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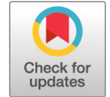
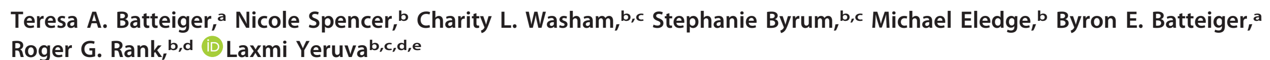


Endocervical miRNA Expression Profiles in Women Positive for *Chlamydia trachomatis* with Clinical Signs and/or Symptoms Are Distinct from Those in Women Positive for *Chlamydia trachomatis* without Signs and Symptoms



Teresa A. Batteiger,[a] Nicole Spencer,[b] Charity L. Washam,[b,c] Stephanie Byrum,[b,c] Michael Eledge,[b] Byron E. Batteiger,[a] Roger G. Rank,[b,d] Laxmi Yeruva[b,c,d,e]

[a]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA
[b]Arkansas Children's Hospital Research Institute, Little Rock, Arkansas, USA
[c]Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA
[d]Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA
[e]Arkansas Children's Nutrition Center, Little Rock, Arkansas, USA

ABSTRACT *Chlamydia trachomatis* is the leading cause of sexually transmitted infections that may progress to pelvic inflammatory disease and infertility. No effective vaccine exists for *Chlamydia*, nor are there biomarkers available that readily predict disease progression. In this cross-sectional pilot study, we recruited symptomatic and asymptomatic women with *C. trachomatis* (CT) infection and asymptomatic, uninfected control women from an urban sexually transmitted disease clinic to determine if there were differences in microRNA (miRNA) expression. Infected women with signs and/or symptoms (CTSS) have distinct miRNA profiles compared to asymptomatic infected women (CTNS). In the CTSS group, miR-142 and -147 showed 2.2- to 6.9-fold increases in expression. In the CTNS group, miR-449c, -6779, -519d, -449a, and -2467 showed 3.9- to 9.0-fold increases in expression. In the CTNS group, cyclins and cell cycle regulation and IL-17 pathways were likely downregulated, while the same signaling pathways were upregulated in the CTSS group. In addition, in the CTSS group, additional inflammatory pathways associated with TNFR1 and IL-8 appear to be upregulated. The miRNA expression patterns differ between CT-infected symptomatic and asymptomatic women, and these differences may warrant further study.

KEYWORDS *Chlamydia*, miRNA, signs and symptoms, endocervix, inflammation

Chlamydia trachomatis (CT) is the most common bacterial cause of sexually transmitted infections (STI) worldwide, with over 1.7 million cases reported in the United States in 2018 (1). Infection rates are highest among adolescents and young adults aged 15 to 24 years. Additionally, higher rates are reported among women and African-Americans. Some CT infections in women are asymptomatic and require screening efforts to diagnose. A proportion of CT infections will progress to pelvic inflammatory disease (PID). PID may be symptomatic, but may also present with vague or nonspecific symptoms or may be asymptomatic. Diagnosis is based on imprecise clinical findings, which may vary by provider and experience of the provider. Given these diagnostic limitations, mild or asymptomatic episodes of PID may not be recognized or treated, which may result in ectopic pregnancy, tubal factor infertility, and chronic pelvic pain (2–6). There are currently no known biomarkers in humans that predict risk for progression to upper genital tract disease. Furthermore, if there are

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Address correspondence to Laxmi Yeruva, vlyeruva@uams.edu.

