Comparative evaluation of microRNA detection in plasma, urine and liquid-based cytology for high-grade cervical intraepithelial neoplasia

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Abstract. Liquid biopsy offers a minimally invasive approach for cancer diagnosis by detecting circulating biomarkers, such as microRNAs (miRNAs), in different types of specimens. The present study aimed to identify the most effective specimen for miRNA detection in cervical cancer precursor lesions by comparing plasma, urine and liquid-based cytology (LBC). A total of 798 miRNAs were analyzed using NanoString technology in 24 women, equally divided between those with high-grade cervical intraepithelial neoplasia (2/3) and controls with negative Pap and human papillomavirus tests. Comparative analyses revealed that LBC exhibited the highest detection efficiency, with only 1.75% of miRNAs demonstrating low counts, compared with 20.68% in plasma and 15.79% in urine. Additionally, LBC exhibited 21.3% of miRNAs above the 9th decile of expression, compared with 5.89% in plasma and 2.88% in urine. LBC revealed the most consistent detection performance across samples, establishing its potential as the most effective specimen for detecting cervical cancer precursor lesions biomarkers. Plasma also showed promise as a detection medium, whilst urine exhibited higher variability and lower detection consistency. In conclusion, LBC demonstrated the highest efficacy for miRNA detection among the specimen types tested. Plasma remains a viable alternative, whilst urine presents challenges due to its inherent variability. These results underscore the importance of specimen selection for optimizing diagnostic sensitivity in cervical cancer precursor lesions screening.

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Introduction

Cervical cancer (CC) ranks as the fourth most common cancer worldwide (1). Persistent infection of high-risk (hr)-human papillomavirus (HPV) is the principal cause of CC, initiating carcinogenic processes that involve the accumulation of genetic and epigenetic changes (2).

Liquid biopsy (LB) has emerged as an innovative cancer diagnosis and prognosis approach, offering a non-invasive alternative to traditional tissue biopsies (3). By detecting circulating tumor components such as circulating tumor cells, cell-free DNA, cell-free RNA and exosomes in biological fluids (blood, urine, feces and saliva), LB has demonstrated utility in early cancer detection, personalized treatment decisions and prognostic assessment (3,4). In CC, LB enables the detection of molecular alterations associated with precursor lesions and disease progression, potentially before morphological changes are evident (5).

Identifying molecular markers at an early stage is crucial to improving screening strategies and clinical outcomes (6). Among the sample types used in this context, liquid-based cytology (LBC), which contains exfoliated cervical cells, is widely employed for conventional cytological screening (Pap smear) (7). Whilst it is not traditionally considered a LB, LBC offers a minimally invasive means to collect cellular material and has proven useful for molecular analyses (8).

Plasma is the most utilized specimen in LB research for detecting biomarkers in several types of cancers due to its easy accessibility (9). Although several studies have focused on identifying biomarkers in LBC or blood (9,10), urine can

be easily self-collected recurrently in relatively large volumes and it has greater patient compliance, suggesting that it could be a promising non-invasive alternative for CC screening and monitoring (11). However, technical limitations, such as low RNA yield, concentration variability and enzymatic degradation, may affect its analytical performance (12,13). Given its non-invasive nature, LB using these types of specimens allows the detection of molecular biomarkers, such as HPV DNA (13-15), DNA methylation (12), circulating tumor cells (16) or dysregulated microRNAs (miRNAs/miRs) (10,11), which may indicate cervical carcinogenesis before the progression to high-grade lesions or invasive cancer (5).

miRNAs are small non-coding RNAs that serve essential roles in regulating gene expression at the post-transcriptional level by inhibiting mRNA translation or promoting mRNA degradation (17). They are stable and easily detected in LB samples, and certain studies have demonstrated their role in several biological pathways in CC (18,19). miRNAs identified in LBC (10), plasma (20,21) or urine (11) samples have been directly associated with the diagnosis, treatment monitoring and progression of CC and its precursor lesions. However, most studies have explored the expression of miRNAs in a single specimen using reverse transcription-quantitative PCR (11,20,21), which has limitations such as low throughput, amplification bias and complex normalization steps (22). This highlights the potential of high-throughput digital platforms, such as the NanoString nCounter® Analysis System, which enables comprehensive miRNA profiling without amplification. It also offers high sensitivity, even from degraded samples, such as those obtained from formalin-fixed paraffin-embedded tissues. Such technologies simultaneously facilitate detection of multiple targets, making them valuable for large-scale biomarker studies (22). To date, only one study has employed NanoString technology in this context, to the best of our knowledge (10).

Therefore, the aim of the present study was to identify the most suitable specimen for miRNA-based detection of CC precursor lesions using NanoString nCounter technology to compare miRNA profiles across three specimen types, LBC, plasma and urine, from women with and without high-grade cervical intraepithelial neoplasia (CIN).

Materials and methods

Study population and sample collection. An overview of the methods and key steps of the present study are presented in Fig. 1. The present study included 24 women, with paired LBC, plasma and urine samples collected from each participant at the Prevention Department of Barretos Cancer Hospital, Barretos, Brazil from June 2021 to November 2022. The present study was approved by the Research Ethics Committee of the Barretos Cancer Hospital (Barretos, Brazil; approval no. 3.926.525). All patients signed an informed consent form for their samples to be used in the research.

