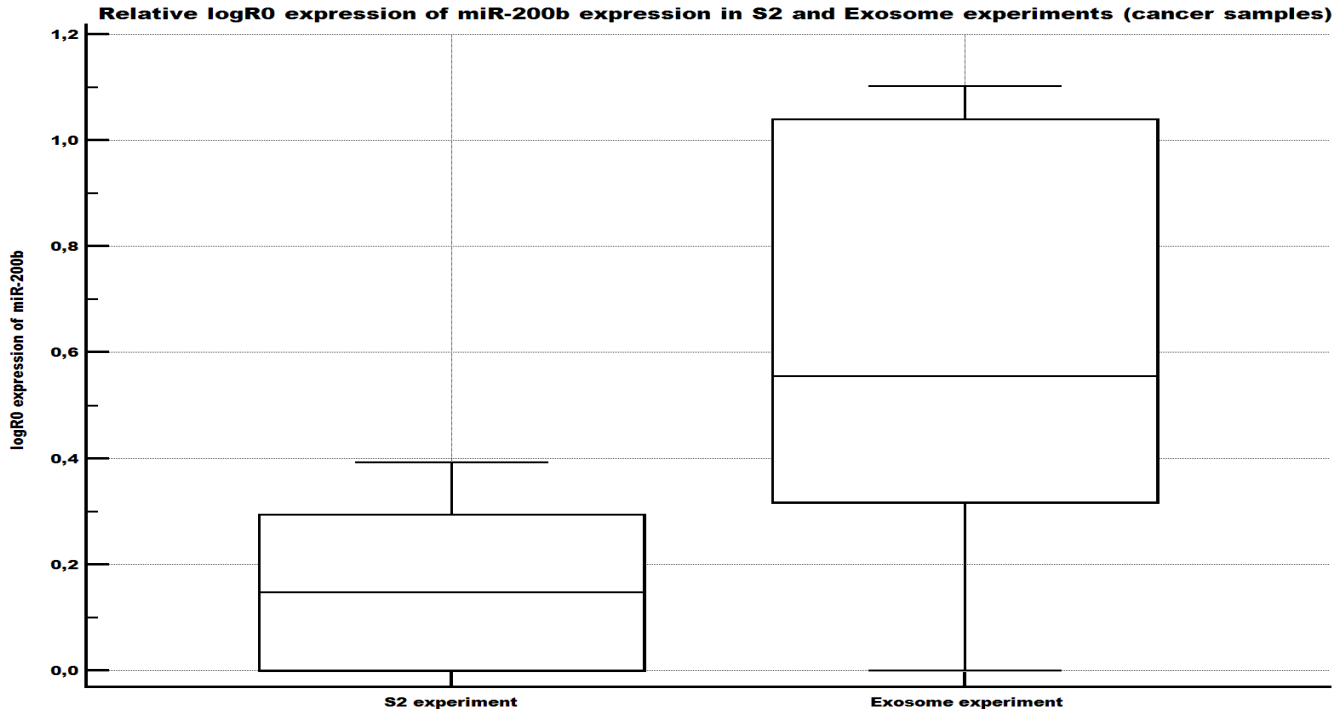
Geometric mean of miR-106b and miR-16 was selected as the normalization factor for the experiment Exosome. Similarly as in the geometric mean normalization method, no significant differences could be found both in endometrial and ovarian cancer groups compared with the control samples.



