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Differential levels of circulating RNAs prior to endometrial cancer diagnosis

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Abstract

Endometrial cancer (EC) is one of the most common female cancers and there is currently no routine screening strategy for early detection. An altered abundance of circulating microRNAs (miRNAs) and other RNA classes have the potential as early cancer biomarkers. We analyzed circulating RNA levels using small RNA sequencing, targeting RNAs in the size range of 17–47 nucleotides, in EC patients with samples collected prior to diagnosis compared to cancer-free controls. The analysis included 316 cases with samples collected 1–11 years prior to EC diagnosis, and 316 matched controls, both from the Janus Serum Bank cohort in Norway. We identified differentially abundant (DA) miRNAs, isomiRs, and small nuclear RNAs between EC cases and controls. The top EC DA miRNAs were miR-155-5p, miR-200b-3p, miR-589-5p, miR-151a-5p, miR-543, miR-485-5p, miR-625-p, and miR-671-3p. miR-200b-3p was previously reported to be among one of the top miRNAs with higher abundance in EC cases. We observed 47, 41, and 32 DA miRNAs for EC interacting with BMI, smoking status, and physical activity, respectively, including two miRNAs (miR-223-3p and miR-29b-3p) interacting with all three factors. The circulating RNAs are altered and show temporal dynamics prior to EC diagnosis. Notably, DA miRNAs for EC had the lowest

q-value 4.39–6.66 years before diagnosis. Enrichment analysis of miRNAs showed that signaling pathways Fc epsilon RI, prolactin, toll-like receptor, and VEGF had the strongest associations.

KEYWORDS

cell-free nucleic acids, endometrial neoplasms, RNA, RNA-sequencing

What's new?

While endometrial cancer (EC) is common among postmenopausal women, early detection is lacking, owing to an absence of effective screening strategies. Here, the authors explored circulating microRNAs (miRNAs) and changes in their abundance as markers for early EC detection. Analyses of blood samples collected prior to EC diagnosis reveal distinct changes in circulating miRNAs in females who eventually developed EC. Relationships between EC and miRNAs were modified by body mass index, physical activity, and smoking status. The findings indicate that circulating miRNAs are susceptible to alterations and temporal fluctuations prior to EC onset, with implications for disease detection and progression.

1 | INTRODUCTION

Uterine corpus cancer, generally known as endometrial cancer (EC), is the sixth most prevalent cancer in women worldwide and is the most common gynecological cancer in countries with a high or very high development index.[1,2] EC occurs predominantly in postmenopausal women. There has been an increase in the global incidence of EC, also among premenopausal women, which has been attributed to increased obesity in the population, together with changes in other reproductive and lifestyle exposures (e.g., parity, exogenous hormone use).[3] There is sufficient evidence to suggest that the absence of excess body fat prevents EC.[4] Traditionally, EC is classified into type I and II. Type I is more frequent (80%–90% of EC cases), more prevalent in European populations, metastasis is rare and prognosis is favorable.[5] Routine screening for EC is not currently practiced, except for women with Lynch syndrome who are at substantially increased risk of EC but could be facilitated by integration of relevant molecular and imaging data.[6]

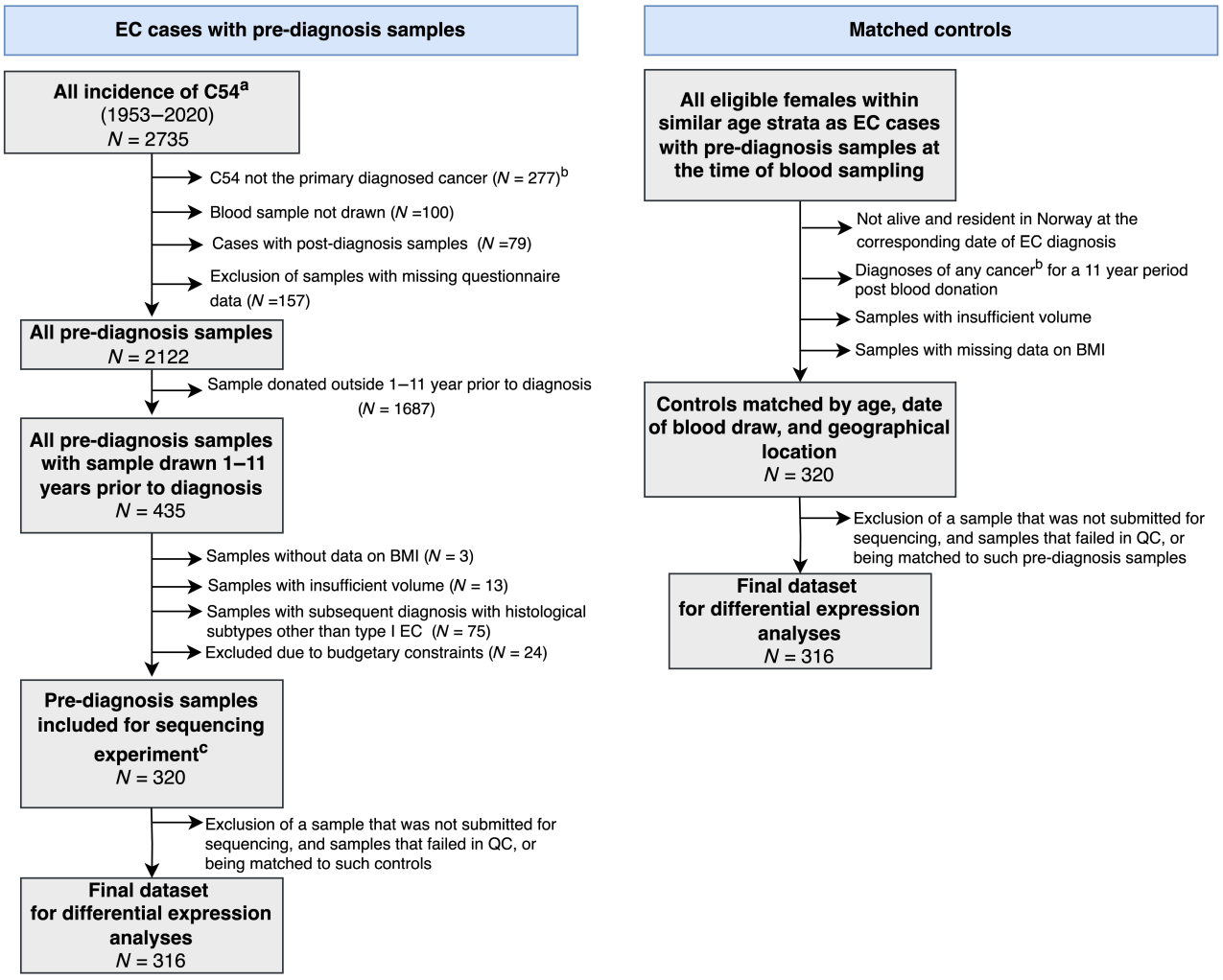
Non-coding RNAs (ncRNAs) affect cancer progression through proliferation, migration, metastasis, and apoptosis.[7] Extracellular RNAs refer to RNAs that are present elsewhere than the cell involved in its transcription. The origin of extracellular RNAs is both blood cells and tissue. The latter is released due to passive leakage, and active secretion independent or dependent on microvesicles.[8] Circulating ncRNAs have potential as early diagnostic and prognostic cancer biomarkers.[7,9,10] Some ncRNAs such as transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) have mainly structural roles, whereas others regulate gene expression at the transcriptional and post-transcriptional level, such as microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and long non-coding RNAs (lncRNAs).[11] miRNAs have an average length of 22 nucleotides and exert their regulatory influence by mRNA degradation and translation inhibition.[12] miRNAs are known to be associated with all hallmarks of cancer.[13]