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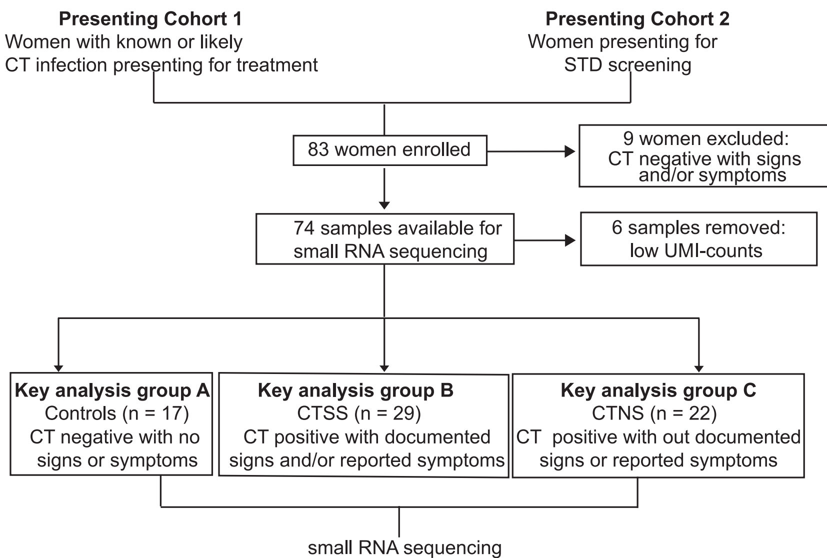

FIG 1 Study design. Cross-sectional study design shows the recruitment strategy to populate the control, CTSS, and CTNS groups.

specific biomarkers that are associated with progression of disease, it is not known how these could be implemented clinically.

One potential biomarker of interest is noncoding micro (~22-nucleotide) RNAs (miRNAs or miRs). These are stable, widely distributed molecules associated with inflammatory responses to human pathogens (7). The role of miRNAs in chlamydial pathogenesis has been demonstrated in both animal models and chlamydia-infected humans (7–10). Additionally, in a murine model of chlamydial genital infection, we have demonstrated that a unique set of miRNAs present in cervical tissue early in infection is associated with progression to upper tract disease (hydrosalpinx). These particular miRNAs were associated with infection by virulent but not avirulent chlamydial strains (8). However, it is not known if miRNAs can be measured from a human cervical swab and whether miRNA patterns detected in cervical samples from women with chlamydial infection are associated with signs and symptoms. Therefore, for this pilot study we evaluated the patterns of miRNAs in women with CT infection with symptoms and/or signs, women with CT infection without symptoms or signs, and women without CT infection and without symptoms or signs. Moreover, we utilized material obtained directly from the endocervical region, which is more likely to accurately reflect the physiological processes.

RESULTS

Cohort characteristics. There were 83 participants enrolled in the study. Nine were excluded due to the presence of signs and/or symptoms with a negative CT test. Of the 74 which remained, 6 samples did not yield RNA sequencing results (Fig. 1). The remaining 68 participants were included in this analysis. There were 29 participants included the CTSS group, 22 in the CTNS group, and 17 in the control group. Study participants' age ranged from 18 to 34 years. The majority of participants were African-American ($n = 38$, 55.8%), followed by Caucasian ($n = 23$, 33.8%). The most commonly reported symptom was vaginal discharge ($n = 14$, 48.3%) and endocervical mucopus ($n = 6$, 20.7%) was the most commonly documented sign. Combinations of symptoms and signs were recorded in 13.8% of the women. There were 3 *Neisseria gonorrhoeae* (NG) and 4 *Trichomonas vaginalis* (TV) coinfections found in the cohort (Table 1).

miRNA expression profile using small RNA sequencing. We performed miRNA expression analysis on samples from women infected with CT with and without signs and symptoms and compared to the chlamydia-uninfected women without signs and symptoms. In the CTSS group, 379 miRs were differentially expressed, with 114 and 127

TABLE 1 Participant characteristics of the control, CTSS, and CTNS groups

Characteristic	Controls ($n = 17$)	CT^+ with signs and/or symptoms (CTSS, $n = 29$)	CT^+ without signs and/or symptoms (CTNS, $n = 22$)
Median age, yr (range)	26 (19–34)	21 (18–33)	22 (18–33)
No. by race (%)			
African-American	8 (47%)	19 (65.5%)	11 (50%)
Caucasian	4 (23.5%)	8 (27.6%)	11 (50%)
Other (including Hispanic)	4 (23.5%)	2 (6.9%)	0
No. by STI test[a]			
CT	0	30	22
NG	2	1	0
TV	0	3	1[b]
No. by reason for visit (%)			
Screening	13 (76.5%)	2 (6.9%)	0
+ CT test	0	24 (82.8%)	20 (90.9%)
CT contact	4 (23.5%)	3 (10.3%)	2 (9.1%)
No. by symptoms[e] (%)			
Discharge	–	14 (48.3%)	–
Abdominal pain	–	4 (13.8%)	–
Dysuria	–	3 (10.3%)	–
Vaginal bleeding (alone)	–	0	–
Multiple symptoms[c]	–	4 (13.8%)	–
No. by signs[e] (%)			
Mucopus	–	6 (20.7%)	–
Ectopy	–	4 (13.8%)	–
Combination of signs[d]	–	4 (13.8%)	–