Eligible participants were between 25 and 64 years old, with no history of cervical precursor lesions, no previous cervical procedures or prior cancer diagnoses. The case group included 12 women with histologically confirmed high-grade cervical intraepithelial lesion (CIN 2/3) (23), confirmed by histopathological examination of the excised tissue obtained through the Loop Electrosurgical Excision Procedure (LEEP). All patients in the case group were tested for hr-HPV. The control group also comprised 12 women, selected based on negative results for both Pap smear cytology and high-risk HPV testing, performed using the Cobas x480™ system (Roche Diagnostics) (24). Patients were matched by age (±2 years). The epidemiological and clinicopathological characteristics of the study participants are summarized in Table I.

LBC cervical samples were collected from each participant immediately before the LEEP (case group) or Pap test (control group). Samples from cervical cell scraping were collected using the Cervex-Brush® (cat. no. 36825G; Rovers Medical Devices B.V.). A total of two cervical smear samples were collected from each participant: One sample was preserved in BD SurePath™ Preservative Fluid (cat. no. 491337; Becton, Dickinson and Company) for morphological and HPV tests, and the other sample was preserved in ThinPrep® Pap test (cat. no. 70097-001; Hologic, Inc.) for miRNA analysis. Blood samples for plasma were collected in anticoagulant tubes, whilst urine samples were collected without any restrictions on time or preparation. All fluids were stored at Barretos Cancer Hospital Biobank (25).

Biological fluids RNA isolation. LBC, plasma and urine total RNA isolation were performed using the miRNeasy Mini Kit (cat. no. 217004; Qiagen GmbH), miRNeasy Serum/Plasma Kit (cat. no. 217184; Qiagen GmbH) and Urine Cell-Free Circulating RNA Purification Mini Kit (cat. no. 56900; Norgen Biotek Corp.), respectively, according to the manufacturer's protocol. The total RNA extracted was stored at -80˚C until use. The total RNA concentration and purity of each sample were measured using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Inc.).

nCounter analysis assays. miRNA expression profiling was performed using the nCounter miRNA Expression Assay Kit (cat. no. CSO-MIR3-12; NanoString; Bruker Spatial Biology, Inc.), according to the manufacturer's protocol, using the nCounter Human v3 miRNA that evaluates 798 miRNAs. In summary, 100 ng total RNA was subjected to tag binding and hybridization with the Reporter CodeSet and Capture ProbeSet for 24 h at 65˚C. Subsequently, the samples were processed using the NanoString PrepStation and immobilized on the nCounter cartridge, which was transferred to the nCounter Digital Analyzer for image capture (555 field-of-views) and data acquisition.

Statistical analysis. Statistical analyses were performed using R version 4.2.3 in RStudio 2023 (26), with the ggplot2 library (27) for constructing graphical analyses. Normalization was performed using the NanoStringNorm R package (version 1.2.1) (28). Background correction and positive control normalization were not applied. Sample content normalization was based on the geometric mean of housekeeping miRNAs, which were selected for their low coefficient of variation (CV) across all samples, as detailed in Table SI. Log transformation was applied to the normalized values. The CV was calculated as the ratio of the standard deviation (SD) to the mean (CV=SD/mean), providing a dimensionless measure of dispersion across datasets with different scales. A reduction in CV