Several studies have reported differentially abundant (DA) circulating RNAs for EC.[14–16] Most of the literature on ncRNAs and EC have focused on disease-associated miRNAs which is based on both tumor tissue and bodily fluid samples collected at or after diagnosis. Far less is known about the abundance profiles of miRNAs and other RNA classes in samples collected prior to diagnosis from EC patients which are more relevant for discovery of potential early detection cancer biomarkers. The aim of the study was to discover DA RNAs in females who subsequently developed EC and had samples collected 1–11 years prior to diagnosis, compared to matched cancer-free female controls. For DA miRNAs, we did two subsequent analyses: (a) enrichment analysis to further elucidate their biological context; and (b) network analyses to investigate their relationship to genes in molecular interaction pathways. We also aimed to investigate the effect of body mass index (BMI), smoking, and physical activity on RNA levels and EC.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

We used a nested case–control design within the Janus Serum Bank (JSB), a population-based cancer biobank with serum samples from 318,628 individuals donated between 1972 and 2004.[17] The cases were identified by linking the JSB to the Cancer Registry of Norway (CRN) using the unique Norwegian national identity number, with follow-up time until December 31, 2020. Since 1952, CRN has systematically collected mandatory notifications on cancer occurrence in the Norwegian population. The registration is close to complete.[18] Information on BMI and other lifestyle variables was obtained from health examination surveys at the Norwegian Institute of Public Health and represents baseline variables at the time of blood collection.[19] An overview of data source linkages is provided in Figure S1.



a. C54, Malignant neoplasm of corpus uteri
b. with the exception of benign tumours and non-melanoma skin cancer
c. all cases with pre-diagnosis samples had complete data on BMI, smoking, and physical activity.

FIGURE 1 Selection of endometrial cancer (EC) cases (type I) with samples collected up to 11 years prior to diagnosis and matched controls.

A flowchart of the case–control selections is provided in Figure 1 showing that a total of 2735 incident EC cases were identified in JSB during the follow-up period 1953–2020. Cases who donated samples outside the 1–11 year range prior to the EC diagnosis were excluded. Only type I EC was selected as cases.

To ensure that the distribution of key baseline variables was comparable between cases and controls, we used a 1:1 matched case–control design, where the matching criteria were participant's age at blood sampling, year of blood sampling (±2 years), and county of residence at blood sampling. The CALIPMATCH Stata module (distribution date: 20170511)[20] was used for the matching. All controls were alive and residing in Norway at the year of EC diagnosis in the matched cases. In addition, controls were cancer-free (except non-melanoma skin cancer) up to 11 years after they donated the blood sample. We included 320 cases and 320 matched controls for sequencing. Finally, we excluded four case-control pairs because one or both samples failed in sequencing/QC steps. The final study sample included 316 cases and 316 controls.

Due to comparatively lower proportions of samples in the underweight and obese groups, we combined WHO levels underweight and normal weight into "low BMI" category, and overweight and obese into "high BMI" category. Similarly, for physical activity, we defined "active" as participants who reported low, medium, or high physical activity versus those who reported being inactive. For smoking status, we combined current and former smokers into a category of ever smokers (Table 1).

