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RNA stability indicates endoplasmic reticulum stress as a novel therapeutic target in endometritis

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Article

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Abstract

Endometritis is a persistent inflammatory disorder of the endometrium, associated with infertility, recurrent pregnancy loss and significant economic losses. Despite numerous studies that focused on transcriptome analysis on endometritis, still its molecular pathogenesis is not completely understood. We hypothesized that pathogenesis of endometritis may be associated with stability changes in the expression of genes. RNA stability pattern of genes between healthy and infected samples were investigated using RNA-Seq dataset. Then, a gene co-expression network was constructed by WGCNA approach and a novel propagation-based algorithm was developed to assess identified module consistency. Finally, the results were subjected to functional enrichment and protein-protein interaction (PPI) network analysis. Of the nine identified modules, brown module showed an increase in RNA stability after infection and was mainly associated with endometritis. This module contained 158 genes that were significantly enriched in the pathways related to endoplasmic reticulum (ER) stress and immune response. In this regard, one important finding from recent studies is that ER stress is a key pathway involved in endometritis development. These findings provided further evidence that ER stress and related pathways are important components contributing to modulation of gene regulatory networks involved in endometritis development. Moreover, network propagation and PPI network analysis revealed the functional connectivity of the genes within the module. Hub gene detection analysis resulted in the identification of 28 hub genes that were also significantly related to biological pathways associated with endometritis pathogenesis. Interestingly, several hub genes were found (including *CD4*, *S100A4*, *CSF3*, *CCR10*, *KLF10*, *CD48*, *CD19*, *CXCL3*, *CXCL5*, *PDGFRB*, *PTPRCAP*, *NR4A2* and *HBEGF*) that have been reported previously as biomarkers in endometritis. Notably, most of genes in brown module were of particular interest, because of their known roles in immune response or ER stress. This study achieved the goal of showing that key genes and pathways that modulate cellular response to endometritis development, can be identified by considering stability of genes as a post-transcriptional regulatory mechanism. The investigation of RNA stability in diseases, not only enhances our understanding of the molecular mechanisms behind the infection, but also opens up new avenues for therapeutic intervention.

Background

Typically, between 35 to 50% of dairy cattle are infected with at least one type of pathological inflammation of the reproductive term, between 3 and 7 weeks postpartum [1]. In this context, one of the most common postpartum diseases is endometritis. Endometritis is a chronic and estrogen-dependent inflammatory disorder, without systemic clinical signs of postpartum uterine disease, which characterized by inflammation of the endometrium [2–4]. Bacterial infection is the primary and most common cause of bovine endometritis, which is developed by poor nutrition and genetic factors [5]. This disease can cause infertility or subfertility, reduce calving rates and increase the elimination rate in dairy cows [6]. This prevalent disease also results in significant economic losses due to reduced milk production and fertility [6, 7]. Since, the pregnancy of dairy cows at a normal calving interval is one of the main goals of programs of reproductive management, better understanding of the etiology of endometritis can be useful in the efforts to prevent and treat this disorder. Undoubtedly, genetic factors

play important roles in the development of endometritis, however, the exact mechanisms underlying development and progression of this disorder are not fully understood yet [8].

Several studies have been conducted with a primary focus on transcriptome analysis to identify differences between infected and healthy cows, in terms of gene expression patterns. For example, a recent study showed upregulation of *IL1B*, *IL6*, *IL17A*, *CXCL8*, *PTGES*, *PTGS1*, *PTGS2* and *INHBA* in the endometrium of cows with clinical endometritis compared to healthy ones [9]. A previous study reported a higher expression of immune factors such as *C3*, *C2*, *LTF*, *PF4*, and *TRAPPC13* in the endometrium of cows with subclinical endometritis [10]. Gene expression profiling in the caruncular and intercaruncular endometrium revealed that *CCL5*, *CXCL8*, *IL6*, and *IL1B* were upregulated in cows with more severe inflammation compared to healthy cows [11]. Also, in our previous study an integrated regulatory network related to bovine endometritis was constructed and two co-expression modules along with several sub-networks were identified. We reported several candidate genes, including mRNAs, lncRNAs and miRNAs, as potential biomarkers or therapeutic targets that may contribute to the progression of bovine endometritis [12]. While the transcriptome analysis has been the subject of several studies in bovine endometritis, like our previous works [12, 13], the findings have often been conflicting or limited. On the other hand, these studies have focused on the identification of differentially expressed genes or gene regulatory network construction based on gene expression patterns. However, the etiology of diseases or molecular mechanisms underlying various biological processes cannot be explained solely by gene expression profiling (based on total RNA), without considering the complexity of cellular gene expression programs [14].