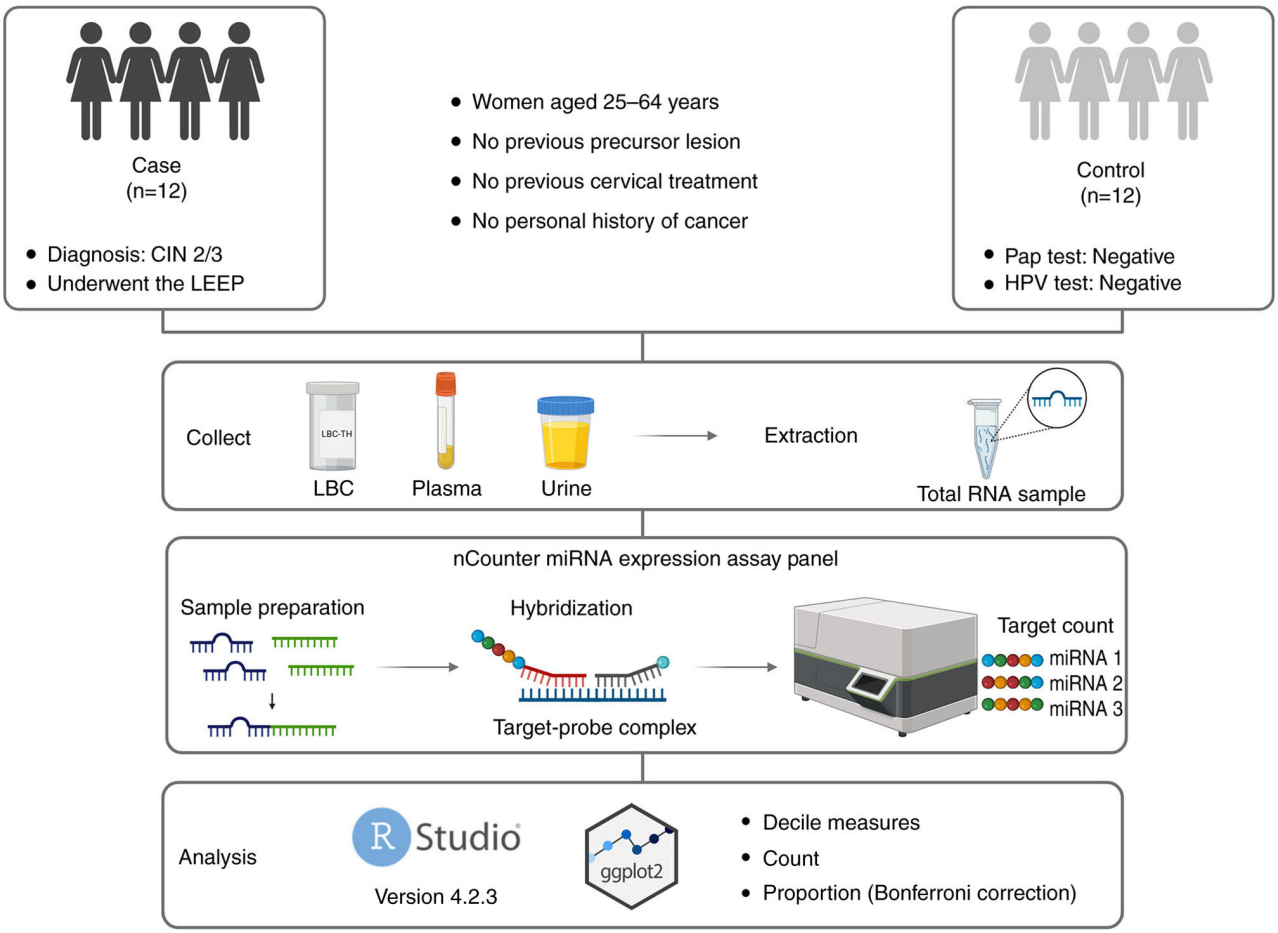

Figure 1. Study overview. Figure created using BioRender (https://www.biorender.com/). CIN, cervical intraepithelial neoplasia/lesion; LEEP, Loop Electrosurgical Excision Procedure; HPV, human papillomavirus; LBC, liquid-based cytology; miRNA, microRNA.

after normalization indicated effective removal of technical noise and improved reproducibility. As CV can be sensitive to small means, the SD was also assessed as an alternative dispersion metric (29).

For data exploration, miRNA deciles, counts and proportions were evaluated. Low-count miRNAs were defined as those with <10 counts in >50% of samples. Bonferroni correction was applied to compare the proportions of low-count miRNAs between case and control groups within each specimen type, as well as across different specimen types.

As no differential expression analysis was performed, and the aim was to assess general detection capacity rather than evaluate specific miRNAs, statistical comparisons of individual miRNAs between groups were not performed. Therefore, no multiple testing correction was applied.

Results

NanoString technology comparison of raw data counts. The distribution of the counts of 798 miRNAs across LBC, plasma and urine samples was analyzed using the raw data. Median miRNA counts were similar between the case and control groups (Tables SII-SIV).

In the raw data analysis, a cutoff was established using the NanoStringNorm package to distinguish between low and high counts. Counts <10 were defined as low, whilst those ≥10 were considered high. Initially, raw data counts were compared in three specimens without stratifying by clinical group. Due to the characteristics of the raw data, statistical tests were not applied and the analysis remained descriptive. The detected miRNA counts in plasma, urine and LBC were analyzed, which demonstrated that LBC exhibited proportionally higher levels of detected miRNAs than plasma and urine (Fig. 2). These results suggest a greater abundance of miRNAs in LBC compared with the other specimens. By contrast, the comparison between plasma and urine revealed no notable difference in the proportions of high and low counts of miRNAs, suggesting comparable levels of miRNA detection between these two fluids.

The technical threshold was set at counts of <10, which were defined as low. A miRNA was considered to have a low count if its expression was <10 in >50% of samples. According to this analysis, LBC revealed a lower percentage of detected miRNAs with low counts (1.75%) compared with plasma (20.68%) and urine (15.79%). This indicates a higher overall detection efficiency of miRNAs in LBC compared with the other fluids. Although plasma and urine demonstrated similar proportions of low-count miRNAs, this indicates comparable levels of detectable miRNAs in these fluids. However, further analysis is needed to determine if similar miRNA species are being captured.

Table I. Participant characteristics and characteristics in case and control groups.