2.2 | Lab protocols

As previously described,[21] RNA was extracted from 400 µL serum using phenol-chloroform phase separation and the miRNeasy Serum/Plasma kit (Cat. no 1071073, Qiagen). Small RNA-seq libraries were prepared using NEBNext Multiplex Small RNA Library Prep Set for Illumina (Cat. No E7300, New England Biolabs Inc.). RNA fragments

TABLE 1 Baseline characteristics of the study participants.[a]

Characteristics	EC pre-diagnosis samples ($n = 316$)	Matched controls ($n = 316$)
Age at sample donation (mean ± SD)	47.4 (8.4)	47.3 (8.4)
BMI[b] (mean ± SD)	26.3 (5.9)	24.8 (4.1)
BMI category,[b,c] *n* (%)		
Low	163 (51.6)	192 (60.7)
High	153 (48.4)	124 (39.3)
Physical activity,[d] *n* (%)		
Inactive	73 (23.5)	71 (22.6)
Active	238 (76.5)	243 (77.4)
Smoking, *n* (%)		
Never	161 (50.9)	145 (45.9)
Ever	155 (49.1)	171 (54.1)

[a]Total cholesterol, triglycerides, and systolic and diastolic blood pressure were comparable between cases and controls and are not reported in this table.
[b]Baseline characteristics were compared between cases and controls using Student's *t* test, and the Chi-square test. At *P*-value threshold .05, only BMI was different between cases and controls. Note that BMI was not a criterion for matching between cases and controls and it is associated with EC.
[c]WHO BMI levels were recoded to represent "low BMI" (underweight and normal weight) and "high BMI" (overweight and obese).
[d]"Active" physical activity represents participants who reported to have low, medium, or high physical activity. Seven individuals had missing data on physical activity.

with length 17–47 nucleotides were selected using Pippin Prep automated size selection (Labgene Scientific SA), and the samples were sequenced on a NovaSeq 6000 platform (Illumina Inc.).

2.3 | Small RNA-seq processing pipeline

The mean sequencing depth was 22.9 million reads. The sequencing coverage and quality statistics are summarized in Figure S2. Pre-processing of RNA was performed using two pipelines:

1. sncRNA workflow, as described previously.[21] Briefly, the pipeline specifications are: trimming of adapters with AdapterRemoval v2,[22] mapping reads to the human genome (hg38) with Bowtie2.[23]
2. smrnaseq nf-core pipeline.[24] We used two databases for miRNA, miRBase and MirGeneDB.[25,26] We used miRBase annotation for miRNA for both pre-processing pipelines (sncRNA, and smrnaseq) but additionally used MirGeneDB annotation only for smrnaseq.

2.4 | Differential abundance analysis

JSB samples were collected over three time periods: 1972–1978 (group 1), 1979–1986 (group 2), and 1987–2004 (group 3). The serum processing methods were different between groups (with iodoacetate added, no additives, and separating gel tubes, for groups 1–3, respectively). This has been shown to be a source of sample batch variation, as described previously.[27] We observed differences in abundance patterns of miRNAs based on the serum sample collection period and processing (Figure S3). Prefiltering of RNAs with low levels was performed using the edgeR package (version 3.30.0)[28] based on the design matrix case–control status and the default minimum count (10). Differential abundance (DA) analysis was performed using Limma-voom (Limma 3.44.1)[29] based on a linear mixed effects model with sample batch groups (3 levels) as a random effect. (voom) and (duplicateCorrelation) functions in Limma were used to compute the correlation between sample batch groups. Using the (makeContrasts) function in Limma, we constructed the contrast matrix based on coefficients from the linear model fit and evaluated DA miRNAs for EC case–control status across the levels of BMI, smoking status, and physical activity groups in Models 1–3, respectively. We also evaluated overall DA miRNAs for EC in the three Models, as well as the interaction term, that is, miRNAs that showed a difference in DA for EC between BMI, smoking status, and physical activity groups in Models 1–3, respectively (detailed script available, see Data Availability Statement).

TABLE 2 Models evaluating differential abundance of RNAs for endometrial cancer (EC).

Models	Variables
Model 1	Case–control status, BMI level, age at sample collection, sample batch group
Model 2	Case–control status, smoking status, age at sample collection, BMI, sample batch group
Model 3	Case–control status, physical activity, age at sample collection, BMI, sample batch group

Table 2 summarizes the variables used in the three Models to evaluate DA of RNAs. Given the importance of age in RNA levels, all Models included age at the time of sample collection as a continuous variable (centered and scaled using scale (base) function in R). Similarly, given the importance of BMI in EC and some associations to RNAs,[30] we included BMI in all Models either as a categorical/binary variable (Model 1), or continuous (centered and scaled) variable (Models 2 and 3). There is less knowledge about the association of smoking status and physical activity with EC. By including BMI in Models 2 and 3, we wanted to ensure that the association of EC with smoking status (Model 2), and physical activity (Model 3) on RNA levels were not influenced by BMI.

Benjamini–Hochberg's method was used to control the False Discovery Rate (FDR), and RNAs with q-value <0.05 were the main focus of the DA analyses. Note that for the interpretation of top DA signals, we relied our focus mainly on q-values rather than fold changes. This is because Limma does not use ordinary t-tests and it favors large fold changes over small fold changes.

EC cases were not equally distributed in sample collection time in the 1–11 year period prior to diagnosis and the median was 6.7 years (interquartile range: 4.3 years) (Figure S4). To evaluate the temporal dynamics of DA patterns, we split the sampling time period prior to

TABLE 3 Number of DA RNA classes for EC with q-value <0.05 based on Model 1 in Table 2.

RNA type	Input RNA number	DA EC for low BMI and high BMI	DA EC different between low BMI and high BMI	DA EC low BMI	DA EC high BMI
miRNA	256 (of 2653)	18	47	35	35
isomiR	1040 (of 59,302)	94	146	71	140
miscRNA	83 (of 1279)	1	0	3	2
tRNA	415 (of 649)	1	0	0	1
tRF	353 (of 34,324)	0	0	0	3
scaRNA	5 (of 48)	1	1	1	1
snRNA	174 (of 1836)	2	15	21	6
snoRNA	29 (of 793)	0	1	1	0
piRNA	404 (of 32,235)	1	4	1	3
lncRNA	609 (of 16,850)	0	1	0	0
mRNA	3366 (of 19,928)	0	0	0	0

diagnosis into quartiles (79 pairs per quartile, Time Frame 1 (1.16-4.39], Time Frame 2 (4.39-6.66], Time Frame 3 (6.66-8.67], and Time Frame 4 (8.67-11] years).