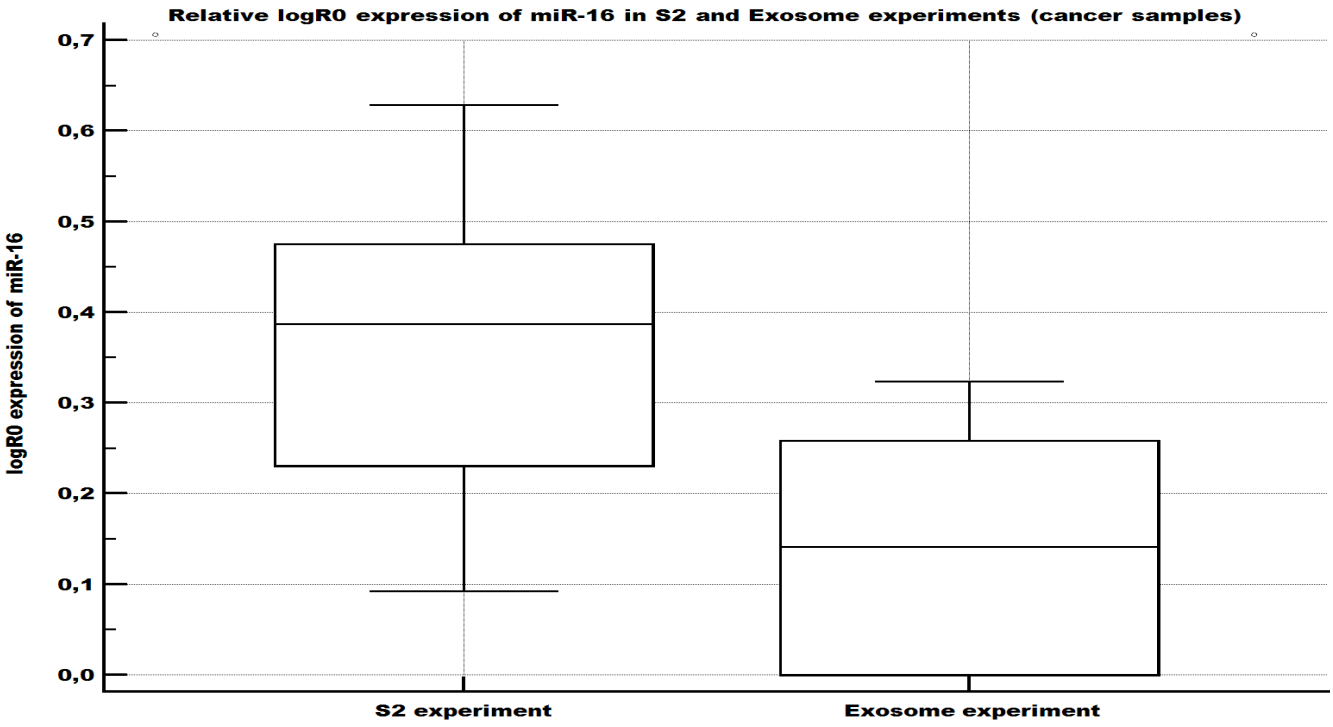
Supplementary Figure 1.



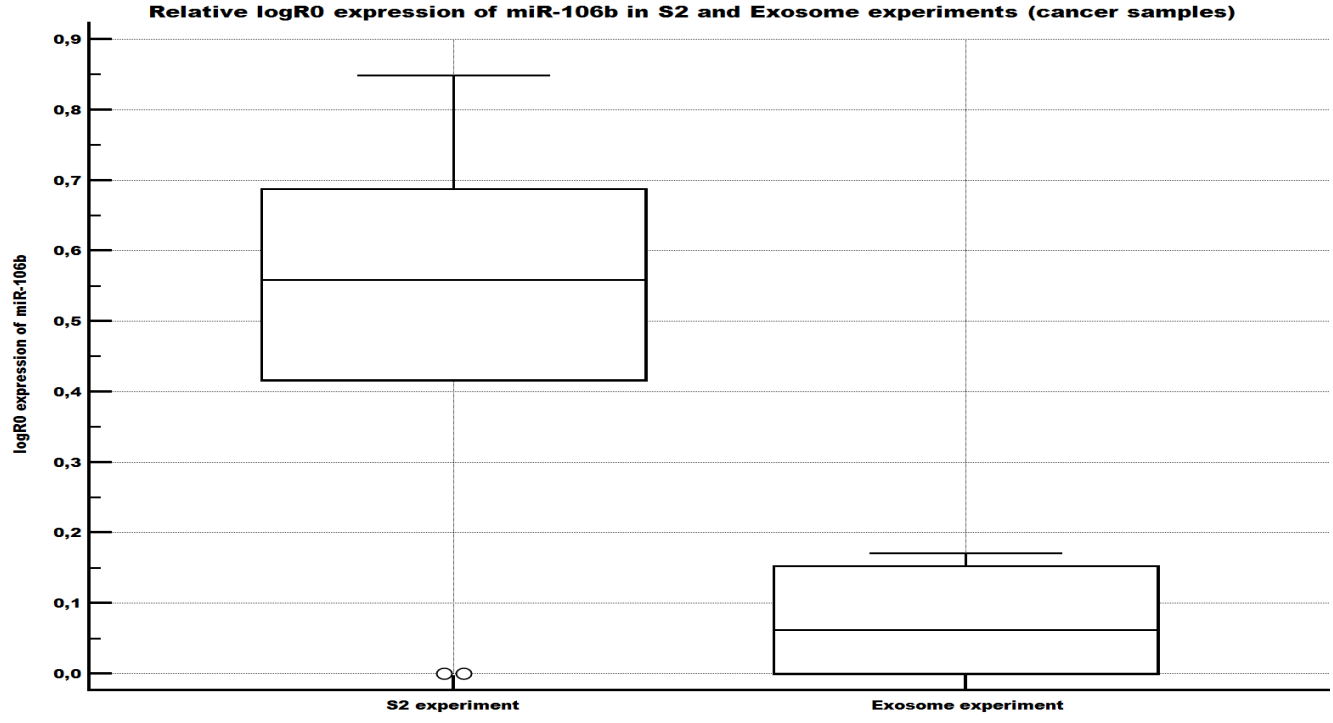
Supplementary Figure 2.