Characteristic	Case group (n=12)	Control group (n=12)
Age, years		
Mean (SD)	32.41 (7.02)	32.91 (6.87)
Median (min-max)	29.00 (25-48)	30.50 (25-48)
Smoking		
Yes	1 (8.33)	0 (0.00)
No	10 (83.33)	11 (91.67)
Former smoker	1 (8.33)	1 (8.33)
Alcohol consumption		
Yes	10 (83.33)	6 (50.00)
No	1 (8.33)	2 (16.67)
Former consumption	1 (8.33)	4 (33.33)
Menopausal status		
Pre-menopause	11 (91.67)	11 (91.67)
Post-menopause	1 (8.33)	1 (8.33)
Use of hormonal contraceptives		
Never	0 (0.00)	2 (16.67)
Currently	6 (50.00)	5 (41.67)
Former	6 (50.00)	5 (41.67)
HPV vaccine		
Yes	0 (0.00)	1 (8.33)
No	12 (100.00)	11 (91.67)
Cytology		
ASC-US	1 (8.33)	0 (0.00)
ASC-H	0 (0.00)	0 (0.00)
LSIL	0 (0.00)	0 (0.00)
HSIL	11 (91.67)	0 (0.00)
Negative	0 (0.00)	12 (100.00)
Consolidated diagnosis[a]		
CIN 2	1 (8.33)	0 (0.00)
CIN 2/3	2 (16.67)	0 (0.00)
CIN 3	9 (75.00)	0 (0.00)
HPV status		
HPV 16	5 (41.67)	0 (0.00)
HPV 18	1 (8.33)	0 (0.00)
Other hr-HPV types	6 (50.00)	0 (0.00)

Categorical data are presented as n (%). [a]Indicates the highest-grade result, whether biopsy or anatomopathological of Loop Electrosurgical Excision Procedure. SD, standard deviation; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells, cannot exclude high-grade intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CIN, cervical intraepithelial neoplasia/lesion; HPV, human papillomavirus; hr-HPV, high-risk HPV.

Comparison of raw data counts between case and control groups. Additionally, a comparative assessment of raw data counts between the case and control groups for each specimen type was performed (Fig. 3). The results demonstrated differences in the proportions of low-count miRNAs between the case and control groups in LBC (case, 1.75%; control, 3.26%) and urine (case, 16.04%; control, 18.05%). This suggests that certain miRNAs presented a higher abundance in the case group compared with in the control group. However, in plasma, the proportions were similar between the case (22.06%) and control (22.43%) groups, indicating no difference in low-count miRNAs for this specimen type.

Comparison of normalized data counts. The distribution of the counts of 798 miRNAs across LBC, plasma and urine samples was analyzed, using the normalized data. Median miRNA counts were similar between the case and control groups

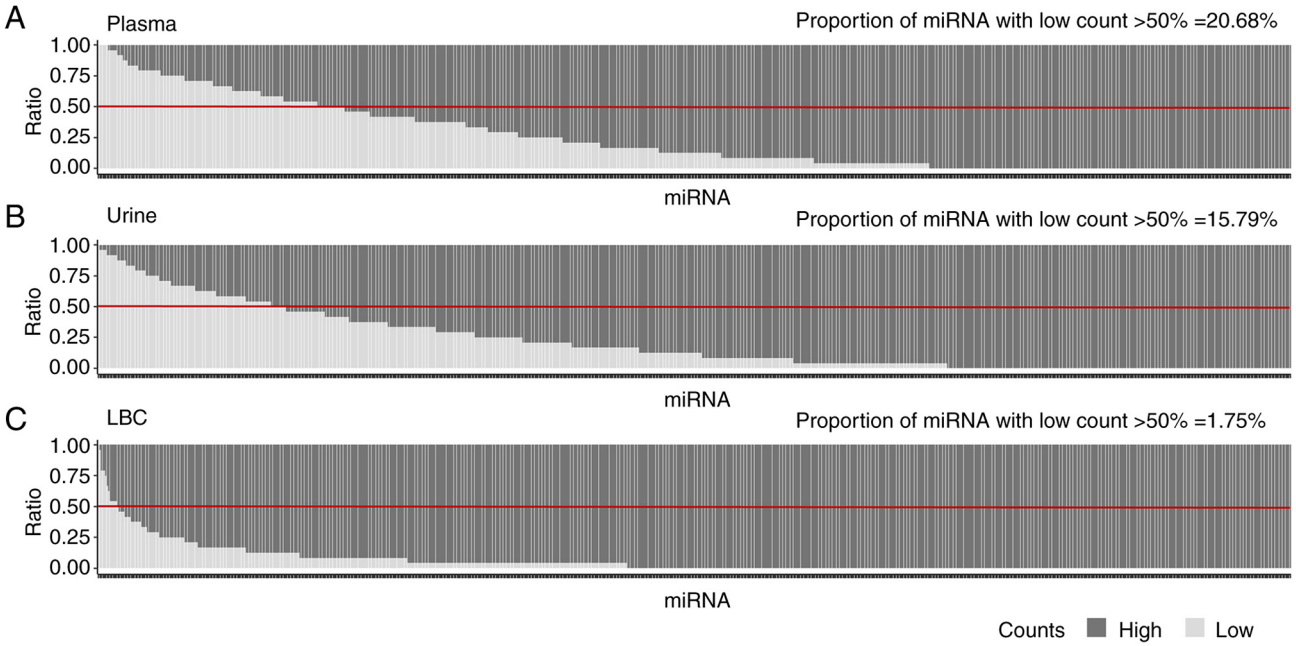

Figure 2. Raw data count comparison chart of 798 miRNAs between the three specimens. (A) Plasma. (B) Urine. (C) LBC. The y-axes present the proportions of counts (<10) of each miRNA in the raw data, and the x-axes present the 798 miRNAs. The red lines indicate 50% of the samples. miRNA, microRNA; LBC, liquid-based cytology.