2.5 | Enrichment and network analysis

The miRNA Enrichment Analysis and Annotation Tool (miEAA 2.0)[31] was used for miRNAs with P-value <.05 in the DA analysis, with the following specifications: over-representation analysis (ORA), category: pathways (KEGG), P-value adjustment method: FDR adjustment, P-value adjustment scope: adjust P-values for each category independently, significance level: 0.1, minimum required hits per subcategory: 1.

In addition, RBiomirGS, a logistic regression method, was used for gene set enrichment analysis.[32] The method depends on mapping miRNAs to mRNAs. Gene set analysis was evaluated for three GSEA gene sets—H: Hallmark gene sets, C7: immunologic signature gene sets, and C8: cell type signature gene sets.

For miRNA regulatory network analysis, we used miRNet 2.0,[33] a weighted network analysis, without specifying tissue type, based on genes (miRTARBASE v8.0),[34] and we included all DA miRNAs with q-value <0.05.

3 | RESULTS

Table 1 summarizes the baseline characteristics of the study participants. Age at EC diagnosis ranged from 36 to 79 years, with a median 52 and a mean 54 years (Figure S5).

3.1 | Differential abundance of miRNA

Analyses based on sncRNA preprocessing, miRNA with miRBase annotation, and removal of very low abundant miRNAs resulted in 256 miRNAs as the input to the Models (Table 3).

In Model 1 (Table 2), we evaluated DA miRNAs for EC case-control status across the levels of BMI. Overall, 18 miRNAs were DA for EC. We identified 35 DA miRNAs in the low BMI level, and 35 DA miRNAs in the high BMI level. In the interaction analysis, 47 miRNAs were DA between the low and high BMI levels (Figure 2A–D,M, Tables 3, Table S1).

In Model 2 (Table 2), we evaluated DA miRNAs for EC case–control status across the levels of smoking. Overall, 19 miRNAs were DA for EC. We identified 24 and 31 DA miRNAs in the ever smoker and never smoker groups, respectively. In the interaction analysis, 41 miRNAs were DA between ever smoker and never smoker groups (Figure 2E–H,N, Table S2).

In Model 3 (Table 2), we evaluated DA miRNAs for EC case–control status across the levels of physical activity. Overall, 18 miRNAs were DA for EC. We identified 35 and 21 DA miRNAs in the inactive and active physical activity groups, respectively. In the interaction analysis, 32 miRNAs were DA between inactive and active physical activity groups (Figure 2I–L,O, Table S3). Eleven DA miRNAs for EC were overlapping between all three Models (Figure 2C,G,K,P).

We did a sensitivity analysis, removing samples that showed the most noticeable sample batch effect (Group 1; Figure S3). Based on Model 1 (Table 2), we noticed that no miRNAs remained with q-value <0.05 which is likely due to reduced statistical power. However, all 18 DA miRNAs in Model 1 for EC (Figure 2C) were present in the top 37 miRNAs sorted by the lowest q-value (Tables S1 and S7). These are indicated by red highlight for Model 1 in Table S7. There was high correlation for logFC of these 18 DA miRNAs between the analysis with all samples and the sensitivity analysis (Spearman's correlation of 0.98). For Model 2, 19 DA miRNAs for EC in the analysis with all samples appeared in the top 26 miRNAs in the sensitivity analysis. miR-200b-3p remained DA even in the sensitivity analysis (Tables S2 and S7). For Model 3, 18 DA miRNAs for EC in the analysis with all samples appeared in the top 35 miRNAs in the sensitivity analysis (Tables S3 and S7). Four miRNAs (miR-4488, miR-200b-3p, miR-150-3p, and miR-181d-5p) remained DA even in the sensitivity analysis.

We wanted to ensure that the DA miRNAs for EC were robust when raw counts were produced by different small RNAs pre-

