Original Article



# The role of estrogen in circular RNA and metabonomics in a *Neisseria gonorrhoeae* infection model

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**Background:** Previous study shows that estrogen exerts both immunosuppressive and immunostimulative effects.

**Methods:** In this study, estrogen was added to a *Neisseria gonorrhoeae* infection model, and transcriptome sequencing and metabolomics studies were performed to clarify the changes in circular RNA (circRNA) and metabolic pathways regulated by the addition of estrogen.

**Results:** The results showed that following the addition of estrogen to the gonococcal infection model, the expression of circRNAs was up-regulated and the expression of circRNAs was down-regulated. In the metabolic group, it was found that after the addition of estrogen, the expression of nine metabolites was down-regulated and 61 metabolites were up-regulated. Furthermore, through network interaction analysis of differentially-expressed circRNAs and differentially-expressed metabolites, we found that the top 10 significantly related metabolites and circRNA were *2-Epoxybutane/novel_circ_0024520; 1,2-Epoxybutane/novel_circ_0061793; 2-Imino-4-methylpiperidine/novel_circ_0012178; 2-Imino-4-methylpiperidine/novel_circ_0056959; Acetone oxime/novel_circ_0012178; Adifoline/novel_circ_0012178; CARBETAPENTANE/novel_circ_0054387; CARBETAPENTANE/novel_circ_0056959; deoxy-PF1140/mmu_circ_0000397;* and *Methyl (2E,6Z)-dodecadienoate/novel_circ_0012178*. Among these, *CARBETAPENTANE/novel_circ_0054387* and *CARBETAPENTANE/novel_circ_0056959* were positively correlated, while the remaining metabolites were negatively correlated.

**Conclusions:** In this study, high-throughput sequencing and metabolomics mass spectrum were applied to screen the differentially-expressed circRNAs and metabolites regulated by estrogen, which will help to provide new research ideas and indicators for asymptomatic infections in women, and can be meaningful for the relevant study in the future.

**Keywords:** Estrogen; *Neisseria gonorrhoeae*; circular RNA (circRNA); transcriptome sequencing; metabolic group

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## Introduction

Estrogen is a fat-soluble steroid hormone mainly composed of estradiol, estrone, and the metabolite, estriol, which is mainly secreted by the ovary (1). The secretion of estrogen determines menstrual cycle changes, and acts to produce and maintain secondary sexual characteristics in women (2). However, recent studies have shown that it also plays a role in immune regulation (3). It has been found that estradiol affects the function of polymorphonuclear leukocytes in clearing *Neisseria gonorrhoeae* (4). Moreover, it has been demonstrated that leukocyte infiltration in the abdominal cavity decreases significantly following intraperitoneal injection of *Neisseria gonorrhoeae* in mice treated with estradiol, which may be due to the monocyte chemotaxis-inhibiting effect of estradiol in mice with gonococcal infection (5). The aforementioned experimental results suggest that increased estrogen reduces the number of neutrophils in reproductive tract secretion, indicating that estrogen can inhibit the occurrence of inflammation to a certain extent.

Estradiol inhibits the messenger RNA (mRNA) expression of interleukin 6 (IL-6), interleukin 8 (IL-8), and NLRP3 in monocytes of HeLa cells infected with *Neisseria gonorrhoeae*, suggesting that estrogen might reduce the body's response to infection by inhibiting the expression of some chemokines and inflammation-related genes (6-8). At present, some researchers have successfully established an experimental infection model of estrogen-dependent *Neisseria gonorrhoeae*, which further illustrates that estrogen can inhibit immunity, thereby increasing the likelihood of *Neisseria gonorrhoeae* infection (9). Among women infected with *Neisseria gonorrhoeae*, 30–50% are asymptomatic; however, whether estradiol inhibits the expression of inflammatory factors requires further study.

Circular RNA (circRNA) is a closed circular non-coding RNA-regulated gene expression formed by covalent bonds (10). CircRNA molecules have a closed ring structure and are not affected by RNA exonuclease, leading to a stable existence *in vivo*. CircRNA molecules are also rich in microRNA (miRNA) binding sites, which act as a miRNA sponge, participate in the occurrence and development of a variety of diseases, and are expected to become disease biomarkers (11). CircRNA, as a miRNA sponge, may alter the expression level of miRNA target genes by blocking the inhibition of miRNA on its target genes, and is involved in regulating the immune and inflammatory responses of allergic diseases at the post-transcriptional level, thereby manipulating the occurrence and progression of various inflammatory diseases (12). However, the anti-infection immunity regulation of circRNAs has not yet been fully elucidated.