One of the most important mechanisms controlling the regulation of gene expression is post-transcriptional events, which modulate RNA biogenesis and stability. In this line, it has been demonstrated that regulation of the stability of a transcript is more important than its transcription to determine the final expression level of that transcript [15, 16]. The interplay of RNA synthesis and degradation determine the RNA half-life (or RNA stability) of a gene in a cell. Stability of genes can be modulated directly, through the activities of RNA binding proteins or small RNAs [17, 18]. Hence, to better understand the mechanisms underlying gene expression regulation, we need to identify changes in the rates of RNA synthesis and degradation, which determine the kinetics of RNA metabolism and RNA stability. In fact, differences of RNA abundance between two conditions, such as healthy and infected samples, are due to dysfunctional synthesis or degradation of RNA transcripts, which can be used to help explain the regulatory mechanisms behind it [14]. Therefore, RNA stability is a key post-transcriptional regulatory mechanism in terms of regulation of transcript abundance and plays an important role in shaping the kinetics of gene induction in gene regulatory networks in mammalian cells [19]. Although RNA metabolism mechanism has long been known, but the role of RNA stability in various biological and molecular pathways as well as in different diseases are largely unknown and are starting to be considered only recently [14]. Thanks to next generation sequencing technology and comprehensive bioinformatic analysis, RNA stability can be estimated directly from RNA-Seq data, under the assumption that RNA processing rate is constant. Then, RNA stability, as a ratio of RNA synthesis

and degradation, is estimated from intronic (as unspliced and pre-mature transcripts) and exonic (as spliced and mature transcripts) aligned reads to genes [20, 21].

Recent studies have reported regulatory strategies that are involved in the etiology of cancers and diseases through modulation of RNA stability (22,23) It is well-known that RNA stability plays a critical role in regulating immune and inflammatory responses. For example, *HuR* (an RNA binding protein) stabilize cytokines, such as *IL6* and *TNF-α*, that results in enhancing immune responses [22]. In this regard, eight RNA-binding proteins (*TTP*, *AUF1*, *KSRP*, *TIA-1*/*TIAR*, *Roquin*, *Regnase*, *HuR*, and *Arid5a*) have been reported as regulators of inflammatory responses through modulation of mRNA stability [23]. Previous studies have demonstrated the crucial connection between RNA stability and neurodegeneration in amyotrophic lateral sclerosis (ALS) patients [24, 25]. Furthermore, it has been found that *TDP-43* aggregation causes targeted RNA instability in ALS and frontotemporal dementia. This instability may ultimately lead to cell death by disrupting pathways involved in energy production and protein synthesis [26]. Recently, we investigated the RNA stability dynamics during bovine mastitis and showed that stability of mRNAs changed following infection as a response mechanism. Different RNA stability profiles were found between healthy and infected samples that led to the identification of two disease-related module genes. Our recent study highlighted the importance of RNA stability in modulating immune pathways to mastitis, which reinforce the potential of RNA homeostasis in the molecular mechanisms underlying diseases [14]. Together, these studies provide insights into the potential mechanisms by which RNA stability may contribute to gene regulatory networks underlying complex traits and diseases. On the other hand, despite these studies, research on RNA stability has received considerably less attention and the extent to which RNA stability contributes to regulatory mechanisms associated with the pathogenesis of endometritis remains largely unknown.

In our previous study, stability of genes, weighted gene regulatory network based on WGCNA approach and a novel application of network propagation algorithm were integrated to investigate the hypothesis that RNA stability dynamics can be changed during bovine mastitis [14]. Herein, this approach was applied to assess the repeatability of this hypothesis in bovine endometritis and investigate whether RNA stability contribute to the regulation of the immune response to this disorder. Our results not only emphasize the importance of RNA stability, as an extra layer of information, in modulating gene regulatory networks underlying endometritis, but also presents a computational pipeline to study how RNA stability may influence molecular pathways behind complex traits and diseases. This study established a foundation for future work on analyzing how RNA stability may regulate the immune pathways in bovine endometritis.

Results

RNA-Seq Data Analysis

A total of 1,157,662,598 read pairs related to 30 RNA-Seq samples were processed based on our stepwise bioinformatic pipeline (Fig. 1). The average number of reads per sample was 38,588,753. After

quality control and trimming, 1,115,383,994 read pairs remained. On average, 91.49% of the reads aligned to the bovine reference genome (Fig. 2).

To create the RNA stability matrix, the aligned reads to exonic and intronic regions of genes were quantified per sample and were analyzed using REMBRANDTS tool. In total, 474,825 RNA stability values (ranged from − 5.067 to 4.40) for 10,939 genes across the 30 samples were obtained and were subjected for down-stream analysis.