[a]TV, *Trichomonas vaginalis*; NG, *Neisseria gonorrhoeae*.
[b]1 positive TV and one indeterminate result for TV.
[c]Combinations of symptoms included discharge + dysuria ($n = 1$, 3.4%); discharge + genital itching ($n = 1$, 3.4%); dysuria + vaginal spotting ($n = 1$, 3.4%); other + back pain ($n = 1$, 3.4%).
[d]Combinations of signs included ectopy + mucopus ($n = 3$, 10.3%), ectopy + mucopus + friability ($n = 1$, 3.4%).
[e]-, no symptoms or signs.

unique miRs up- or downregulated, respectively, relative to the control group (Fig. 2A to C). In the CTNS group relative to control, 244 miRs were altered (Fig. 2A). Of these, 71 were upregulated and 35 were downregulated only in the CTNS group (Fig. 2A to C). There were 139 common miRs between the CTSS and CTNS groups, with 13 upregulated and 125 downregulated relative to the control group (Fig. 2A to C).

We used Ingenuity Pathway Analysis (IPA) to determine which of the identified miRs have experimentally validated gene targets. The IPA tool identified 24 unique miRs in the CTSS and 11 in the CTNS groups relative to controls (Fig. 3A). Of these, 10 were upregulated and 13 were downregulated in the CTSS group (Fig. 3B). In the CTNS group, 8 were upregulated and 2 were downregulated (Fig. 3B). The heat map shows all the miRs with significantly different expression in the CTSS and CTNS groups relative to controls (Fig. 3C and D). Within the CT-infected groups, there were 14 miRs in common (2 miRs upregulated and 14 miRs downregulated), suggesting these miRs likely were altered due to infection. Tables S2 and S3 in the supplemental material provide the full list of miRs differentially expressed in the CTSS and CTNS groups relative to the control group.

miRNA expression validation. We conducted real-time PCR to confirm some of the unique miRs altered in the CTSS and CTNS groups relative to the control group. We found miR-142, -520, and -147 to be significantly upregulated relative to control in the CTSS group (Fig. 4A). In addition, in the CTNS group, miR-449a, -449c, -6779, and -2467 were found to have significant upregulation, while miR-519d trended toward increased expression ($P < 0.08$) in comparison to the control group (Fig. 4B).