There is a specific relationship between estrogen and circRNAs, which manifests mainly via the role of circRNAs as a molecular sponge in affecting estrogen decrease, or to alter the expression of related circRNAs when the estrogen receptor is affected. However, issues such as whether there is a similar phenomenon in infection immunity and what kind of circRNA regulation of estradiol affects anti-infective immunity require further exploration. In order to further analyze estrogen regulation of circRNA and metabolism, we detected differentially-expressed circRNAs and metabolites by transcriptome sequencing and LC-MS (liquid chromatography mass spectrometry) in a *Neisseria gonorrhoeae* infection model, and the regulatory network was also analyzed. By discussing the interactions between estrogen, circRNAs, and metabolites, studying the relationship between pathogen and host, and comprehensively revealing the regulatory mechanism of estrogen on *Neisseria gonorrhoeae* infection, this study will be hugely important in the screening of anti-infection biomarkers as well as research into asymptomatic infection in female mucosa. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3127/rc).

## Methods

### *Sample information*

Mouse monocytes were purchased from the American Tissue Culture Collection (ATCC). Monocytes were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum (FBS) in 5% $CO_2$ (carbon dioxide) incubator at 37 ℃. The monocytes were used in the experiment when they were in the logarithmic growth phase. The cell fluid was changed every 2 days, and the cell morphology, adhesion, and growth were observed before the fluid change. When the cells were covered and adhered to the wall intact, the cells were sub-cultured, digested by 0.25 gL trypsin Ethylenediaminetetraacetic acid (EDTA), and the cell concentration was adjusted to $2\times10^6$/L. Each 5 mL bottle was inoculated into a 100 mL cell culture flask. After the cells were fused for 80%, they were divided into four groups:

 

a blank control group (cultured with RPMI 1640 medium containing 10% FBS for 24 hours), an estrogen group (treated with $10^{-8}$ mol/L estradiol for 24 hours), a gonococcal disease group (treated with $2\times10^{7}$ cfu/mL *Neisseria gonorrhoeae* suspension for 24 hours), and a gonococcal disease + estrogen treatment group (treated with $10^{-8}$ mol/L estradiol for 2 hours, and then treated with $2\times10^{7}$ cfu/mL gonococci for 24 hours).

### *RNA extraction and quality control*

Total RNA was extracted using a TRIzol kit, and the RNA concentration of each sample was measured by Nano Drop. The absorbance (Optical Density $OD2_{60/280}$) was used as the RNA purity index. The Rib-Zero ribosomal RNA (rRNA) Removal Kit was used to remove the rRNA from the total RNA. The Tru Seq Stranded Total RNALibrary Prep Kit was used to preprocess the RNA and construct the sequencing library. The BioAnalyzer2100 instrument was used to control and quantify the library. According to the Illumina sequencing instructions, the 10 pM library was denatured into single-stranded DNA, which were captured by Illumina flowcell and amplified into clusters *in situ*. One hundred and fifty cycle sequencing was carried out on the Illumina HiSeq sequencer in double-terminal mode.

### *CircRNA sequencing data analysis*

The circRNAs were sequenced by Illumina Hi Seq4000 sequencer, and the annotation reference multiple change (fold change, FC) and P value of the identified circRNAs were screened by the circBase database and circ2Traits. FC ≥2.0 (P<0.05) was selected as the threshold for screening differential circRNAs.

### *Detection and analysis of LC-MS metabonomics*

The non-target metabolic group of monocytes was detected by the Ultra-high Performance Liquid Chromatography-quadrupole Time-of-flight Mass Spectrometry (UHPLC-TOF-MS) (AB)/Q-Exactive Focus (Thermo)/Xevo G2-XS (Watres) detection platform. The acetonitrile (B)-water (0.1% formic acid) (A) mobile phase system was used, the analysis time was 30 min, the column temperature was 25 ℃, the flow rate was 0.4 mL/min, the post-column split ratio was 2 μL, the injection volume was 5 μL, and the Ultraviolet (UV) detection wavelength was 286 nm.