(Tables SIV-SVII) and box plots demonstrated variability across the sample types (Fig. S1).

Subsequently, the mean normalized counts of each detected miRNA were compared across the three specimen types using the normalized data (Fig. 4). Density plots were generated to visualize the distribution of mean miRNA counts in LBC, plasma and urine (Fig. 4). The 9th decile (5.225) was used as a reference to identify regions with extreme values of mean miRNA counts, allowing for a clear comparative analysis of miRNA count distributions between the different specimens. This approach highlighted differences and extreme values in the density plots, facilitating the visualization of distinctive characteristics between the analyzed fluids. Additionally, the proportions of miRNA counts above the 9th decile were calculated for each specimen: LBC vs. plasma, LBC vs. urine, and plasma vs. urine (Table II). The comparisons revealed that LBC was superior to the other specimens, with a significantly higher concentration of miRNAs with counts >9th decile (21.3%), compared with plasma (5.89%) ($P<0.01$; Fig. 4A; Table II) and urine (2.88%) ($P<0.01$; Fig. 4B; Table II). Furthermore, the difference in miRNAs with high counts >9th decile between plasma (5.89%) and urine (2.88%) was significant ($P<0.01$; Fig. 4C; Table II).

Discussion

The early detection of CC precursor lesions is crucial for improving patient prognosis, as timely intervention can markedly reduce progression to invasive cancer (5). LB provides a unique opportunity to identify molecular changes at an early stage, even before morphological alterations become evident in conventional cytology or histopathology (6). In this context, circulating miRNAs have emerged as promising biomarkers, allowing the detection of early oncogenic alterations and enhancing the sensitivity of current screening strategies (30,31). However, selecting the most appropriate specimen for LB-based diagnostics remains a critical challenge (32). Therefore, the present study performed a comparative analysis of plasma, urine and LBC as potential specimens for CC precursor lesions detection, using raw and normalized counts of detected miRNAs.

The initial analysis revealed substantial differences in the levels of detected miRNAs between plasma, urine and LBC. LBC exhibited higher levels of detected miRNAs than plasma and urine, suggesting its potential superiority in capturing miRNA biomarkers for the detection of CC precursor lesions. This observation is in-line with previous studies highlighting LBC as a potential specimen for LB-based identification of CC precursor lesions (10,33). By contrast, plasma and urine demonstrated comparable levels of detected miRNAs, indicative of a relative similarity between these two specimens. Although plasma and urine demonstrated comparable levels of detected miRNAs, plasma remains the preferred specimen for LB because it directly reflects systemic processes and captures miRNAs released from circulating cells (34).

Although there was no primary aim to compare diagnostic groups in the present study, an exploratory analysis of raw miRNA counts was performed to assess differences in detection between the case and control groups. However, no differences were observed, suggesting that interindividual variability, rather than biological factors, may underlie the similar detection rates. This is consistent with previous studies reporting that circulating miRNA profiles are affected by factors such as cellular origin, sample handling and inherent physiological variation between individuals (35,36). Given the small sample size and early stage of the disease, such variability is expected and has been cited as a challenge in LB interpretation (37,38). These findings are therefore