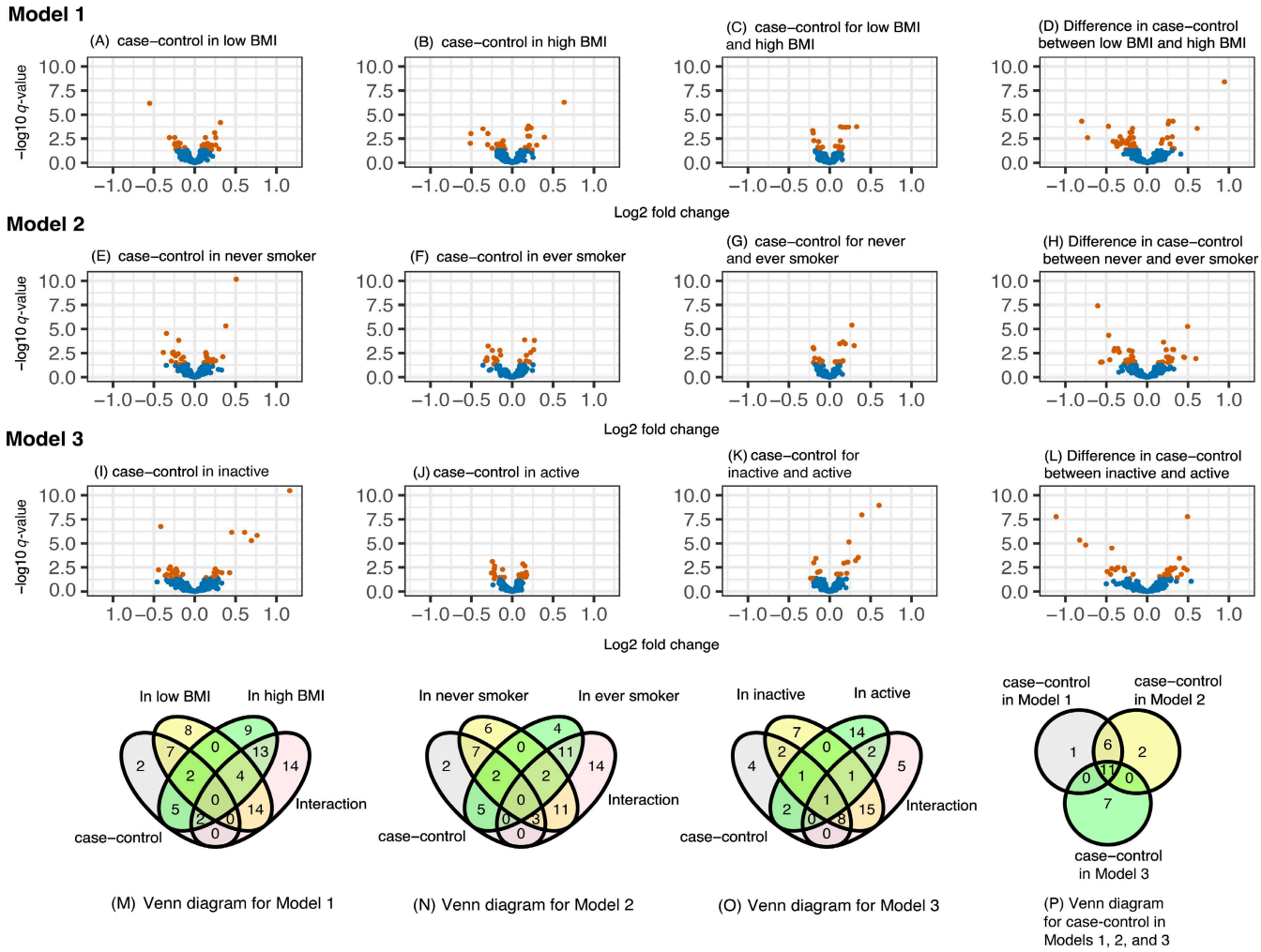


FIGURE 2 Volcano plots, based on Log2 fold change and −log10 *q*-value, representing differential abundance of miRNAs for endometrial cancer (EC) cases with samples collected prior to diagnosis and matched controls across the levels of BMI (Model 1), smoking status (Model 2), and physical activity (Model 3). The upper panel shows Model 1: (A) Differential abundance for EC in the low BMI level, (B) Differential abundance for EC in the high BMI level, (C) Differential abundance for EC for low BMI and high BMI, (D) interaction: difference in differential abundance for EC between low BMI and high BMI levels. The middle panel shows Model 2: (E) Differential abundance for EC in never smokers, (F) Differential abundance for EC in ever smoker, (G) Differential abundance for EC for never and ever smoker, (H) interaction: difference in differential abundance for EC between never and ever smoker. The lower panel shows Model 3: (I) Differential abundance for EC in inactive physical activity, (J) Differential abundance for EC in active physical activity, (K) Differential abundance for EC for inactive and active physical activity, (L) interaction: difference in differential abundance for EC between inactive and active physical activity. Venn diagrams (M), (N), and (O) summarize the number of DA miRNAs that were overlapping between the four contrasts for EC in Models 1–3, respectively. Venn diagram (P) summarizes DA miRNAs for EC that were overlapping between Models 1–3 (Figure 2C, G, K). Differential abundance Models were based on a mixed model with sample batch group as random effect, and included age at sample collection (scaled and centered). For Models 2 and 3, BMI was included as a scaled and centered variable. For Model 1, WHO BMI levels were recoded to represent "low BMI" (underweight and normal weight) and "high BMI" (overweight and obese). For Model 3, we defined "active' physical activity" as participants who reported to have low, medium, or high physical activity. In a sensitivity analysis, in addition to the *q*-values produced by Limma, we also used qvalue Bioconductor package (github.com/StoreyLab/qvalue) to calculate *q*-values at different lambda for DA miRNAs for EC in Model 1 (Figure 2C). At lambda 0.01, similar to the number of DA miRNAs in Figure 2C, 18 had *q*-value <0.05, whereas at 0.1, we saw 22 DA miRNAs. Given that there were only four more DA hits at this threshold, we concluded that the results are not changed considerably. Therefore, we only reported q-values calculated by the Limma package. In a sensitivity analysis (Figure S10), we evaluated the Spearman's correlation of 11 DA miRNAs shared for EC in Models 1–3 (Figure 2P, and Tables S1–S3), BMI, and age at sample collection. There was no strong correlation between BMI and age at sample collection with any of the 11 miRNAs. In a sensitivity analysis (Figure S11), we evaluated hierarchical clustering heatmap with samples labelled for EC case–control status, BMI levels, smoking levels, and physical activity levels. We presented heatmaps for 11 DA miRNAs shared for EC in Models 1, 2, and 3 (Figure 2P, and Tables S1–S3). Overall, there was no specific pattern for sample clustering. In a sensitivity analysis (Figure S12), we evaluated the Spearman's correlation of 11 DA miRNAs shared for EC in Models 1–3 (Figure 2P, and Tables S1–S3), and sample collection year prior to diagnosis for EC cases. There was no strong correlation between year before diagnosis and any of the 11 miRNAs. In a sensitivity analysis, we evaluated the Spearman's correlation of logFC between 11 miRNAs that were shared for EC in Models 1–3 (Figure 2P, and Tables S1–S3). The Spearman's correlation between Model 1 and Model 2 was 0.99, and for that of Model 1 and Model 3 was 0.88.