WGCNA Analysis

Considering the fact that most of the previous researches investigated gene expression patterns in endometritis, there is a need to study other molecular mechanisms that may help to better understand the etiology of disease. Here, a network-based approach based on RNA stability patterns was used to further explore the molecular mechanisms behind the endometritis. After initial processing of the RNA stability matrix, sample 29 detected as outlier and removed from further analysis. The soft-thresholding power (β) was set to 16 to satisfy the scale-free topology of the network (R^2 value ~ 85%) (Supplementary Information, Figure S1). Setting this threshold results in nine modules (Supplementary Information, Figure S2) with gene numbers ranging from 234 (turquoise module) to 36 genes (magenta module). Genes that were not assigned to other modules (including 557 genes) were clustered into grey module (Supplementary File, Table S1). Then, RNA stability trend analysis performed based on heatmap plot and three modules were found, according to the described groups in Material and Methods. Actually, these modules represented the genes whose stabilities were changed similarly between healthy and infected samples. As members of group 1, turquoise (including 234 genes) and yellow (including 134 genes) modules showed higher RNA stability in healthy in comparison to infected samples (Fig. 3). On the other hand, brown module (including 158 genes) as a member of group 2, showed higher stability in infected samples than healthy ones. Figure 4 shows the heatmap plot related to stability of genes in this module. Obviously, stability of genes is increased after infection in both times (7 and 21 days postpartum).

Propagation Network Analysis

If our hypothesis is correct, it is expected that rate of propagated genes in functional modules (here brown) be significantly higher than non-functional modules (reference groups including grey module and random set). To investigate this, our previous propagation network analysis approach used (Material and Methods). Of 158 genes in brown module, 40 genes were randomly selected as seed, during 100 iterations. The average number of the propagated genes per iteration was 13 genes that was significantly higher than seven genes in grey module and random set group. Notably, difference between the two reference groups was not significant, in terms of number of the propagated genes (Fig. 5).

Functional Enrichment Analysis

The modules of interest (turquoise, yellow and brown) were functionally analyzed and clustered based on biological processes and KEGG pathways. Genes in turquoise modules were enriched (p-value < 0.05) in 112 biological processes and nine KEGG pathways (Supplementary File, Table S2). In total, 71 and two biological processes and KEGG pathways were enriched (p-value < 0.05) in yellow module, respectively (Supplementary File, Table S3). As expected, most of the enriched terms in both modules were involved in general cellular processes including “Positive Regulation of Cell Differentiation”, “Transcription by RNA Polymerase II “, “Positive Regulation of Transcription”, “RNA Polymerase II Preinitiation Complex Assembly”, “DNA Repair”, “Organelle Assembly” and “Spliceosome”. In brown module, 154 biological processes and 16 KEGG pathways were identified (p-value < 0.05). Of these, three biological processes (“Response to Endoplasmic Reticulum Stress”, “Regulation of T-helper 17 Cell Differentiation” and “ERAD Pathway”) and one KEGG pathway (“Protein processing in endoplasmic reticulum”) were significantly enriched at FDR < 0.05 (Supplementary File, Table S4). Based on our hypothesis, brown module is of great importance and considered for further analysis, as it is expected to contain functionally related genes as well as include key genes controlling immune response to the infection.

PPI Network Analysis

To further investigate the functional inferences of the genes in brown module, PPI network was constructed using STRING database. The results showed a significant PPI network (p-value < 1.0e-16) and revealed a dense functional interaction network among the genes within the module, including 148 nodes (genes) and 176 edges (interactions) (Fig. 6). Hub gene detection method enabled us to identify 40 highly connected genes (overlapped between two methods, as described in Material and Methods) that were centrally located in the PPI network. Of these, 28 genes showed kME > 0.5 and were considered as final hub genes (Supplementary File, Table S5). Functional enrichment analysis of the hub genes revealed 22 biological processes and 175 KEGG pathways (p-value < 0.05). Of these 10 biological processes and 14 KEGG pathways were significantly enriched at FDR < 0.05 (Supplementary File, Table S6).

Discussion

Over the past 25 years, comparative analysis of gene expression has emerged as an approach to discover genes that regulate various biological processes and pathways involved in different traits/diseases. For example, significantly up- or down-regulated genes in the infected cells have been reported as diseases-related genes and used as a proxy for identifying pathological cell states. However, the quantification of absolute expression (based on total RNA) and identification of differentially expressed genes provide only a glimpse of the complexity of cellular gene expression programs. Hence, other molecular mechanisms, especially post-transcriptional regulations, have to be considered to better explain the regulatory mechanisms underlying various biological processes or diseases.