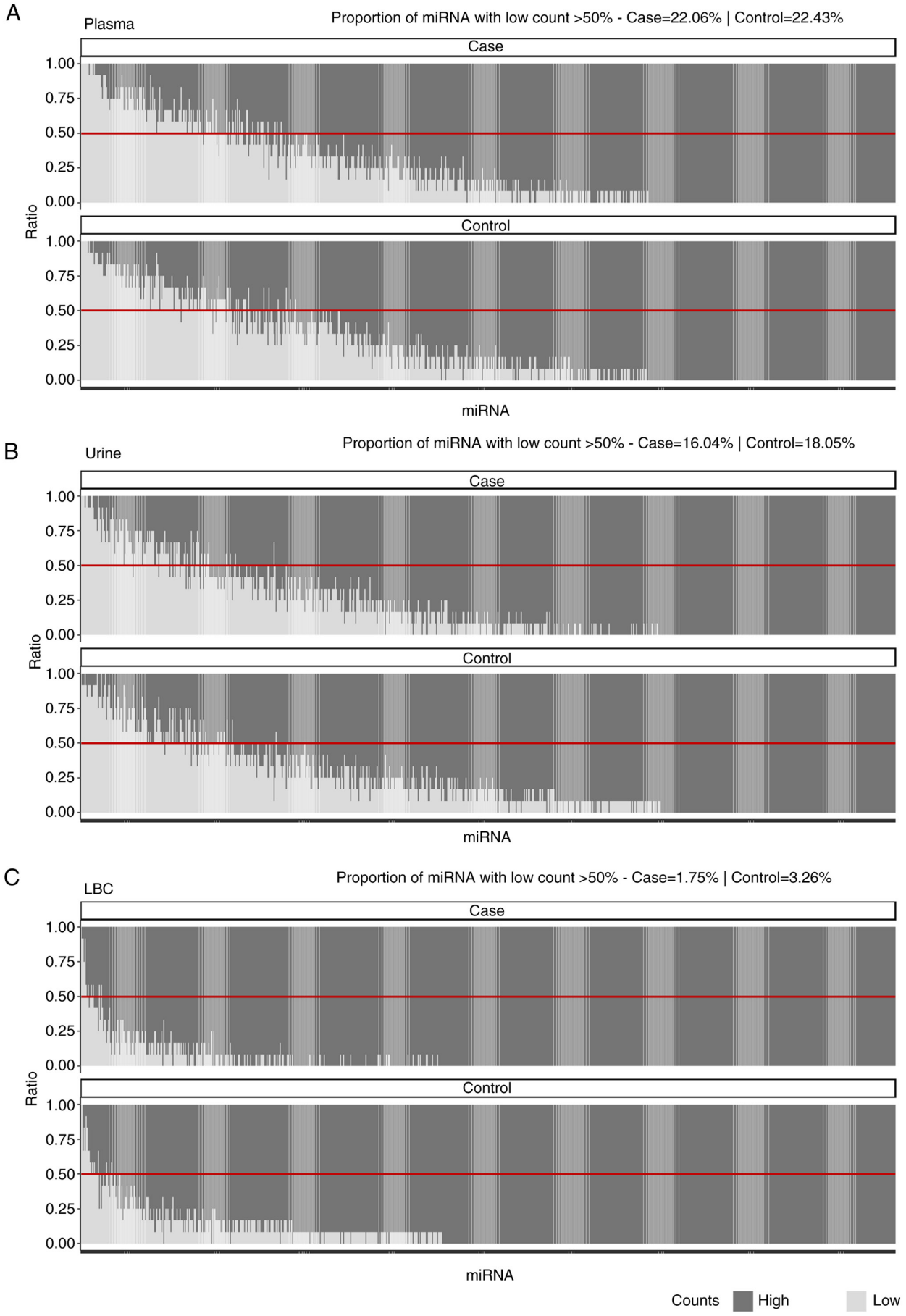

Figure 3. Raw data count comparison chart of 798 between the case and control groups for each specimen. (A) Plasma. (B) Urine. (C) LBC. The y-axes present the proportions of counts (<10) of each miRNA in the raw data, and the x-axes present the 798 miRNAs. The red lines indicate 50% of the samples. LBC, liquid-based cytology; miRNA, microRNA.

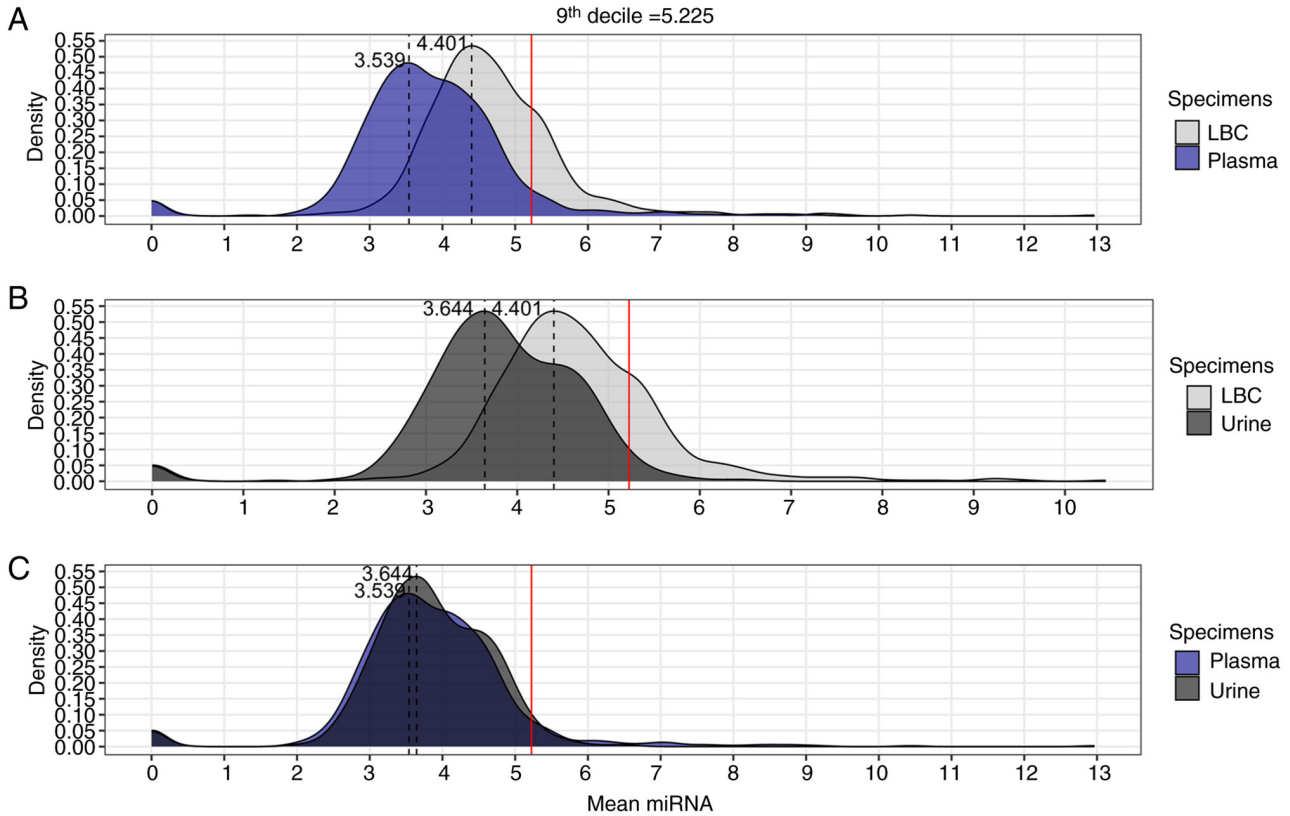

Figure 4. Based on normalized data, density curves show the mean count values for the 798 miRNAs. (A) LBC vs. plasma. (B) LBC vs. urine. (C) Plasma vs. urine. The y-axes present the density values, whilst the x-axes present the sample mean values of the detected miRNAs. The red lines indicate the 9th decile. miRNA, microRNA; LBC, liquid-based cytology.