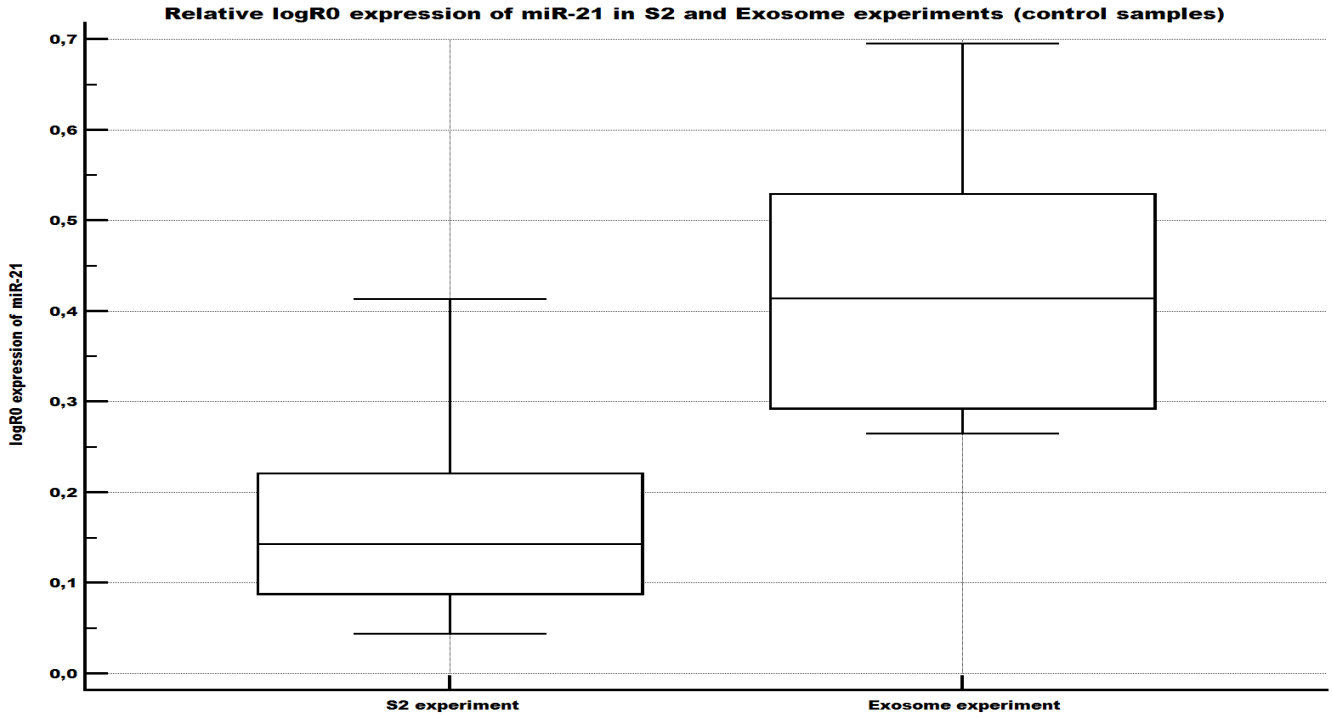
Supplementary Figure 3. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-200b expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