In the positive ion mode of electrospray, the mass scanning range was 70–1,000, the capillary voltage was 4,000 V, the atomization gas pressure was 40 psi, the drying gas speed was 10 L/min, the drying gas temperature was 350 ℃, the fragment voltage was 160 V, and the reference calibration solution was selected for real-time mass correction. The experimental data were processed by Qualitative Analysis software. Before measuring the sample, the tuning liquid was used to calibrate the quality axis.

### *Statistical analysis*

The original data are converted into. mzXML format by Proteo Wizard, and peak alignment, retention time correction, and peak area extraction were then carried out by XCMS software. The structure of metabolites was identified by accurate mass number matching (<25 ppm) and secondary spectrum matching, and the local database was searched. After Pareto-scaling processing, the data were analyzed by multi-dimensional statistical analysis such as unsupervised principal component analysis (PCA), supervised partial least square discriminant analysis (PLS-DA), and orthogonal partial least square discriminant analysis (OPLS-DA). Two samples were tested by SPSS 240. the difference was statistically significant. Under the conditions of variable projection importance value (variable importance for the projection, VIP) >1 and P<0.1, the mass-charge ratio was screened and the differential metabolites were searched. The possible differential metabolites were searched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The level of metabolite enrichment in each pathway was analyzed by the Fisher test, and the affected metabolic and signal transduction pathways were finally determined.

## Results

### *Transcriptome sequencing to screen differentially-expressed circRNAs*

The blank control group, estrogen treatment group, gonococcal disease group, and gonococcal disease + estrogen treatment group samples were sequenced, and the differentially-expressed circRNAs were analyzed by cluster analysis (*Figure 1*) and Wayne diagram analysis (*Figure 2*). In addition, we also analyzed the results of K-means cluster diagram (Figure S1), and Self-Organizing Map (SOM) cluster (Figure S2) of differentially-expressed circRNAs among the groups. Further analysis of the differentially-

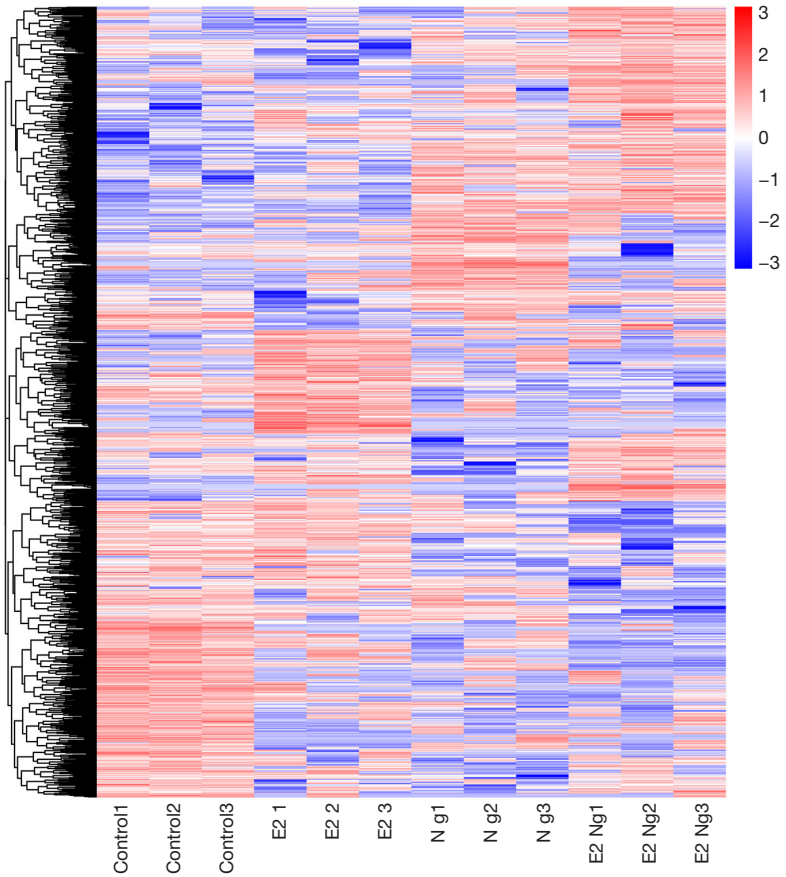


**Figure 1** Differential circRNA cluster map, clustered according to the $\log_{10}$ (TPM +1) value. Red indicates high expression of circRNA and blue indicates low expression of circRNA. Control1–3 is the blank control group; E2 1–3 is the estrogen treatment group; N g1–3 is the gonococcal disease group; and E2 Ng1–3 is the gonococcal disease + estrogen treatment group. CircRNA, circular RNA; TPM, transcripts per kilobase of exon model per million mapped reads.