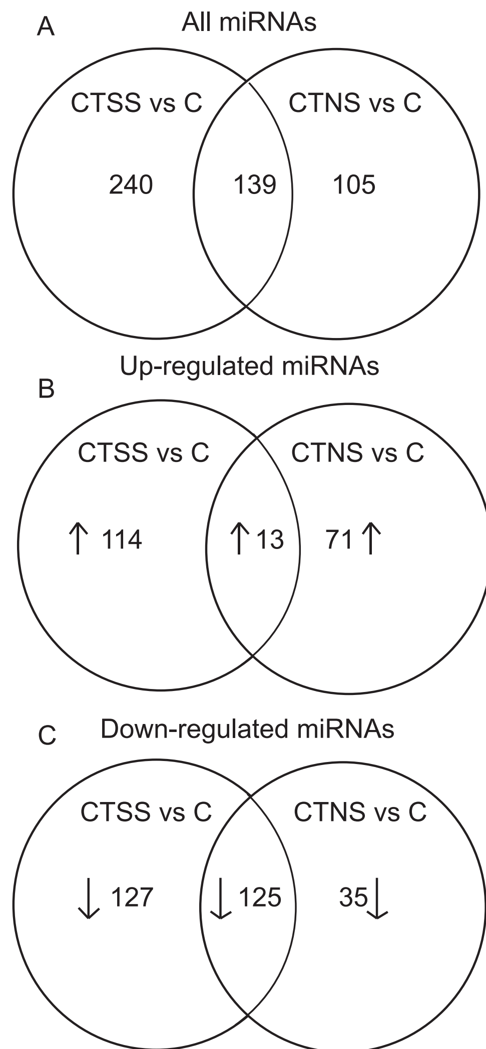

FIG 2 Endocervical miRNA expression in chlamydia-infected patients with (CTSS) and without (CTNS) symptoms, in comparison to controls with no chlamydia infection. (A) Venn diagram of the unique and shared miRNAs in CTSS and CTNS women relative to controls. (B) Venn diagram of the upregulated miRNAs in CTSS and CTNS women in comparison to controls. (C) Venn diagram of the downregulated miRNAs in CTSS and CTNS women relative to controls. The data represent values from 17 controls, 23 CTNS, and 29 CTSS women.

Functional analysis of target genes. IPA-predicted gene numbers for upregulated miRs are shown in Fig. 5. We observed 102 unique genes in the CTSS group and 72 unique genes in the CTNS group from the target prediction of upregulated miRs (Fig. 5A). To further understand the pathways possibly regulated in the CTSS and CTNS groups, pathway analysis was conducted by IPA. The top 25 pathways are shown in Fig. 5B and C. In addition, 293 unique genes in the CTSS group and none in the CTNS group were observed from the target-prediction analysis of downregulated miRs (Fig. 6A), and the pathways are shown in Fig. 6B. The full lists of genes and pathways regulated by miRs are presented in Tables S4 and S5. The common pathways likely impacted in the CTSS and CTNS groups are presented in Fig. S1.

DISCUSSION

To explore the possibility that an miRNA expression profile from a local site of infection could be used as a prognostic indicator of signs and symptoms, the miRNA profile in women infected with CT with and without signs and symptoms was evaluated. In this pilot study, we observed distinct miRNA profiles in the

FIG 3 miRNAs sorted based on experimentally confirmed target genes in CTSS and CTNS patients in comparison to controls. (A) Venn diagram showing unique and shared miRNAs based on experimentally confirmed gene targets in CTSS and CTNS groups relative to controls. (B) The sorted miRNAs differential expression (up and down) in CTSS and CTNS women relative to controls. (C) The heat map shows all the miRs significantly differentially expressed in the CTSS relative to controls. (D) The heat map shows all the miRs significantly differentially expressed in the CTNS relative to controls. The data represent values from 17 controls, 23 CTNS, and 29 CTSS women.

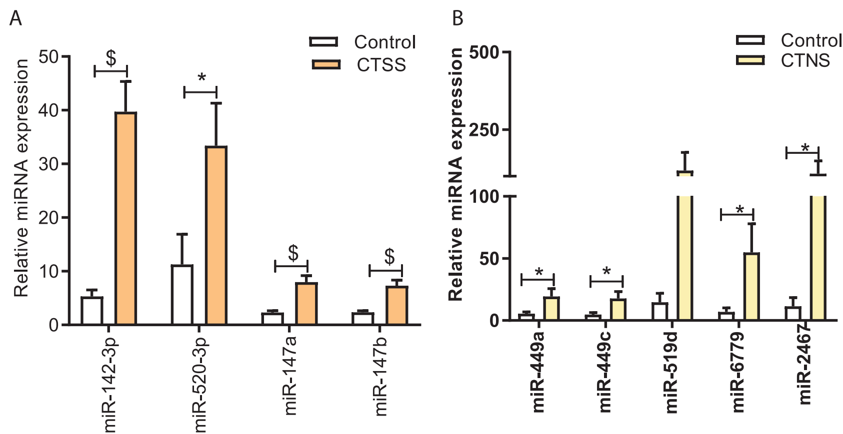

FIG 4 miRNA expression confirmation by real-time PCR. (A) The graph shows the miRNA expression in CTSS relative to controls. (B) The graph shows miRNA expression in CTNS relative to controls. The data are presented as mean ± standard error of the mean (SEM) and the values represent 17 controls, 23 CTNS, and 29 CTSS women. Data were analyzed by two-tailed *t* test between control and CTSS or CTNS women ($^{\$}$, $P < 0.0001$; *, $P < 0.05$).