Postpartum uterine disease, like endometritis, has a detrimental effect on the reproductive performance of dairy cows. Cows with these diseases require more services to conceive, which increase the risk of culling and cause significant economic losses to the dairy industry [27]. While molecular mechanisms underlying bovine endometritis are very complex, studies have mainly focused on transcriptomic profiling and differential gene expression analysis. As explained above, recent studies have shifted their focus to post-transcriptional control mediated by different molecular mechanisms. Therefore, although there is a wealth of information about gene expression variation between infected and healthy samples, but much less is known about post-transcriptional regulation mechanisms involved in bovine endometritis, such as RNA stability. A comprehensive literature review showed that a systematic investigation of the role of RNA stability in the pathogenesis of endometritis and the extent to which it shapes the gene expression patterns has not been performed, despite strong evidence in support of the importance of stability of genes in external stimuli. Considering RNA stability, as a post-transcriptional regulation mechanism, enable us to identify the novel pathways and regulators that may participate in the pathogenesis of endometritis, as was performed in the present study. Our results suggested that RNA stability can be accepted as a potential mechanism involved in bovine endometritis development. In this context, stability of genes estimated and used to assign genes to the modules. Out of the identified modules, brown module was found with higher stability in infected samples than healthy ones. According to our hypothesis, it is expected that genes within this module be functionally related and involved in immune response and endometritis progression. Hence, three different approaches were applied to investigate the functional connections and relatedness of these genes, including 1) propagation network analysis, 2) functional enrichment analysis and 3) PPI network analysis. Findings of these analyses underscore the importance of RNA stability to explore molecular mechanisms behind the endometritis and discussed as follow.

1) Propagation network analysis

As described in details in our previous study [14], we developed a novel network propagation framework to quantify the extent to which a set of genes are interconnected within a PPI network (here, PCNet), by calculating propagation Z-scores derived from an iterative RWR algorithm. Therefore, it is expected that if the genes of interest be functionally related, they are more likely to be directly/indirectly connected in the network and have higher and significant propagation scores than random genes. Results revealed that, while distribution of propagation values of brown module was significantly higher than both random gene sets, propagation values between grey and random set was not significant. It is worth noting that, two distinct random gene sets were used to show that the higher propagation values in brown module are reproducible and consistent with our hypothesis that functionally related genes have higher network connectivity than random gene sets. This finding emphasized that topologically connected genes can be clustered based on the stability of genes, which indicate that another layer of complexity should be considered in gene regulatory network construction. Notably, this finding reinforces the utility of our network propagation approach, as a downstream analysis, to investigate the functional connections of gene modules resulting from co-expression analysis.

2) Functional enrichment analysis

This analysis supported the involvement of brown module in immune and inflammatory responses during endometritis. Interestingly, this module was significantly enriched in biological processes/KEGG pathway related to endoplasmic reticulum (ER) stress, ERAD pathway and immune response associated terms. These functional terms are directly related to endometritis as well as play important roles in immune response and inflammation regulation. ER is a multifunctional organelle in eukaryotic cells, which is responsible for synthesis, folding and transport of protein, lipid synthesis, calcium regulation and interactions with other cellular organelles [28]. Various external stimuli, such as hypoxia, protein misfolding and change in Ca levels may result in ER stress. Then, the unfolded protein response is triggered by the accumulation of unfolded/misfolded proteins, during ER stress, to repair ER homeostasis by promoting ER-associated degradation (ERAD) pathway and clearing unfolded proteins. In fact, ER stress is a defense mechanism for survival and rescue the cell under stress conditions. However, a prolonged or too strong ER stress may result in the activation of apoptosis, to prevent damage to the surrounding tissue [29]. Recent studies have demonstrated the relationship between ER stress and inflammatory immune responses [30–32]. Interestingly, ER stress, as a novel pathway, has attracted increasing interest in the identification of genetic mechanisms that may be involved in endometritis. In this context, it is well known that ER stress can be occurred in bovine endometrial epithelial cells, as a consequence of diverse post-natal stressors [33], like oxidative stress [34]. Furthermore, it has been shown that a prolonged state of ER stress affects uterine involution and contribute to the pathogenesis of endometriosis [35]. Because of the importance of ER protein homeostasis in cellular functions, disruptions in these processes play vital roles in the pathogenesis of various diseases, such as endometritis, and reviewed in [36]. Several genes were found in brown module that are involved in response to ER stress including *TRAM1*, *ANKZF1*, *UBE2J1, ATP2A2*, *DNAJB9*, *ERO1A*, *LMAN1*, *PPP1R15A*, *RNF121*, *SEC23B*, *SEC24D*, *SELENOS*, *TBL2* and *UFD1*. The previous studies have discussed the functions of these genes in immune response to infection/diseases and ER stress-related pathways. Based on our hypothesis, cells may increase the stability of these genes, through a regulatory mechanism, to activate the immune system-related pathways and response to the infection. For example, *TRAM1* is one of the proteins involved in the transport of newly synthesized proteins into ER via the Sec61 channel. This gene activates in conditions of accumulation of misfolded proteins and participates in ER stress response [37]. It has been found that *TRAM1* protects the cells and alleviate ER stress through ER stress-JNK pathway [38]. The potential role of *TRAM1* in the management and treatment of inflammatory, by activation of *TLR4*-dependent pathway and *NF-κB* nuclear translocation, has been reported [39]. Moreover, *TRAM1* has been suggested as powerful biomarkers for early and non-invasive diagnosis of bladder cancer [40]. A previous study showed that *ANKZF1*, as an important ER stress-related gene, contributes to the progression and metastasis of colon cancer [41]. It has been demonstrated that *UBE2J1* inhibits the apoptosis of endometrial cancer cells via the *PI3K/AKT* and *MDM2/p53* pathways [42]. Also, potential role of *UBE2J1* in the immunotherapy response has been highlighted. It has also been reported that overexpression of this gene in ovarian cancer may stimulate