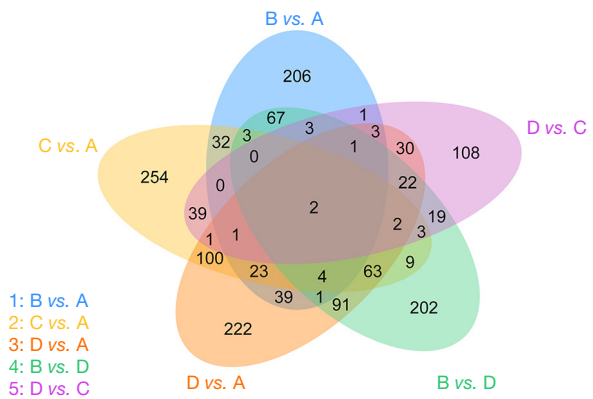


**Figure 2** CircRNA differential expression. Wayne diagram among different treatment groups, in which A represents blank control group; B represents estrogen treatment group; C represents gonococcal disease group; D represents gonococcal disease + estrogen treatment group. circRNA, circular RNA.

expressed circRNAs between the *Neisseria gonorrhoeae* disease group and the gonococcal disease + estrogen treatment group (Table S1) showed that after adding estrogen to the gonococcal infection model, there was up-regulated expression of 111 circRNAs expression, and down-regulated expression of 124 circRNAs (*Figure 3A*). Gene Ontology (GO) analysis showed that the differentially expressed circRNAs were mainly concentrated in metabolism-related and protein binding-related pathways (*Figure 3B*).

### *Screening differentially-expressed metabolites by LC-MS*

The blank control group, estrogen treatment group, gonococcal disease group and gonococcal disease + estrogen treatment group samples were detected by LC-MS, and

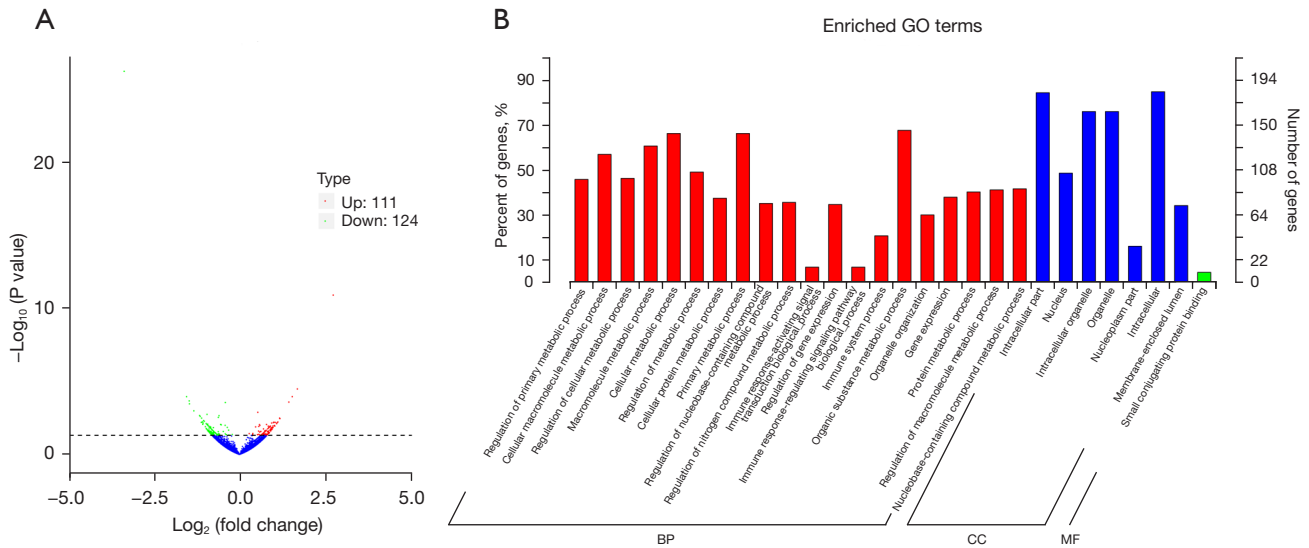


**Figure 3** CircRNA differential expression. Volcano map (A) and differential gene enrichment analysis map (B) between the Neisseria gonorrhoeae disease group and the gonococcal disease + estrogen treatment group. CircRNA, circular RNA; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