endocervical samples of CTSS and CTNS groups. To the best of our knowledge, this is the first time an endocervical swab miRNA profile was associated with clinical signs and symptoms. Moreover, miRNAs are quite stable and are involved in regulating the signaling pathways, so there is the potential to identify specific miRNAs in future longitudinal studies that could be predictive of chronic inflammatory and/or fibrotic events.

In the CTSS group, downregulation of genes responsible for T and B cell receptor signaling, gap junction, activated and anergic T lymphocytes, and IL-1 was observed. This is consistent with previously published results from patients with PID, where T cell, B cell, NK cell, lymphocyte proliferation and activation, and protein synthesis genes were downregulated in blood samples (9). The data suggest that the transcriptome blood profile observed in patients with PID is similar to the CTSS group of women in our study. The miRs observed in the CTNS group have been shown to downregulate genes that impact cyclins and cell cycle regulation and Th1 and Th2 activation pathways (Fig. 5B), while the same pathways are upregulated in the CTSS group

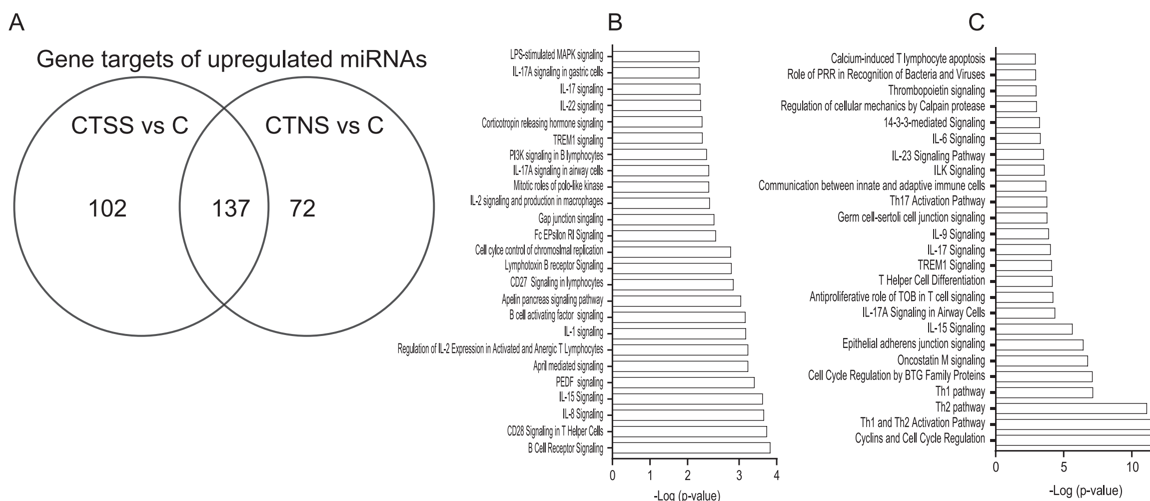

FIG 5 Target predicted genes and enriched pathways of upregulated miRs in CTSS and CTNS in comparison to controls. (A) A Venn diagram of target predicted genes for upregulated miRNAs in CTSS and CTNS women relative to control. (B) Ingenuity Pathway Analysis of genes for upregulated miRNAs in CTSS and CTNS women relative to control. The data represent values from 17 controls, 23 CTNS and 29 CTSS women.