plasma cell infiltration, which, in turn, targets *UBE2J1* and enhances the anti-tumor immune response [43].

In addition, some interesting functional terms were found that were directly related to endometritis, including “Regulation of T-helper 17 Cell Differentiation“ (p-value < 0.05 and FDR < 0.05), “Cellular Response to Molecule of Bacterial Origin” (p-value < 0.05 and FDR < 0.08) and “Cellular Response to Lipopolysaccharide” (p-value < 0.05 and FDR < 0.08) (Supplementary File, Table S4). For instance, cellular response to molecules of bacterial origin is particularly important, as endometritis is typically initiated by bacterial infection in the uterus. Molecules such as lipopolysaccharide (LPS) from gram-negative bacteria activate the innate immune system through cytokine production and stimulation of pattern recognition receptors (e.g., TLRs), which are essential for infection control and tissue repair. Collectively, these processes affect the progression of endometritis by regulating inflammatory responses, immune activity and cellular stress [12]. Disruption in these pathways can increase disease severity or delay uterine recovery. Several genes were found in brown module that have been reported directly as important regulators involved in endometritis development-related pathways including *NFKBIZ*, *CNR2*, *SLC20A1*, *RAB27A* and *CFD*. One important key gene that plays critical roles in immune and inflammatory signaling pathways is *NFKBIZ*. This gene encodes *IκBζ* protein that is activated in the nucleus of cells and controls the expression of certain genes by binding to *NF-κB*, one of the key factors in regulating immune responses. This protein is activated especially in inflammatory conditions and stimulated by various factors such as *TNF* (tumor necrosis factor) and *IL-17* (interleukin-17). Studies have shown that *IκBζ* plays a role in the production of inflammatory cytokines such as *IL-6* and *IL-17*, which are well known in inflammatory processes and immune response to infections. This protein, *IκBζ*, also participates in regulating T and B cell differentiation [44, 45]. Studies have also reported *NFKBIZ* as an important regulator of anti-inflammatory pathways, which helps to reduce the production of *IL-6* and, as a result, controls inflammatory responses [46, 47]. Furthermore, *NFKBIZ* has been reported as a candidate gene involved in inflammatory signaling pathways and immune response in bovine endometrial stromal cells exposed to LPS [48]. *CNR2* gene is a receptor and mainly expressed in immune cells, such as lymphocytes, macrophages and NK cells. This receptor plays a key role in immune response and is effective in regulating cytokine production, cell migration and proliferation. The findings showed that mice lacking *CNR2* had significant changes in immune responses [49]. *SLC20A1* is a sodium-dependent phosphate transporter that plays a critical role in maintaining cellular phosphate homeostasis and is associated with the *NF-kB* pathway [50]. *RAB27* gene is active in the secretory pathway and has two isoforms *RAB27A* and *RAB27B* that regulates the secretory function of endometrium. It has documented that *RAB27* may contribute to the development and progression of endometritis by regulating secretion of extracellular vesicles and affecting immune and angiogenic pathways [51]. *CFD* has been reported as a potential biomarker for the diagnosis of endometriosis. Studies have also shown that high expression of *CFD* can affect inflammatory processes and is associated with the progression of inflammatory diseases such as endometriosis [52]. Altogether, these findings support the hypothesis that RNA stability may serve as a molecular mechanism to modulate diseases development through their target genes and associated pathways.