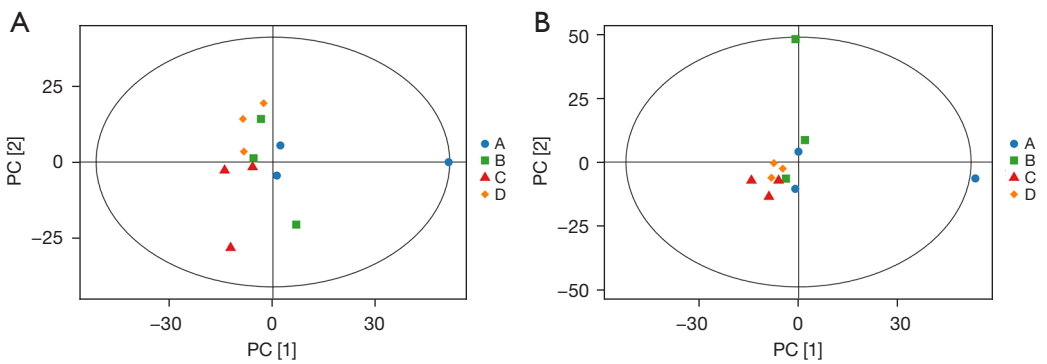


**Figure 4** The PCA of all samples could be divided into scattered points: the abscissa PC [1] and ordinate PC [2] in the negative ion model result map (A) and the positive ion model result map (B) represent the scores of the first and second principal components, respectively. The color and shape of the scatter represent the experimental grouping of the sample, and the sample is basically within 95% confidence interval. A represents the blank control group; B represents the estrogen treatment group; C represents the gonococcal disease group; and D represents the gonococcal disease + estrogen treatment group. PCA, principal component analysis; PC, principal component.

the PCA dispersion points of all samples are shown in *Figure 4*. Further analysis of the differentially-expressed metabolites of the *Neisseria gonorrhoeae* disease group and the gonococcal disease + estrogen treatment group showed 70 differentially-expressed metabolites (Table S2). For the volcano map of these 68 differentially expressed metabolites, the displacement test results of the PCA dispersion plot and OPLS-DA model are shown in *Figure 5*, showing the detection results of differential metabolites in the negative and positive ion modes, respectively.

In addition, the GO (Table S3) and KEGG (Table S4) databases were used to analyze the differential metabolites of each treatment group, which showed that the pathway mainly focused on the metabolism of various acids. The Metabolite Mapping statistical results of different metabolites among the various treatment groups showed that the addition of estrogen to the *Neisseria gonorrhoeae* model could lead to the abnormal expression of multiple

 