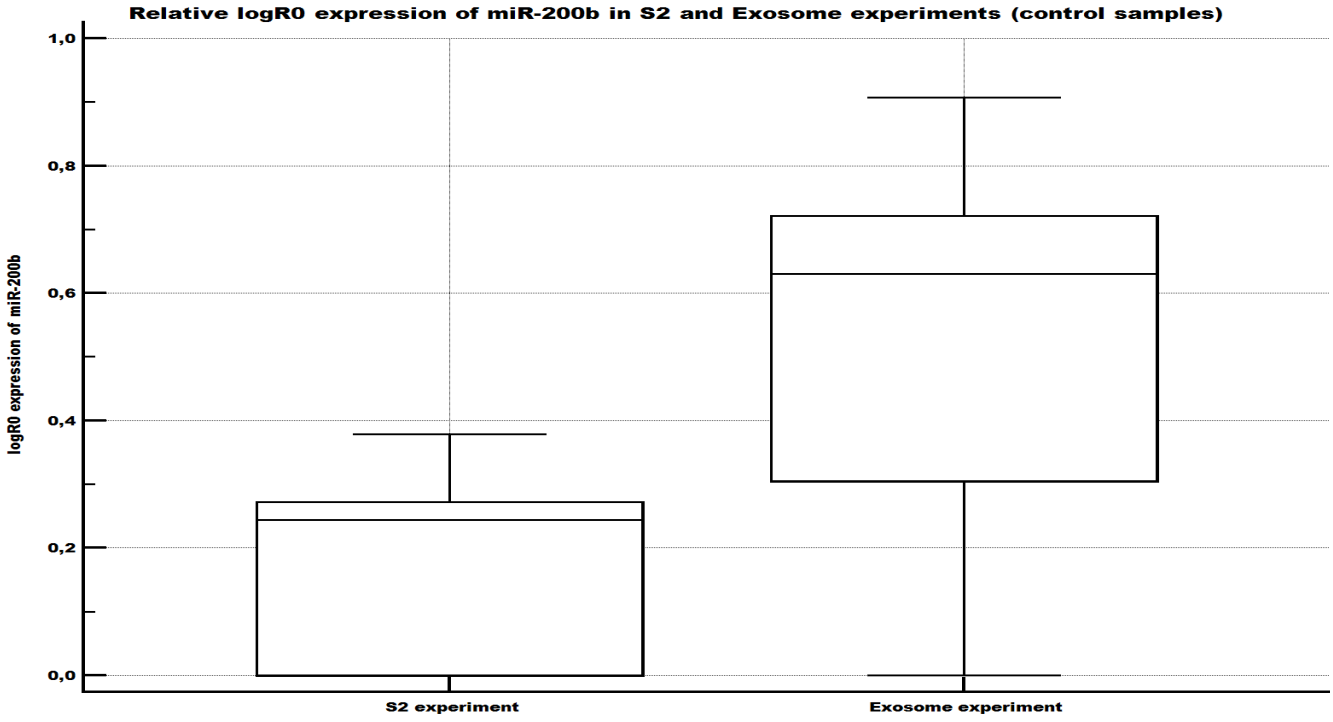
Supplementary Figure 4. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-16 expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



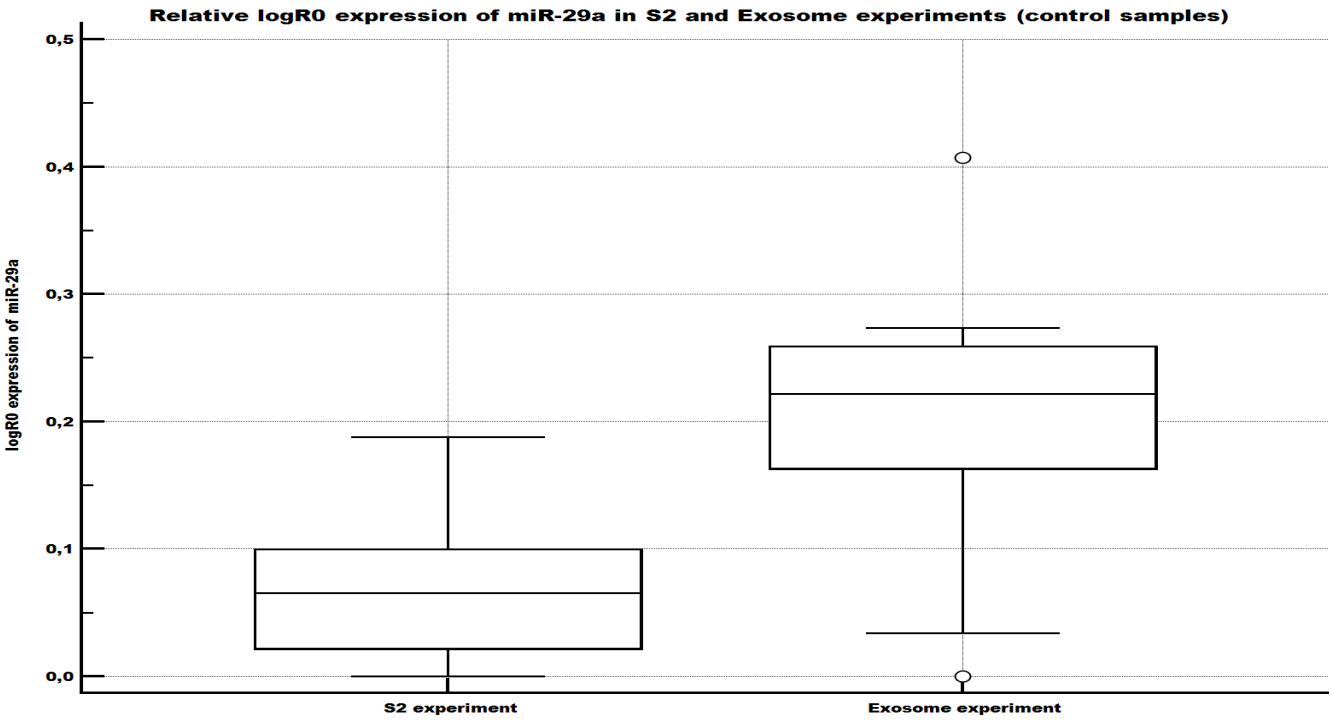
Supplementary Figure 5. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-106b expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