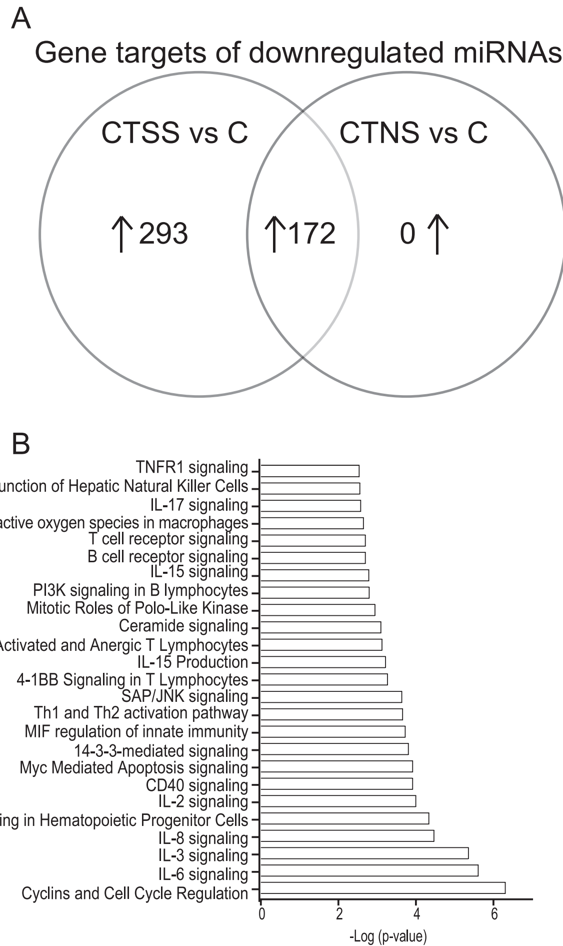

FIG 6 Target predicted genes and enriched pathways of downregulated miRs in CTSS and CTNS in comparison to controls. (A) A Venn diagram of target predicted genes for downregulated miRNAs in CTSS and CTNS patients relative to control. (B) Ingenuity Pathway Analysis of genes for downregulated miRNAs in CTSS patients relative to control. The data represent values from 17 controls, 23 CTNS and 29 CTSS women.

(Fig. 6B). Furthermore, in CTSS, TNRFR1, IL-17, ceramide, and IL-8 pathways are likely upregulated (Fig. 6B). Previously, the roles of IL-17 and TNFR1 in inflammation and pathology have been shown during chlamydial infection in mouse models (11–13), and the IL-17 pathway was significantly upregulated in endometritis with *Chlamydia* infection (9). IL-8 signaling is observed both in up- and downregulated pathways in the CTSS group of women, suggesting complex regulation of the molecular signaling during inflammation and disease progression.

The Tamsyn et al. study of Gambian children demonstrated that a specific miRNA expression profile was associated with the late-stage ocular disease (trachoma), and expression profiles of miRNAs between trachoma scarring and trachoma scarring with inflammation were different (14). The follow-up study from Tamsyn et al. demonstrated that upregulation of miR-155 was associated with conjunctival inflammation (15). In the current study, miR-16-5p was significantly downregulated in the CTSS group of women. The target gene for miR-16-5p is transcription factor SPI1. SPI1 switches fibroblasts from inflammatory to fibrotic in conjunction with miR-155. Inhibition of both miR-155 and SPI1 suppressed the profibrotic gene expression while still inducing inflammatory and metalloproteinase expression, suggesting the activation of other inflammatory signaling pathways (16). Not surprisingly, inflammatory pathways such as IL-8 and TNFR-1 were predicted to be upregulated by the miRs expressed in the CTSS but not in the CTNS group of women. Furthermore, Derrick et al. showed an association of miR-147

expression with inflammatory trachomatous scarring in Gambian adults, while miR-155 was associated with ocular chlamydial infection and trachoma development in Guinea-Bissau children (16, 17). Our data showed significantly higher expression of miR-142 and miR-147 in the CTSS group, while miR-155 expression was observed in both the CTNS and CTSS groups. In the mouse model, there is an association of cervical miR-142 and miR-147 expression on day 4 to pathology on day 35 (8). Our data suggest that miR-155 may be a marker of infection and that miR-142 and/or miR-147 may be markers for signs or symptoms. Recently it has been shown that GU-rich miRs (i.e., miR-142 and miR-147) bind to TLR7/TLR8 and induce inflammation in cancer, arthritis, and ischemia models (18–20). We saw that miR-142 and -147 are upregulated in the CTSS but not in the CTNS group of women. It is possible that these miRs play a role in inflammation in human infection. Previously, Igietseme et al. (21) demonstrated that *C. trachomatis* infection-induced miRs control epithelial mesenchymal transition (EMT) and fibrosis in a mouse model, further supporting our results. Our data, along with the published literature from the animal model and clinical studies, suggest that inflammatory and fibrotic genes are tightly regulated by a complex network of transcription and post-transcriptional events. Additionally, these data warrant future studies to determine if miR-16 along with miR-142 and miR-147 could serve as marker(s) of progression for fibrosis.