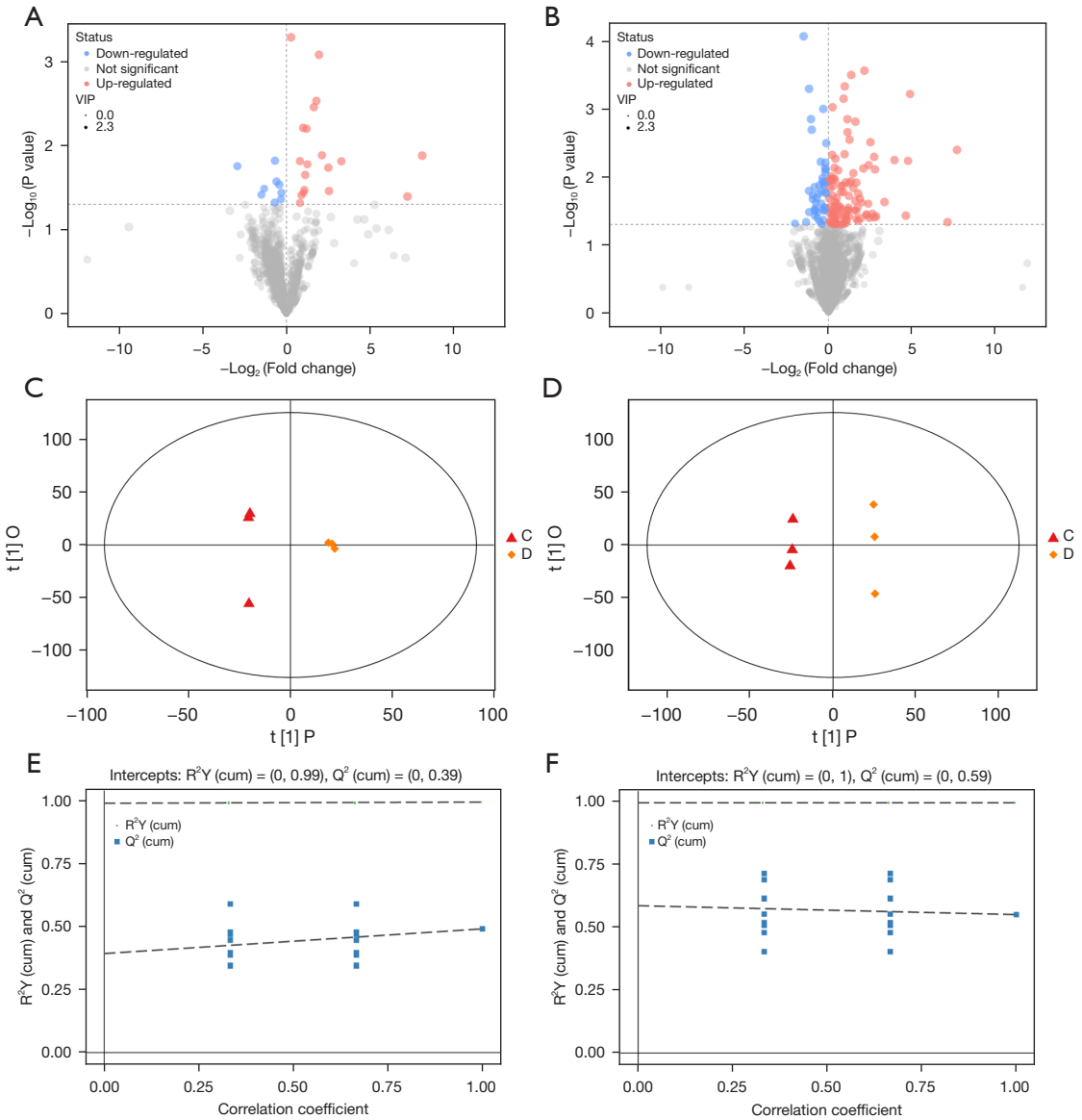


**Figure 5** The difference volcano map of the metabolites of Neisseria gonorrhoeae and Neisseria gonorrhoeae estrogen groups. The PCA dispersion point map and the replacement test results of OPLS-DA model: (A) and (B) represent the difference volcano map of the negative ion and positive ion models, respectively. (C) and (D) represent the PCA dispersion point diagram of the negative ion and positive ion models, respectively, in which the abscissa PC [1] and the ordinate PC [2] represent the scores of the first and second principal components, respectively, and the color and shape of the scatter represent the experimental grouping of the sample. The sample is basically within 95% confidence interval, where C represents the gonococcal disease group and D represents the gonococcal disease + estrogen treatment group. (E) and (F) represent the permutation test results of the OPLS-DA model of negative and positive ion models, respectively. The abscissa represents the permutation retention degree of the permutation test (in proportion to the order of Y variables of the original model; the point where the permutation retention is equal to 1 is the $R^2Y$ and $Q^2$ values of the original model), the ordinate represents the value of $R^2Y$ or $Q^2$, and the green dot represents the $R^2Y$ value obtained by the permutation test. The blue square dot represents the $Q^2$ value obtained by the permutation test, and the two dotted lines represent the regression lines of $R^2Y$ and $Q^2$ values, respectively. $Q^2$, fraction of the total variation of the X block (PCA) or the Y block (PLS) that can be predicted by each component. $R^2Y$, fraction of the total variation of the Y block that can be explained by each component. VIP, variable importance for the projection; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; PC, principal component; PLS, partial least square.

kinds of circRNAs and metabolites, and that there might be an interaction between these circRNAs and metabolites.