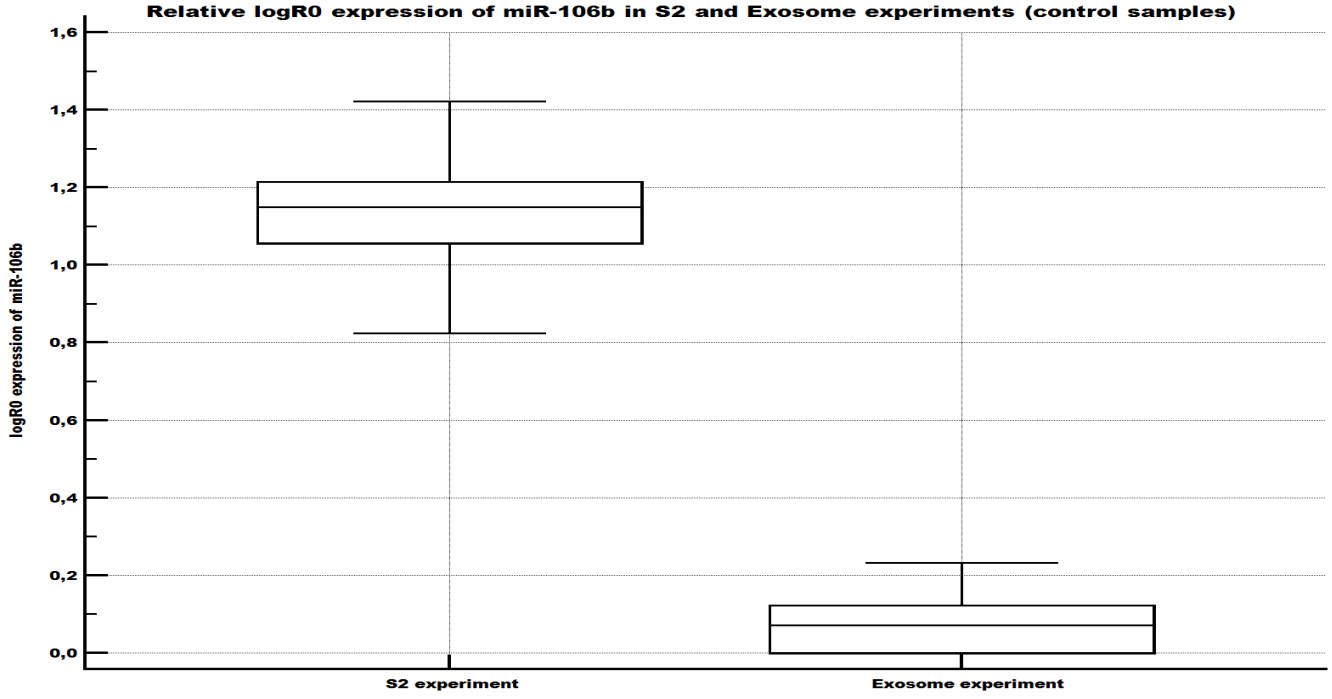
Supplementary Figure 6. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-21 expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



Supplementary Figure 7. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-200b expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



Supplementary Figure 8. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-29a expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.



Supplementary Figure 9. Box and Whisker plot showing log-transformed and geometric mean normalized data of the urinary miR-106b expression in the S2 experiment and Exosome experiment, based on Real-time PCR Miner data (R0). The central box represents the values from the lower to upper quartile (25 to 75 percentile). Median is represented by the middle line. A line leads from the minimum to the maximum value.