There are several limitations of this study. This was a cross-sectional study of women with and without chlamydial infection. There was no longitudinal follow up to assess changes in miRNAs following treatment, documentation of duration of infection prior to enrollment in the study, history of chlamydial infection, or possible alterations associated with hormonal changes. We captured coinfections with gonorrhea and trichomonas but did not capture concomitant yeast infections or the presence of bacterial vaginosis, which may also have impacted miRNA expression profiles.

This study generated additional questions which warrant further research, including the question of why the miRNA expression profile is different in the CTNS group compared to the CTSS group? This could potentially be due to host response to infection, inoculum size at initial exposure, or possibly due to the virulence of the infecting *Chlamydia*. Data from a guinea pig model showed infection outcome was dependent upon the ratio of attenuated versus virulent chlamydial variants (22). It is currently unknown if the CT-infected women have different genetic compositions of chlamydiae. It is also possible that microbiota changes (23–25) and hormonal changes at the time of infection could dictate the miR expression pattern or result in differences in the development of symptoms. Future studies should include women with signs and symptoms without *Chlamydia* infection and also coinfection groups (NG/TV/bacterial vaginosis) to determine if miRNA expression patterns are specific to chlamydial infection.

In conclusion, miRNA expression is different in chlamydia-infected groups compared to the control group. Additionally, there were differences in miRNA expression between the chlamydia-infected group with signs and/or symptoms and the chlamydia-infected group without signs and/or symptoms. Further longitudinal studies are needed to assess changes in miRNA expression following treatment and to determine whether biomarker development is useful or feasible.

MATERIALS AND METHODS

Study population. We prospectively recruited women aged 18 to 35 years who presented to the Marion County Public Health Department Bell Flower STD Clinic (BFC) in Indianapolis, IN, from March 2016 to October 2017 for enrollment in this cross-sectional study. These women were presenting to the clinic for treatment of known CT infection or as a contact to a man with known CT infection within the last 30 days (Presenting Cohort 1) or for screening for STIs (Presenting Cohort 2). Women were excluded if they were pregnant, living with HIV, had known coinfection with *N. gonorrheae* or *T. vaginalis* prior to enrollment, were receiving immunosuppressive therapy, participated in a clinical trial in the last 30 days, received systemic antimicrobials within the prior 30 days, or had known renal, hepatic, or hematological disease. Women with symptomatic or asymptomatic bacterial vaginosis were not excluded.

Study design. Women were recruited based on likelihood of CT infection to populate the three Key Groups of subjects (Fig. 1). Cohort 1 was considered to have a higher likelihood of CT infection and

Cohort 2 was less likely to have CT infection. Key Analysis Group A was the control population, women who were CT uninfected without reported symptoms and no signs on a physical exam. Key Analysis Group B (CTSS) was defined as women who were CT infected with reported symptoms and/or signs documented on a physical exam. Key Analysis Group C (CTNS) was defined as women who were CT infected without reported symptoms and no signs documented on a physical exam. All study subjects provided written consent before study enrollment.

Study procedures. Study-related procedures included symptom assessment, a speculum and bimanual pelvic examination to assess for signs consistent with infection, vaginal swabs for GC/CT/TV NAAT testing, and endocervical swabs for miRNA analysis. A study data collection form documenting reported symptoms and signs noted by the research nurse was completed. Reported symptoms included abnormal vaginal discharge, mid-cycle bleeding, abdominal pain, and dysuria. Participants could report more than one symptom. Signs recorded included cervical ectopy, cervical friability, endocervical mucopus, cervical motion tenderness, uterine fundal tenderness, and adnexal tenderness. More than one sign could be documented by the research nurse. Laboratory data recorded included CT/GC/TV amplification tests. Written informed consent was obtained from the participants. This study was approved by the Indiana University/Purdue University-Indianapolis Institutional Review Board.