processing pipelines. This provided a certain level of reliability of DA miRNAs despite that the sncRNA and smrnaseq pipelines have different specifications, for instance in mapping with Bowtie. Differential abundance of miRNA for Model 1 (Table 2) based on pre-processing of RNAs with smrnaseq pipeline[24] are presented in Figure S6 and Table S4. Among the top 18 DA miRNAs for EC, which were based on pre-processing with the sncRNA pipeline,[21] 8 were also DA based on pre-processing with the smrnaseq pipeline (miRBase annotation)[24] (Tables S1 and S4, Figures 2C and S6C). Results for Models 2 and 3 based on miRNA counts from smrnaseq pipeline (miRBase and MirGeneDB annotations) are listed in Table S4.

3.2 | Differential abundance of other RNA

Other RNA types (isomiR, miscRNA, tRNA, tRF, scaRNA, snRNA, snoRNA, piRNA, lncRNA, and mRNA) and their DA for EC were evaluated (Table 3). For Model 1, a summary of the RNA type, input RNA number in the Model (after pre-filtering of low abundant RNAs), and the number of DA RNAs is provided. Generally, for all three Models, DA RNAs for EC were mainly found for miRNAs, isomiRs, and snRNAs (Tables 3 and Tables S1–S3).

3.3 | Temporal dynamics of miRNA differential abundance

Using RNA counts for miRNA from the sncRNA pipeline, Model 1 in Table 2 was constructed and the DA for EC was evaluated at 4 Time Frames prior to diagnosis. Figure S7 shows that the DA miRNAs for EC was dynamic in time. No miRNAs were DA. However, the DA signals had the lowest *q*-value in the Time Frame 2 (4.39-6.66 years] (Figure S7). Among the top 10 DA miRNAs in each Time Frame (labeled), only miR-3180-3p and miR-3180 were present in more than one Time Frame (twice in Time Frames 3 (6.66–8.67] and 4 (8.67–11] years (Figure S7 and Table S5)). Among the 11 DA miRNAs for EC shared between Models 1, 2, and 3 (Figure 2P), there was a stronger correlation for logFC (Spearman's correlation of 0.93) between Time Frame 4 and the analysis with all samples (Figure S13). The hierarchical clustering heat map for these 11 DA miRNAs did not show specific clustering patterns for different Time Frames prior to diagnosis (Figure S14). One miRNA was DA for EC in Model 2 in Time Frame 4 (miR-10527-5p). For Model 2, among the top 10 DA miRNAs for EC in each Time Frame, only miR-939-5p was present in more than one Time Frame (Time Frames 2, and 3), and had opposite directions of abundance for EC, that is, higher and lower abundance in EC cases in Time Frame 2 and 3, respectively (Table S5). For Model 3, among the top 10 DA miRNAs for EC in each Time Frame, miR-10527-5p, miR-3180, and miR-3180-3p were present in more than one Time Frame, although with inconsistent directions of abundance: miR-10527-5p was lower in cases in Time Frame 1, but was higher in cases in Time Frame 4; miR-3180 was lower in cases in Time Frame 3, but was higher in cases in Time Frame 4; and miR-3180-3p was lower in cases in Time Frame 3, but was higher in cases in Time Frame 4 (Table S5). We did a sensitivity analyses and included all controls in all Time Frames (Figure S7 and Table S6).

3.4 | Enrichment and network analysis

We evaluated functional aspects of DA miRNAs for EC by focusing on biological pathways. As input to miEAA 2.0, we selected 53 miRNAs with DA *P*-value <.05 for the EC (Model 1 in Table 2 and Figure 2C). The miRNA to category heatmap for the top 100 by *P*-value is shown. The top signaling pathways are Fc epsilon RI, prolactin, toll-like receptor, and VEGF (Figure S8).

For RBiomirGS gene set analysis, the input was logFC and *P*-value of all 256 miRNAs for EC using Model 1 (Table 2, Figure 2C). Among the top signals for hallmark gene sets were signaling pathways including TNFA, TGF-B, and Notch (Figure S9A). We also evaluated RBiomirGS for C7: immunologic signature gene sets, and C8: cell type signature gene sets (Figure S9B,C). The top signals for cell type signature gene sets included pancreas beta cells, gastric immune cells, and adult kidney interstitium.

As input for miRNA network analysis, DA miRNAs for EC (Model 1 in Table 2, Figure 2C, and Table S1) was used and a degree filter of 2 was specified. The miRNA network shows the DA miRNAs for EC and the associated genes. KEGG pathways in cancers had the strongest association (q-value = 3.03e−8; Figure 3).

4 | DISCUSSION

Differential abundance of nine RNA classes was evaluated for EC patients with samples collected prior to diagnosis, and matched controls. DA RNAs for EC were mainly found for miRNAs (miRNAs and isomiRs), and snRNAs. In addition, between BMI, smoking, and physical activity levels, RNAs showed differences in differential abundance for EC. There were 47, 41, and 32 miRNAs between BMI levels, smoking status, and physical activity groups in Models 1–3, respectively, that showed difference in differential abundance for EC. This included two DA miRNAs for EC (miR-223-3p and miR-29b-3p) that showed interaction with all three factors.

We noticed that DA patterns for RNAs were temporally dynamic: the top DA miRNAs for EC were dissimilar in the different Time Frames, and the associations had the lowest *q*-value in Time Frame 2 (4.4–6.7 years) which is not closest to diagnosis. Biomarkers might be more strongly associated with cancer at specific stages of development. The temporal dynamics might be related to activation of specific cancer hallmarks and fluctuation in export of miRNAs to circulation.