Supplementary Table 1. Summary of clinicopathological data for patients and samples used in the Exosome experiment (additional diagnoses)

Type of sample	Code	FIGO stage	Grade	Age at surgery
Mixed colorectal carcinoma, carcinoma mammae, fallopian tube carcinoma	UCP3	n/a	n/a	77
Malignant diffuse large B-Cell lymphoma infiltrating extensively ovaries and fallopian tubes	UCP4	n/a	n/a	66
Malignant granulosa tumor of ovary	UCB331A (pre-surgery), UCB331B (post-surgery)	1a	n/a	66
Undifferentiated carcinoma from transervical resection of submucosal endometrial myoma	UCP20	n/a	n/a	44
Krukenberg carcinoma of the ovary (uncertain origin)	UCP23	n/a	n/a	45

Supplementary Table 2. List of control samples and their use in the S1, S2 and Exosome experiments

Code	Age at sampling	S1 experiment	S2 experiment	Exosome experiment
Post-menopausal patients				
UN9	61	▪	▪	▪
UN10	54	▪	▪	▪
UN11	66	▪	▪	▪
UN13	62	▪	▪	▪
UN24	50		▪	
UN28	52			▪
UN31	62	▪		
UN32	62	▪	▪	▪
UN33	54	▪	▪	▪
UN36	57	▪	▪	
UN38	51	▪		▪
UN41	60	▪	▪	▪
UN42	54	▪	▪	▪
UN43	58	▪	▪	
UN44	52	▪	▪	▪
UN46	54			▪
UN47	56			▪
Pre-menopausal patients				
UN66 (contraception yes)	30			▪
UN68 (contraception yes)	28			▪
UN70 (contraception yes)	29			▪
UN73 (no contraception)	26			▪
UN79 (no contraception)	29			▪
UN81 (no contraception)	29			▪

▪ – included in the analyses

Supplementary Table 3. List of microRNA assays used in the experiments

Assay name	miRBase ID	Catalogue number (Life Technologies)	Localization on chromosome	Experiment		
				S1	S2	Exosome
hsa-miR-223[1]	hsa-miR-223-3p	4427975/002295	Chr X	●	●	n/a
hsa-miR-21	hsa-miR-21-5p	4427975/000397	Chr 17	●	●	●
hsa-miR-200b	hsa-miR-200b-3p	4427975/002251	Chr 1	●	●	●
hsa-miR-367	hsa-miR-367-3p	4427975/000555	Chr 4	●	●	●
hsa-miR-92a[2]	hsa-miR-92a-3p	4427975/000431	Chr 13	●	n/a	n/a
hsa-miR-16	hsa-miR-16-5p	4427975/000391	Chr 13	●	●	●
hsa-miR-29a	hsa-miR-29a-3p	4427975/002112	Chr 7	●	●	●
hsa-miR-106b	hsa-miR-106b-5p	4427975/000442	Chr 7	●	●	●
hsa-miR-100[3]	hsa-miR-100-5p	4427975/000437	Chr 11	●	●	partially
hsa-miR-20a	hsa-miR-20a-5p	4427975/000580	Chr 13	●	●	●
hsa-miR-1228	hsa-miR-1228-3p	4427975/002919	Chr 12	●	●	●

[1] miR-223 was not assessed in the Exosome experiment, [2] miR-92a was not assessed in S2 and Exosome experiments,)[3] miR-100 was partially functional in Exosome experiment, n/a – not-available, ● – applied in the analyses

Supplementary Table 4. Logistic regression model and preliminary ROC analyses for the S1 experiment

Logistic regression model (MedCalc)			Classification table	Preliminary ROC analysis		
miRNA	**Sample size (pre-surgery cancer A and controls)**	**Overall Model Fit [1]**	**Percent of cases correctly classified [2]**	**Area under the ROC curve (AUC)**	**Standard Error**	**95% CI [3]**
miR-21	18	0.0996	61.11	0.708	0.121	0.450 to 0.894
miR-223	18	0.5107	72.22	0.500	0.185	0.260 to 0.740
miR-92a	18	P < 0.0001	100	1.000	0.000	0.815 to 1.000
miR-200b	18	P < 0.0001	100	1.000	0.000	0.782 to 1.000
miR-16	18	0.8670	72.22	0.538	0.152	0.293 to 0.771
miR-29a	18	0.1450	61.11	0.708	0.120	0.450 to 0.894
miR-367	18	0.0457	77.78	0.769	0.119	0.514 to 0.931
miR-106b	18	0.0001	94.44	0.969	0.0384	0.764 to 1.000
miR-100	18	0.0420	66.67	0.846	0.0945	0.601 to 0.970
miR-20a	18	0.0667	72.22	0.792	0.106	0.539 to 0.944
miR-1228	18	0.3078	61.11	0.754	0.114	0.498 to 0.922
miR-106b/miR-100 combination	18	P < 0.0001	100	1.000	0.000	0.815 to 1.000
miR-100/miR-92a combination	18	P < 0.0001	100	1.000	0.000	0.815 to 1.000

[1] significance level P, [2] cut-off value 0.5, [3] confidence interval

Supplementary Table 5. Ovarian cancer percentage abundance of microRNAs (Exosome experiment)