complementary and do not support differential expression analysis.

Data normalization provided more information about the diagnostic utility of plasma and urine. Scatterplots demonstrated uniformity and similarity in detected miRNA levels between plasma and LBC, highlighting the potential of plasma as a promising specimen for miRNA detection. By contrast, urine revealed greater variability in miRNA levels, posing challenges to diagnostic accuracy. Density plots accentuated the disparities between plasma and urine, with plasma demonstrating a distribution profile more similar to LBC. This observation underlines the diagnostic superiority of plasma over urine, as the latter exhibited notable differences with the standard specimen. Moreover, the findings of the present study highlight the importance of selecting the most appropriate specimen for LB-based diagnostics in CC precursor lesions. Although LBC has emerged as a pioneer in capturing miRNA biomarkers, plasma exhibited a comparable performance, offering advantages in diagnostic accuracy and clinical utility (39,40). The aforementioned observations align with previous studies that emphasize the diagnostic potential of plasma-derived miRNAs in CC and its precursor lesions (21,41). The robustness of plasma as a specimen for LB can be attributed to its direct reflection of systemic processes and release of miRNA from circulating cells (4,42), making it a valuable resource for early cancer detection and monitoring.

By contrast, urine, despite its non-invasive collection method and potential usefulness in certain scenarios, presents a challenge due to its inherent variability in miRNA levels. This variability can be attributed to contamination and compositional differences (43), which need to be addressed when utilizing urine for LB-based diagnosis. However, despite the aforementioned challenges of using the NanoString technology in the present study, other studies have reported the use of miRNAs as biomarkers in urine using quantitative PCR. Aftab *et al* (11) identified a combination of miR-145-5p, miR-218-5p and miR-34a-5p in urine that achieved notable results. This combination showed 100% sensitivity and 92.8% specificity in distinguishing between patients with pre-cancer and cancer from healthy controls. Moreover, the levels of these miRNAs in urine were associated with those observed in serum and tumor tissues. Notably, the expression of miR-34a-5p and miR-218-5p emerged as independent prognostic factors for the overall survival of patients with CC (11).

Furthermore, the NanoString nCounter system served a key role in improving the reliability of the findings in the present study by delivering consistent and reproducible miRNA quantification across all specimens. Its ability to handle multiple samples simultaneously facilitated a comprehensive comparison of miRNA profiles between LBC, plasma and urine. Additionally, the sensitivity of the platform in detecting miRNAs, even from degraded samples, allowed the generation of robust expression profiles, supporting the exploration of miRNAs as potential biomarkers for CC screening (44).

The present has several notable strengths, particularly its comprehensive approach to evaluating multiple specimens (LBC, plasma and urine) for miRNA detection in CC precursor lesions. It provides valuable insight into identifying the optimal

Table II. Comparison of the proportions of microRNA counts >9th decile between liquid-based cytology, plasma and urine samples.

A, LBC vs. plasma

Specimen	Count	Proportion, %	P-value
LBC	170/798	21.3	<0.01
Plasma	47/798	5.89	

B, LBC vs. urine

Specimen	Count	Proportion, %	P-value
LBC	170/798	21.3	<0.01
Urine	23/798	2.88	

C, Plasma vs. urine

Specimen	Count	Proportion, %	P-value
Plasma	47/798	5.89	<0.01
Urine	23/798	2.88	

Test of Proportions with Bonferroni correction. LBC, liquid-based cytology.