3) PPI network analysis

In addition to the previous analyses (propagation network and functional enrichment analysis) that showed functional association of brown module with endometritis, PPI network analysis was also performed to accurately identify hub genes. This analysis resulted in a significant and dense PPI network, which indicates the functional connectivity of the genes in a module that is constructed based on RNA stability values. Using a stringent hub gene detection method, 28 hub genes were detected, which were significantly enriched in functional terms related to ER stress and immune response, including “Response to Endoplasmic Reticulum Stress “, “Myeloid Leukocyte Differentiation “, “Cytokine-cytokine receptor interaction”, “IL-17 signaling pathway“, “PI3K-Akt signaling pathway“, “JAK-STAT signaling pathway” and “Chemokine signaling pathway” (Supplementary File, Table S5 and S6). What is worth emphasizing is that cytokines and chemokines have been reported as inflammatory factors involved in the pathogenesis of endometritis. On the other hand, it has been demonstrated that ER stress induces the production of inflammatory factors, such as cytokine and interleukins [53]. The fact that these processes are present in the brown module indicates a strong relationship between them. In fact, immune response can be an outcome of ER stress. While the exact mechanisms by which stability of genes contribute to the pathogenesis of endometritis (or other diseases) has not been clearly understood, we identified several hub genes that are reported to be directly related to immune response and endometritis development including *CD4*, *S100A4*, *CSF3*, *CCR10*, *KLF10*, *CD48*, *CD19*, *CXCL3*, *CXCL5*, *PDGFRB*, *PTPRCAP*, *NR4A2* and *HBEGF*. In fact, cells can change the stability of these genes to modulate gene regulatory mechanisms behind the infection progression. One of the most important hub genes, with the highest number of connections in the network (29 edges), is *CD4*. This gene is a chemokine receptor on the surface of T helper cells and typically is considered a marker for T cells. *CD4* is well known for its roles in immunity, as *CD4*-T cells are mainly activated due to inflammation [54]. Several previous studies have consistently shown that *CD4* is the best predictor of disease progression. *CD4* and its related products, such as *CD4 + CD25+Treg*, play important roles in the balance of the immune system. There are several studies reporting the important roles of *CD4* in endometritis [54–56]. For example, it has been reported that the disruption of *CD4*/*CD8* balance, impaired Treg function, and decreased *IL-10* production create a favorable environment for inflammation and disease progression in endometriosis [55]. The other important hub gene, *S100* calcium-binding protein A4 (*S100A4*), is an important member of the *S100* calcium-binding protein family, which is composed of more than 20 members. The important roles of this family in endometritis have been reviewed in [57]. Interestingly, it has been demonstrated that *S100A4* promotes the progression of endometritis (based on a mouse model induced by LPS), as deficiency of *S100A4* reduced uterine pathological reaction. It has also been suggested that the mechanism by which *S100A4* promotes endometritis may be related to facilitating cellular proliferation and decreasing apoptosis in the endometrial stroma during inflammation [58]. In a recent study, *S100A4* was reported as a key driver of cell invasion and disease progression and proposed as a potential therapeutic target for endometriosis, particularly through targeting the gene in endometrial stromal cells to inhibit disease progression[59]. *S100A4* has also been suggested to mediate endometrial cancer invasion [60]. It is interesting to note that a previous study reported that the lower