Sample	miR-21	miR-200b	miR-16	miR-29a	miR-367	miR-106b	miR-100	miR-20a	miR-1228
EXC UCB318C	8.50	48.00	15.03	6.79	n/a	3.70	n/a	2.33	15.64
EXC UCB417A	35.32	49.42	15.25	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCB322A	8.84	32.21	40.55	3.59	0.22	5.02	0.68	6.00	2.88
EXC UCP12	2.73	27.66	0.56	0.56	n/a	n/a	n/a	0.29	68.20

n/a – not-available/no expression detected

Supplementary Table 6. Endometrial cancer percentage abundance of microRNAs (Exosome experiment)

Sample	miR-21	miR-200b	miR-16	miR-29a	miR-106b	miR-20a	miR-1228
EXC UCP8	6.95	26.81	9.81	3.35	3.38	4.57	45.11
EXC UCP9	17.03	82.97	n/a	n/a	n/a	n/a	n/a
EXC UCP11	28.59	53.42	2.72	10.86	3.48	0.92	0.00
EXC UCP13	6.02	n/a	n/a	n/a	n/a	n/a	93.98
EXC UCP15	48.82	n/a	51.18	n/a	n/a	n/a	n/a
EXC UCP16	24.87	5.59	0.90	2.24	1.89	0.60	63.90
EXC UCP17	73.92	11.57	5.17	3.45	1.89	2.85	1.17
EXC UCP19	38.74	41.32	5.98	8.11	1.60	4.24	0.00
EXC UCP21	22.95	41.81	9.59	5.62	2.77	4.21	13.04

n/a – not-available/no expression detected

Supplementary Table 7. Other diagnoses and their microRNAs percentage abundances (Exosome experiment)

Diagnosis	Sample code	miR-21	miR-200b	miR-16	miR-29a	miR-367	miR-106b	miR-20a	miR-1228	
Fallopian tube carcinoma	EXC UCP1	19.26	39.78	0.00	3.52	0.00	0.00	0.00	37.44	
	EXC UCB902B	6.79	28.40	7.21	0.00	0.00	1.33	0.62	55.65	
Krukenberg carcinoma of ovary	EXC UCP23	57.88	26.49	6.71	3.98	0.00	0.00	1.42	3.52	
Ovarian carcinoma + endometrial carcinoma	EXC UCP5	2.12	5.93	0.00	0.81	0.00	0.00	0.00	91.14	
Malignant B-lymphoma	EXC UCP4	52.16	46.90	0.00	0.18	0.00	0.76	0.00	0.00	
Undifferentiated endometrial carcinoma	EXC UCP20	29.06	10.62	0.00	6.35	0.00	3.55	8.60	41.82	
	EXC UCP22	21.92	21.57	2.12	5.32	0.00	1.00	0.94	47.13	
Benign diagnoses	EXC UCP2	24.43	64.78	3.01	6.00	0.00	1.56	0.22	0.00	
	EXC UCP7	6.16	78.58	4.57	0.00	0.00	3.05	2.92	4.73	
	EXC UCP10	30.60	56.63	1.98	1.96	0.00	2.13	1.07	5.62	
	EXC UCP14	2.25	3.38	1.46	0.39	0.00	0.59	0.26	91.67	
	EXC UCP18	13.96	12.76	2.46	2.63	0.00	1.39	0.35	66.45	
	EXC UFB01	49.90	44.98	0.00	5.12	0.00	0.00	0.00	0.00	
Diagnosis	**Sample code**	**miR-21**	**miR-200b**	**miR-16**	**miR-29a**	**miR-367**	**miR-106b**	**miR-20a**	**miR-1228**	**miR-100**
Malignant granulosa cell tumor of ovary	EXC UCB331A	46.07	2.48	49.12	1.06	0.25	0.00	0.20	0.77	0.04
	EXC UCB331B	74.44	2.36	17.53	0.23	0.23	0.29	0.64	3.85	0.43

Supplementary Table 8. Percentage abundance of microRNA in control samples (Exosome experiment)