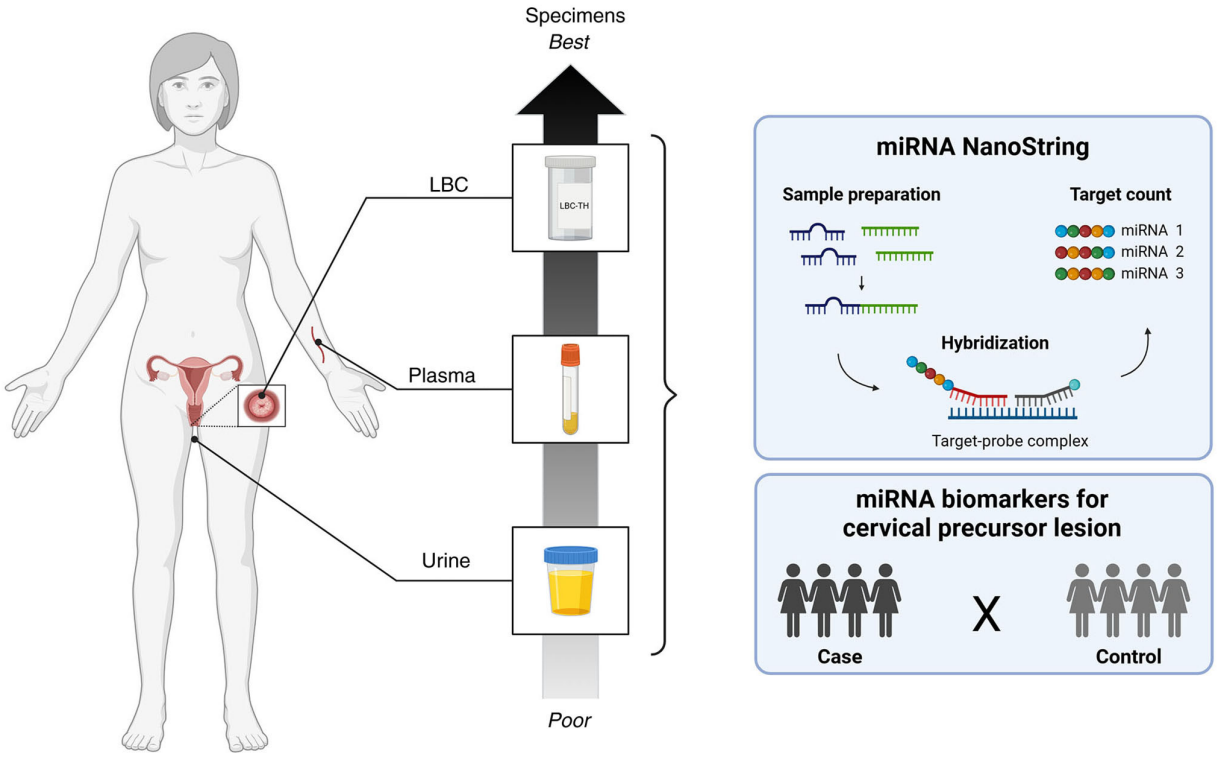

Figure 5. Comparison of LBC, plasma and urine for the detection of miRNA biomarkers in cervical cancer precursor lesions. The gradient arrow indicates the classification of specimens from more to less effective using NanoString technology. Figure created using BioRender (https://www.biorender.com/). LBC, liquid-based cytology; miRNA, microRNA.

medium for non-invasive CC screening. Moreover, the use of NanoString technology to analyze a broad panel of 798 miRNAs strengthens the reliability of the data by providing a comprehensive assessment of miRNA expression levels across specimens. However, despite the valuable insights gained from the comparative analysis of specimens for CC precursor lesions

detection, several limitations restrict the interpretation and generalization of the findings. For example, the present study did not include groups with low-grade lesions (CIN 1) and CC. Their inclusion would have enabled a more comprehensive analysis of miRNA detection across disease stages. Without these groups, conclusions on the effectiveness of LBC, plasma and urine as specimens are limited. The modest sample size and inherent heterogeneity of the study population may also limit the statistical power and robustness of the conclusions. In addition, due to the limited number of samples, no differential expression analysis was performed to identify specific miRNAs between groups. This decision was made to avoid generating potentially unreliable results without adequate statistical power. Considering this, the present study focused on evaluating the overall detection performance of NanoString technology across different specimen types. Future studies with larger cohorts will allow for robust differential expression analyses to explore biomarker candidates with greater statistical confidence. Furthermore, the cross-sectional design of the study precludes the establishment of causal relationships, and the biological variability inherent in circulating miRNA levels may have confounded the results. Addressing these limitations through large-scale studies with standardized protocols and comprehensive biomarker panels will be critical to advancing the field of LB-based diagnostics in CC and its precursor lesions.

In conclusion, the results of the present study demonstrate the superiority of LBC, followed by plasma, over urine when using NanoString technology to detect miRNA biomarkers in CC precursor lesions and control groups (Fig. 5). The comparative analysis highlights the diagnostic superiority of plasma over urine in capturing miRNA biomarkers for CC precursor lesions detection, whilst LBC stands out as the most effective specimen overall. These findings provide valuable information for clinicians and researchers in selecting the most appropriate specimen for LB-based diagnostics, paving the way for greater diagnostic accuracy and improved patient outcomes in treating CC and its precursor lesions.

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Availability of data and materials

The raw and normalized NanoString data generated in the present study may be found in the National Centre for Biotechnology Information Gene Expression Omnibus under accession number GSE302097 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE302097. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

SC and AJAF developed and led the study, performed data reviews and analyses, and prepared the manuscript. JCPR helped with the study design. RLC assisted with the NanoString experiments. WH participated in the data analysis. RDR helped with the study design and critically reviewed the manuscript. RMR helped with the study design, provided advice during the study development and critically reviewed the manuscript. MMCM conceived and guided the development of the study and critically reviewed the manuscript. SC and AJAF confirm the authenticity of all the raw data. All authors have substantially revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Barretos Cancer Hospital (approval no. 3.926.525). Each research participant provided written informed consent for their samples to be used in the research. All information that could be used to identify the study participants was kept confidential and encrypted in a secure database to ensure full confidentiality of clinical information, laboratory findings and the anonymity of each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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