### *Analysis of the relationship between circRNAs and metabolites*

In order to further clarify the relationship between circRNAs and metabolites, we analyzed the network relationship between the detection results of differential metabolites in the negative and positive ion modes and differential circRNA, respectively. The first 10 metabolites and circRNAs were significantly related to each other as follows: circology 0024520, circology 0024520, circology 0061793, excercination 0061793, and 4-methylpiperidine4-methylpiperidine-dodecadienoate/novel_circ_0012178 (Table Slim 0056959-Acetone oxime/novel_circ_0012178; Adifoline/novel_circ_0012178; CARBETAPENTANE/novel_circ_0054387; CARBETAPENTANE/novel_circ_0056959; deoxy-PF1140/mmu_circ_0000397; and Methyl (2Ether 6Z). Among these, CARBETAPENTANE/novel_circ_0054387 and CARBETAPENTANE/novel_circ_0056959 were positively correlated, while the remaining metabolites and circRNAs were negatively correlated.

## Discussion

In this study, transcriptome sequencing and metabonomics were applied to study the effects of estrogen on differentially-expressed circRNA and metabolites in the monocytes of mice infected with *Neisseria gonorrhoeae*. An estrogen-induced circRNA program was affected by the expression of cell cycle-related genes. The results showed that following the addition of estrogen to the gonococcal infection model, the expression of circRNAs was up-regulated and the expression of circRNAs was down-regulated. In the metabolic group, it was found that after the addition of estrogen, the expression of nine metabolites was down-regulated, and 61 metabolites were up-regulated. Based on the network interaction analysis of differentially-expressed circRNAs and differentially-expressed metabolites, it was found that the first 10 metabolites and circRNAs were significantly related to each other. Among them, CARBETAPENTANE/novel_circ_0054387 and CARBETAPENTANE/novel_circ_0056959 were positively correlated, while the remaining metabolites and circRNAs were negatively correlated. The results of this study are helpful in providing new research ideas and indicators for female asymptomatic infection.

Previous studies have shown that estrogen can inhibit the chemotaxis of monocytes in mice with *Neisseria gonorrhoeae* infection, reduce the number of granulocytes in reproductive tract secretion, and inhibit inflammation to a certain extent (1,4). Estrogen not only inhibits the non-specific cellular immunity of neutrophils, but also inhibits local cellular and humoral immunity. Studies have shown that estrogen can promote the adhesion and colonization of Candida albicans in VK2/E6E7 cells by binding to estrogen receptor α and estrogen receptor β to regulate a series of downstream signaling pathways (13-15). Some studies have reported that although there is no significant difference in the prevalence of vulvovaginal candidiasis between women who are taking oral contraceptives and those who are not, the detection rate of vaginal Candida albicans is positively correlated with the content of estrogen in contraceptives (16). These results demonstrate that estrogen can enhance the adhesion and invasiveness of Candida albicans (15,17). Estrogen exerts anti-inflammatory effects, including the inhibition of pro-inflammatory gene transcription and cytokine production, as well as in several disease models such as autoimmunity, atherosclerosis, arthritis, inflammatory intestinal diseases, asthma, and influenza (18,19). In this study, it was found that following the addition of estrogen, the differentially-expressed circRNAs in monocytes were mainly concentrated in metabolism-related and protein binding-related pathways, which may suggest that estrogen affects cell metabolism and adhesion by regulating circRNAs.

The characteristics of circRNA molecules, such as tissue specificity, disease specificity, time sequence specificity, and high stability, reflect their important potential as a biomarker of clinical disease, as well as their roles in anti-infective immunity and inflammation. Regarding influenza A virus (IAV) infection, researchers previously established a mouse model of IAV infection and obtained 12 differentially-expressed circRNAs using bioinformatics software in the lung tissues of the IAV-infected mice (20,21). The results showed that 12 circRNA target genes played a role in the biological process, cell composition, and molecular function, and affected antigen processing and presentation, Tol receptor, retinoic acid-induced gene I-like receptor and myocardial function, as well as other related signaling pathways. Therefore, the researchers speculated that circRNA might participate in the IAV infection process via host immune regulation. CircRNAs is involved in the regulation of a variety of inflammatory responses. In

 