Control sample	miR-21	miR-200b	miR-16	miR-29a	miR-367	miR-106b	miR-100	miR-20a	miR-1228
EXN UN9	25.54	0.00	54.23	20.23	0.00	0.00	0.00	0.00	0.00
EXN UN10	18.23	60.90	9.54	11.33	0.00	0.00	0.00	0.00	0.00
EXN UN11	9.55	34.99	13.33	16.33	0.00	3.21	0.00	3.11	19.48
EXN UN13	37.05	27.30	9.37	7.01	0.00	2.63	0.00	2.57	14.07
EXN UN28	21.11	41.04	6.66	3.00	5.43	2.94	5.21	2.03	12.57
EXN UN32	13.76	48.31	12.29	5.43	0.41	4.83	8.60	6.37	0.00
EXN UN33	51.57	48.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EXN UN38/A	7.55	19.68	0.00	0.00	0.00	0.00	0.00	0.00	72.76
EXN UN41	4.63	3.00	1.28	0.24	1.60	0.00	0.00	0.00	89.26
EXN UN42	31.11	45.24	4.99	7.04	7.39	4.23	0.00	0.00	0.00
EXN UN44	17.95	35.75	4.40	7.08	2.50	1.44	0.00	0.00	30.88
EXN UN46	54.67	28.29	4.97	7.88	0.55	1.12	0.00	0.74	1.78
EXN UN47	27.51	44.61	4.15	5.41	0.48	1.11	0.00	1.34	15.39
Pre-menopausal controls with contraception									
EXN AA	55.22	18.86	14.45	3.43	0.11	0.83	0.00	0.61	6.49
Pre-menopausal control without contraception									
EXN AN	42.02	4.52	24.41	9.85	1.95	6.17	0.00	7.88	3.20

Supplementary Table 9. Fold-differences in microRNA expression in pathological samples in comparison to control samples (Exosome experiment)

Sample	miR-21	miR-200b	miR-16	miR-29a	miR-367	miR-106b	miR-100	miR-20a	miR-1228
EXC UCB315A	n/a	n/a	n/a	n/a	2.66	n/a	n/a	n/a	n/a
EXC UCB318A	n/a	0.24	n/a	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCB318B	0.19	0.41	n/a	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCB318C	0.3	1.24	1.64	0.85	n/a	1.14	n/a	0.67	0.63
EXC UCB322A	0.67	1.76	9.42	0.95	0.13	3.29	0.16	3.67	0.25
EXC UCB322B	1.2	1.9	2.11	0.66	0.14	0.89	n/a	1.2	1.63
EXC UCB331A	11.34	0.44	37.15	0.92	0.51	n/a	0.03	0.41	0.21
EXC UCB331B	15.82	0.36	11.45	0.17	0.39	0.54	0.28	1.1	0.92
EXC UCB417A	0.39	0.39	0.51	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCB417B	0.02	0.4	0.25	n/a	n/a	n/a	n/a	n/a	12.96
EXC UCB902B	0.35	1.7	1.15	n/a	n/a	0.6	n/a	0.26	3.26
EXC UFB01	0.72	0.47	n/a	0.26	n/a	n/a	n/a	n/a	n/a
EXC UCP1	0.35	0.53	n/a	0.23	n/a	n/a	n/a	n/a	0.78
EXC UCP2	1.73	3.32	0.66	1.49	n/a	0.96	n/a	0.13	n/a
EXC UCP3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.37
EXC UCP4	4.1	2.61	n/a	0.05	n/a	0.51	n/a	n/a	n/a
EXC UCP5	0.13	0.25	n/a	0.17	n/a	n/a	n/a	n/a	6.11
EXC UCP7	0.3	2.77	0.68	n/a	n/a	1.29	n/a	1.15	0.26
EXC UCP8	0.26	0.72	1.12	0.43	n/a	1.9	n/a	1.37	1.88
EXC UCP9	0.15	0.52	n/a	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCP10	1.98	2.65	0.39	0.44	n/a	1.19	n/a	0.56	0.41
EXC UCP11	1.29	1.75	0.38	1.72	n/a	1.37	n/a	0.34	n/a
EXC UCP12	0.32	2.36	0.2	0.23	n/a	n/a	n/a	0.28	9.7
EXC UCP13	0.08	n/a	n/a	n/a	n/a	n/a	n/a	n/a	1.46
EXC UCP14	0.41	0.45	0.82	0.25	n/a	0.93	n/a	0.38	18.88
EXC UCP15	0.32	n/a	1.3	n/a	n/a	n/a	n/a	n/a	n/a
EXC UCP16	1.97	0.32	0.22	0.62	n/a	1.3	n/a	0.39	5.72
EXC UCP17	4.71	0.53	1.1	0.77	n/a	1.5	n/a	1.47	0.08
EXC UCP18	1.2	0.67	0.55	0.67	n/a	0.88	n/a	0.2	5.47
EXC UCP19	1.41	1.9	0.67	1.4	n/a	0.51	n/a	1.25	n/a
EXC UCP20	0.82	0.22	n/a	0.63	n/a	0.87	n/a	1.96	1.33
EXC UCP21	0.78	1.3	1	0.67	n/a	0.82	n/a	1.16	0.5
EXC UCP22	1.23	0.87	0.36	1.4	n/a	0.49	n/a	0.43	2.97
EXC UCP23	2.47	0.82	0.88	0.6	n/a	n/a	n/a	0.49	0.17

n/a – not-available/no expression detected