Sample processing for small RNA library, sequencing, and data analysis. RNA was isolated from endocervical swabs using an miRNeasy kit (Qiagen). RNA was subjected to miRNA library preparation using QIAseq miRNA library kit (Qiagen cat. 331502). Briefly, adapter sequences were sequentially ligated to the 3′ and 5′ ends of miRNA in each sample. Adapter-ligated miRNAs were then assigned unique molecular indexes (UMI) and simultaneously transcribed into single-stranded cDNA. This was followed by cDNA cleanup per the manufacturer's instructions, and construction of PCR-amplified illumina-compatible sequencing libraries, which involved ligating a 3′ sequencing adapter and 1 of 68 indexed adapters (QIAseq miRNA NGS 96 index IL) during the amplification process. The sequencing libraries were then subjected to a second library cleanup and validated for fragment size and quantity using an Advanced Analytical Fragment Analyzer (AATI) and Qubit fluorometer (Life Technologies), respectively. Equal amounts of each library were then pooled and sequenced on a NextSeq 500 platform using high output flow cells to generate ~5 to 10 million 75-base single-end reads per sample (1×75bp SE). All sequencing was performed by the Center for Translational Pediatric Research (CTPR) Genomics Core at the Arkansas Children's Research Institute (Little Rock, AR, USA).

Quantitative real-time PCR. miScript II RT kit (Qiagen) was used for cDNA synthesis, and miRNA expression was confirmed by real-time PCR using miScript SYBR green PCR kit (Qiagen). The primer sequences are shown in Table S1. We normalized the miRNA data to SNORDs 95 and 71 with 17 control, 22 CTNS, and 29 CTSS samples. Data were presented as relative miRNA expression to SNORDs.

Pathway analysis. The experimentally verified target gene list of unique miRs in the CTSS and CTNS groups relative to controls was generated using Ingenuity Pathway Analysis (IPA) software (Qiagen). We subjected the target genes to canonical pathway analysis that includes apoptosis, cell cycle regulation, cellular growth and proliferation, cellular immune response, cytokine signaling, humoral immune response, and pathogen influenced signaling. The enriched pathways were presented as a negative log *P* value based on the right-tailed Fisher's exact test (adjusted for false discovery rate at 5%). These pathways indicate the likelihood of the association between a pathway and the number of genes in the CTSS and CTNS groups.

Statistical analysis. Following demultiplexing, miRNA reads were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC (14). This led to the removal of 6 samples (4 control [73, 76, 82, and 83], 1 CTNS [39], and 1 CTSS [42]) due to low sequencing output. The 68 fastq files that passed quality control were then adapter trimmed and human miRNAs (mirBase v21, GRCh38, piRNABank) quantified using Qiagen's primary QIAseq miRNA quantification tool available through GeneGlobe's data analysis center (https://geneglobe.qiagen.com/us/analyze/). miRNAs with low UMI counts were then removed before downstream analysis. To retain the maximum number of interesting features, miRNAs with a minimum of 10 counts-per-million (CPM) in at least 17 libraries were retained for further investigation. The filtered data set was then normalized for compositional bias using a trimmed mean of M values (TMM) (15, 16). The edgeR likelihood ratio test (glmLRT) was used to identify differentially expressed miRNAs between experimental groups (17–19). miRNAs with multiple tests corrected (FDR) *P* values of 0.1 (20) were selected and analyzed by IPA for biological involvement. The control group was used as a reference, as it allowed comparison of the changes in miRNAs relative to those with infection and those who were uninfected. The data with *P* value of 0.1 after Benjamini-Hochberg (BH) adjustment are presented in Table S1 and Table S2. We used the Venny 2.0 online tool to generate the Venn diagrams. Heat maps were generated using Metaboanalyst (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml). Real time PCR data were analyzed by two-tailed *t* tests.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.01 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.1 MB.

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