expression of *S100A4* may play an important role in the development of bovine endometritis, marking it as a pathogenesis-related protein [61]. The other important hub gene with 12 connections, *CSF3*, encodes granulocyte colony-stimulating factor (*G-CSF*), a cytokine that is involved in regulating the growth and differentiation of hematopoietic stem cells into granulocytes (a type of white blood cell). Higher levels of *G-CSF* have been reported in serum and peritoneal fluids of endometriosis mice compared to controls. Also, *G-CSF*, in conjunction with *IL-6*, modulates neutrophils to promote the establishment of early endometriosis [62]. *CSF3* is normally active in the immune system and participates in anti-inflammatory responses and tissue repair. A previous study showed that *CSF3* regulates the embryo implantation and plays a crucial role in promoting endometrial thickening and improving the pathophysiology of endometriosis [63]. This gene has also been suggested as a useful biomarker for the diagnosis of endometriosis-related infertility [64]. The next hub gene, *CCR10*, is a key factor in the recruitment of Th17 cells to the site of inflammation in endometritis patients. In a study, *CCR10* was reported as a marker of Th17 cells along with *CXCR3* and *CCR6* [65]. On the other hand, *CCR10* is a known receptor for the chemokines *CCL27* and *CCL28*. It is demonstrated that knockdown of *CCL28* inhibits endometriosis stromal cell proliferation and invasion via ERK signaling pathway inactivation [66]. It is worth noting that “Regulation of ERK1 and ERK2 Cascade” was enriched (p-value < 0.05 and FDR < 0.08) in the hub genes (Supplementary File, Table S6). *KLF10*, as another hub gene, reduces inflammation by regulating the *CD40/CD154* pathway, thereby reducing the incidence of endometriosis [67]. Recently, *KLF10* has been suggested to participates in the pathogenesis of endometriosis, through inducing the activation of *CD40* and initiating the alternative activation pathway of *NF-κB* [68]. In this context, in a recent study, the suppressive role of this gene in endometriosis development has been reported [69]. *CD48* is known as a B-lymphocyte activation marker (BLAST-1) expressed on the surface of immune cells (such as NK cells) [70] and is involved in T cell activation [71]. It has also been reported that the *CD48* is associated with the molecular and cellular function of activation of myeloid cells in the inflamed endometrium in pig and may be important for maintaining uterine homeostasis and functions [72]. In a study, *CD48* suggested as a novel biomarker for the diagnosis of endometritis [73]. *CD19* is a well-known marker for B cell activation and plays an important role in maintaining immune balance by regulating the immune response of B lymphocytes. In patients with endometriosis, altered expression of *CD19* has been suggested to contribute to the pathological mechanisms of this disease [74]. *CXC* chemokines, a subfamily of chemokines including *CXCL1-17*, participate in both the initiation and progression of endometriosis, as discussed in [75]. Previous studies have shown that overexpression of *CXCL3* and *CXCL5*, play a role in the pathogenesis of bovine [76] and canine [77] endometriosis. Totally, chemokines have been reported to be associated with endometriosis [78]. In this regard, the expression pattern of *CXCL3* and *CXCL5* suggested that they can be considered as biomarkers for the severity of endometriosis [75, 79]. *PDGFRB* gene encodes platelet-derived growth factor receptor beta, which transmits signals critical for cell survival, proliferation, and differentiation. Notably, it is one of the key markers for identifying endometrial mesenchymal stem cells [80]. This gene has also been suggested as a potential therapeutic target for endometriosis, because its knockdown in endometriosis cells inhibited their proliferation, invasion and migration [81]. *PTPRCAP* gene (also known as *CD45*-associated protein or *CD45* protector) encodes a protein that binds to *CD45* and plays a role in

regulating its function. *CD45* gene is known as a surface marker on immune cells, commonly found on most white blood cells, including T lymphocytes, B lymphocytes, macrophages and neutrophils. In a scRNA-Seq study of endometrium of infertile women, higher proportion of *CD45* + cells compared to controls was reported in endometritis, highlighting the active immune response [82]. *NR4A2* is expressed in epithelial and stromal derived endometriotic cells and inhibits NF-κB-driven inflammation. It has been demonstrated that this gene plays an important role in activating endometriosis-driving cellular pathways in human endometriotic epithelial and stromal cells [83]. *HBEGF* gene in the normal endometrium helps improve embryo adhesion to the endometrium by interacting with EGF receptors. Insufficient or abnormal expression of *HBEGF* can lead to problems with the endometrium's receptivity to embryo implantation [84].

In addition to the above genes, the important roles of the other hub genes in the pathogenesis of endometritis have been highlighted in the previous studies such as *EPHA2* [85], *EGR1* [86], *CD93* [74] and *ARG2* [87]. It is worth pointing out that out of the genes involved in ER stress, *ATP2A2*, *LMAN1* and *SEC24D* were hub genes in the PPI network. Roles of these genes in endometritis and immune response-related pathways have been documented. For example, *ATP2A2* encodes type 2 sarcoendoplasmic reticulum calcium-ATPase (*SERCA2*), which is an important candidate protein involved in pathophysiology of endometriosis [88]. There are also recent studies that have demonstrated role of this gene in endometriosis [89, 90]. All this evidence emphasizes the importance of the brown module in the pathogenesis of endometritis. Taken together, the hub genes within the brown module appear to play crucial roles in immune activation, inflammation and tissue repair during endometritis. Therefore, further investigations on these genes may help to elucidate disease mechanisms and facilitate the identification of promising diagnostic biomarkers and therapeutic targets. It is well known that the complexity of the molecular mechanisms underlying various biological processes as well as diseases development arises from elaborate gene regulation rather than an increase in the number of expressed genes. In this regard, results of the present study, in agreement with the previous studies [14, 91–93], showed that RNA stability can be considered as an extra layer of information that modulate gene regulatory networks behind pathogenesis of diseases. With all this in mind, it is concluded that regulation of stability of genes can be a potential mechanism involved in the pathogenesis of endometritis, which should be focused on to highlight genes and pathways that might be overlooked by focusing only on expression levels. These findings lay a foundation for further investigation into the molecular mechanisms governing diseases/infection development through RNA stability.