Fewer miRNAs are included in MirGeneDB than miRBase (~40%). Nevertheless, the number of miRNAs after exclusion of low abundant miRNAs, and the number of DA miRNAs for EC were comparable between the two. After pre-processing by smrnaseq, 8 of 14 DA miRNAs for EC based on MirGeneDB were also present for those in miRBase. This shows that curated miRNAs in MirGeneDB were also DA.

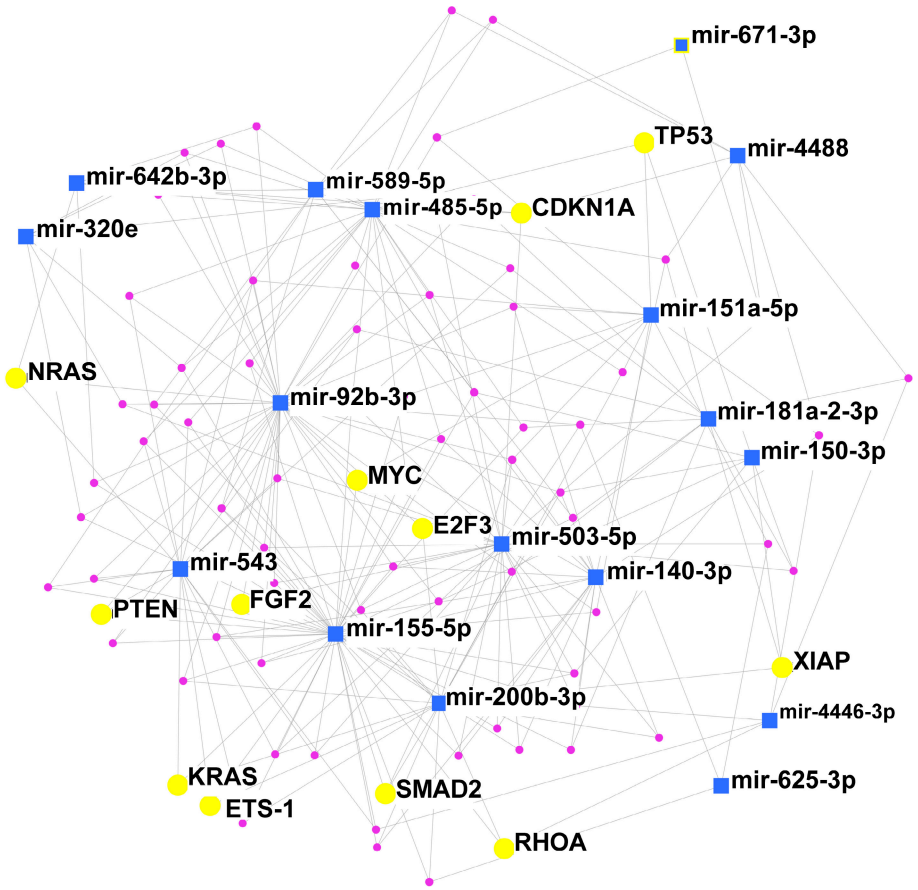


FIGURE 3 Network analysis for DA miRNAs (q-value <0.05) for endometrial cancer (Model 1 in Table 2, Figure 2C) and the associated genes. Blue, miRNAs; Yellow, genes for KEGG pathways in cancers (KEGG pathways in cancers had the highest strength of association, q-value = 3.03e−8); Purple, other genes in the network (names not given).

Comparison of DA RNAs from different studies is not straightforward for several reasons. First, most studies of RNA levels and relevance to EC have not used cases with samples collected prior to diagnosis. RNA abundance patterns are not necessarily similar before and after diagnosis. Second, sample types (e.g., tissue versus circulating) will influence the results and circulating RNA profiles do not directly reflect the specific tissue profile.[35] Third, there could be differences in clinical phenotypes of samples in different studies.

Eight DA miRNAs overlapped for EC in Model 1 based on RNA counts from the two RNA pre-processing pipelines with miRBase annotation. Therefore, we can report these DA miRNAs with greater reliability. Upregulation of oncogenic miR-155-5p and its association with clinical outcomes is well known for several cancers including breast cancer[36] and cervical cancer.[37] In a small study ($n = 27$), miRNAs were evaluated between benign endometrium, endometrial endometrioid adenocarcinoma, and serous adenocarcinoma tissue samples. Association of miR-155-5p with endometrioid adenocarcinoma, and serous adenocarcinoma were reported borderline significant ($P = .053$, and $P = .049$, respectively).[38] miR-155 is involved in controlling expression in genes encoding for immunomodulatory, tumor-suppressor, and inflammatory proteins.[39] In this study, miR-200b-3p had higher level in EC cases compared to controls. Both tumor-suppressor and oncogenic associations of miR-200b-3p have been reported in different studies and cancers (reviewed in Ref. [40]). In one study, miR-200b-3p was among the top 10 miRNAs with higher levels in EC cases.[41] In the current study, miR-589-5p had a higher level in EC cases. In a study using endometrial tissue samples, miR-589-5p had a lower level in EC cases.[42] Different microenvironments (serum vs. tissue) make the studies less comparable but might imply the biomarker potential of miR-589-5p. Similar to our study, miR-543 had a lower level in EC cases in a study using endometrial tissue samples. miR-543 was shown to target FAK and TWIST1, which are known to be associated with metastasis and survival in different cancers.[43] miR-625-3p showed association with colorectal cancer by affecting cell migration and invasion.[44,45]