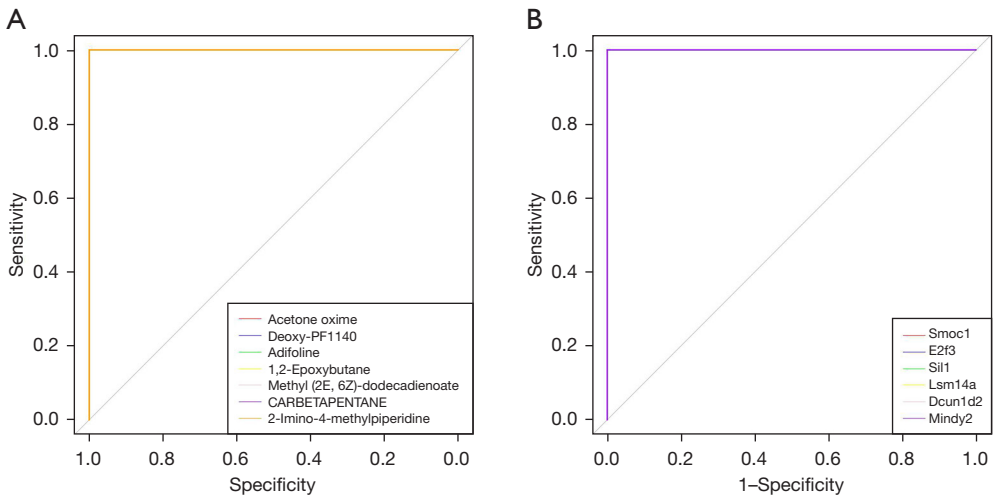


**Figure 6** ROC analysis of top 10 significantly related circRNA and metabolites, show that AUC =1. When AUC >0.5, the result is more reliable, if AUC is closer to 1. This means the differentially-expressed circRNAs and metabolites regulated by estrogen are significant reliable. circRNA, circular RNA; ROC, receiver operating characteristics; AUC, area under the curve.

the preliminary research work, some researchers found that compared with healthy groups, there is a differential expression of circRNAs in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (22,23). The expression of circRNAs is both up-regulated and partly down-regulated; however, whether it is up- or down-regulated will affect the occurrence and development of SLE.

In this study, the interaction between differentially-expressed circRNAs and differential metabolites was also analyzed, and the regulatory network diagram was drawn. ROC analysis of top 10 significantly related circRNA and metabolites shows that AUC =1. When AUC >0.5, the result is more reliable, if AUC is closer to 1. This means the differentially-expressed circRNAs and metabolites regulated by estrogen are significant reliable. (*Figure 6*) Among the top 10 significant relationships between the circRNAs and differential metabolites selected, including *1,2-Epoxybutane/novel_circ_0024520; 1,2-Epoxybutane/novel_circ_0061793; 2-Imino-4-methylpiperidine/novel_circ_0012178; 2-Imino-4-methylpiperidine/novel_circ_0056959; Acetone oxime/novel_circ_0012178; Adifoline/novel_circ_0012178; CARBETAPENTANE/novel_circ_0054387; CARBETAPENTANE/novel_circ_0056959; deoxy-PF1140/mmu_circ_0000397; and Methyl (2E,6Z)-dodecadienoate/novel_circ_0012178*. Among them, C*ARBETAPENTANE/novel_circ_0054387* and *CARBETAPENTANE/novel_circ_0056959* while the remaining metabolites and circRNAs were negatively correlated. Metabolomics is an emerging omics that enables comprehensive analysis of metabolites in biological samples is defined as metabolomics technology, and apply in many areas, such as, disease diagnosis, pharmaceutical research and development, nutrition and food science, toxicology, environmental science, botany. At this stage, it has limitations that metabolomics' databases are not sourced universal and shared. More and more scientific laboratories and organizations have set metabolomics standard documents, which means, the former limitations will be very likely conquered in the future. In the future, we will design further verification tests based on these corresponding relationships and correlations, clarify their regulatory functions, study the relationship between pathogens and hosts, and further explore the interaction between estrogen, circRNAs, and metabolites. This research is hugely important to the screening of anti-infection biomarkers as well as the study of asymptomatic infection of female mucosa.

## Conclusions

In the *Neisseria gonorrhoeae* infection model, we found that the expression of circRNAs was up-regulated and the expression of circRNAs was down-regulated after adding estrogen. In the metabolic group, following the addition of estrogen, the expression of nine metabolites was down-regulated, while that of 61 metabolites was up-regulated. Moreover, through network interaction analysis

 

of the differentially-expressed circRNA and differentially-expressed metabolites, a series of interaction networks between metabolites and circRNAs were identified. In this study, estrogen-regulated differentially-expressed circRNAs and metabolites were screened by high-throughput sequencing, which was helpful in providing new research ideas and indicators for female asymptomatic infection.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3127/rc

*Data Sharing Statement:* Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3127/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3127/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.



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