We compared our DA miRNAs from cases with samples collected prior to diagnosis, to previous studies measuring circulating miRNAs at the time of EC diagnosis.[14–16] Fourteen DA miRNAs for EC from the three Models in the present study were reported in one or more of these three studies (Table S8). However, the direction (higher or lower levels in EC cases compared to controls) were not necessarily similar. In our high BMI group, miR-142-3p had a lower level in EC cases compared to control. This is consistent with reports from several cancer types showing that miR-142-3p is a tumor suppressor miRNA with lower levels in cancer cases.[46] Although, in Bloomfield et al.,[14] EC cases were reported to have a higher level of miR-142-3p. In the present study, EC cases had a lower level of miR-151a-5p (overall in both BMI levels, as well as in the high BMI level), which is the opposite direction to Bloomfield et. al.[14] miR-139-3p had a lower level in EC cases in Zhou et al.,[16] similar to the low BMI group in our study. For high BMI level,

however, EC cases had a higher level. These inconsistencies exemplifying the variability between studies and cohort characteristics. snRNAs are involved in cleavage of pre-mRNAs.[7] We identified several DA snRNAs for EC mainly for U6 snRNAs and some U2 snRNAs. Differential abundance of U6 snRNA has been reported for cervical cancer tissue.[47] Serum biomarker potential of U2 snRNA was reported for lung cancer.[48] We identified EC DA for scaRNA3, a H/ACA scaRNA relevant for several cancers such as breast cancer.[49]

JSB is useful for studying cancer biomarkers given its population-based design and availability of cases with samples collected prior to diagnosis. This study has also taken advantage of linkage to the Cancer Registry of Norway, considering its high quality and completeness of data, as well as linkage to data from health survey.

RNA levels are known to be influenced by age, smoking, BMI, and physical activity.[30] Obesity has the strongest association with EC among 20 of the most prevalent cancer types.[3] By incorporating relevant RNA-related and EC-related variables in our Models, we aimed to identify associated DA circulating RNAs for EC.

There were several sources of non-biological variation in the data. First, there was a notable effect of sample batch, given the different storage times and processing of samples.[27] In all DA models, we used a mixed model and treated sample batch group as a random effect. In this way, we ensured that the variation due to this technical factor was accounted for without allowing the statistical power of the test to be heavily compromised because random effect models use less parameters compared to fixed effect models. Furthermore, high Spearman's correlation for logFC (0.98) for the analysis including all samples, and the sensitivity analysis with group 1 samples removed implies that the DA results are reliable.

In the present study, age at EC diagnosis had the median 52 and mean 54 years. However, median age at EC diagnosis in Norway in the 2017–2021 period was 69 years.[50] BMI, physical activity, and smoking distributions are available for the whole JSB cohort (140,282 individuals) which is fairly representative of general Norwegian female population at the time of sample collection.[19] Overall, individuals in the present study were comparable with regards to physical activity to the whole JSB. However, females in JSB had a higher percentage of individuals in the "low BMI" level (67.1%) compared to both EC cases and controls. Regarding smoking, 57.8% were ever smokers in the whole JSB. This is higher than that of EC cases and controls in the present study. Therefore, with the samples in JSB, and the selection criteria we used, the DA analysis is not necessarily representative of the general Norwegian female population at the time of study. Reproductive factors such as parity, and oral contraceptive and menopausal hormone use are associated with the risk of EC. Data on lifestyle factors such as alcohol use, dietary habits, and drug usage were not available or not reliably reported. Therefore, potential residual confounding due to these factors cannot be excluded.

In conclusion, findings from the present study suggest that circulating RNAs undergo alterations and exhibits temporal fluctuations before the onset of EC. The relationship between RNAs and EC are affected by BMI, smoking status, and physical activity. These findings may have implications for identification of potential pathways associated with EC risk, and progression of pre-neoplastic and early neoplastic disease to clinically evident endometrial cancer.

AUTHOR CONTRIBUTIONS

Sina Rostami: Conceptualization; data curation; formal analysis; methodology; software; visualization; writing – original draft; writing – review and editing. **Trine B. Rounge:** Conceptualization; project administration; resources; software; methodology; supervision; writing – review and editing. **Luca Pestarino:** Methodology; software; visualization; writing – review and editing. **Robert Lyle:** conceptualization; resources; writing – review and editing. **Renée Turzanski Fortner:** Methodology; writing – review and editing. **Øystein Ariansen Haaland:** Methodology; formal analysis; writing – review and editing. **Rolv T. Lie:** Project administration; writing – review and editing. **Fredrik Wiklund:** Project administration; writing – review and editing. **Tone Bjørge:** Conceptualization; project administration; data curation; resources; writing – review and editing. **Hilde Langseth:** Funding acquisition; conceptualization; project administration; methodology; resources; supervision; data curation; writing – review and editing. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

RNA sequencing data generated in this study have been deposited in the database Federated EGA Norway (FEGA Norway) under accession code EGAD50000000391 (https://ega-archive.org/datasets/EGAD50000000391). The data are available under restricted access due to the sensitive nature of data derived from human biological material. The data are available on request provided that the processing has legal basis, and otherwise meets the conditions set out in applicable law. The data may only be used for cancer research purposes, and the recipient

must have ethical approval from the Norwegian Regional Committee for Medical and Health Research Ethics (REC). Access can be obtained by following the procedure described here: Access to ata (kreftregisteret.no) A data Access committee is established at The Norwegian Institute of Public Health, and will evaluate applications for data access. Requests can also be directed to Hilde Langseth, hl@kreftregisteret.no. Script for the differential abundance analysis can be accessed from Github (https://github.com/CancerRegistryOfNorway/ec-rna) and (https://github.com/Rounge-lab/ec-rna). Further information is available from the corresponding authors.

ETHICS STATEMENT

The study was approved by Norwegian Regional Committee for Medical and Health Research Ethics (REC no: 19892). The JSB donors have given broad consent for the use of the samples in cancer research.[17]

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