To provide a better understanding of the genetic basis of endometritis disease, a gene co-expression network based on RNA stability was constructed. The hypothesis is that comparison of RNA stability profiles between normal and infected samples may highlight changes induced by bacterial infection, potentially revealing new molecular targets for improving endometritis treatment and management. Our results revealed that genes with higher stability in the infected samples than healthy ones, were significantly involved in ER stress and immune response pathways. Moreover, what is worth noting is that a PPI network was constructed based on these genes and 28 hub genes were detected, which most of them were reported previously as biomarkers in endometritis or response to infection. This finding

showed effectiveness of our approach to identify related genes/pathways to endometritis based on stability of genes. This study supplied a theoretical basis for considering RNA stability as another level of regulatory control in endometritis, and probably in other diseases, in additional to the various post-transcriptional mechanisms.

Methods

Dataset

In this study, raw data was obtained from the gene expression omnibus (GEO) database at the national center for biotechnology information (NCBI) under the accession number GSE66825. Samples were collected from 15 Holstein cows (nine healthy and six endometritis cows) on days 7 and 21 postpartum. Cows were clinically evaluated postpartum and classified as healthy or endometritic based on established diagnostic criteria. Endometrial biopsies were collected and histopathologically examined to confirm disease status prior to downstream analyses. For gene expression analysis, total endometrial RNA was extracted using the Qiagen RNeasy® Plus Mini kit and RNA quality was assessed with the Agilent 2100 Bioanalyzer. RNAs were converted to cDNA and RNA-Seq sequencing was performed using the Illumina® HiSeq™ 2000 instrument, resulting in the generation of 30 RNA-Seq samples in total (49 bp paired-end) [94].

RNA-Seq Data Processing and Analysis

The quality of raw reads was checked using FASTQC software (version 0.11.9), both prior to and following the trimming process [95]. Adapter trimming and low-quality filtering were performed using Trimmomatic software (version 0.35) (TRAILING:20, MAXINFO:40:0.90 and MINLEN:40) [96]. A key step in many next-generation sequencing analyses is the alignment of reads to the reference genome. In this study, HISAT2 software (version 2.1) was used to align the reads to the bovine reference genome [97]. The bovine genome (Bos_taurus.ARS-UCD1.2.1) and GTF file (version 110) were downloaded from the Ensembl database.

RNA Stability Matrix

RNA synthesis/degradation is an important step in post-transcriptional regulation and changes in RNA stability rate are involved in immune response and the other biological processes [14, 26, 98]. Several methods have been developed to measures RNA stability based on RNA-Seq data. The basic principle behind these methods is comparing the un-spliced and spliced transcripts based on the aligned reads to intronic and exonic regions of genes, respectively [20, 21, 99]. In fact, the exonic read counts indicate steady-state mRNA levels, while intronic read counts represent the transcriptional activity of a gene prior to splicing. Therefore, a high exonic/intronic ratio represents a stable transcript with a longer half-life, while a low ratio indicates more intronic reads and suggests an unstable transcript that is rapidly degraded [20]. In the current study, our previous approach was applied to construct RNA stability matrix, as described in details in [14]. Briefly, REMBRANDTS tool (version 1.0) used with default settings to

estimate stability of RNA transcripts across the samples [21]. HTSeq-count software (version 2.0.2) was used to quantify the aligned reads to exonic and intronic regions of the genes [100], which is applied by REMBRANDTS to calculate relative stability indices as log(Δexon / Δintron). In this formula, Δexon indicates steady-state mRNA, based on exonic read counts, and Δintron represents transcriptional activity according to intronic read counts. Then, to provide unbiased estimates of stability, REMBRANDTS refines this baseline metric (log(Δexon / Δintron)) using several sophisticated algorithms, which result in more precise estimates of stability [21]. Positive and negative values in RNA stability matrix represent higher (longer half-life) and lower (shorter half-life) stability, respectively.

Weighted Co-expression Module Detection

We hypothesized that RNA stability patterns of transcripts can be different between healthy and infected samples. Hence, to obtain the RNA stability patterns of transcripts between the two conditions, weighted correlation network analysis (WGCNA) package of R was used [101]. To do this, the resulting RNA stability matrix used as input for WGCNA analysis. By this approach, the transcripts with similar RNA stability patterns were clustered together in same modules. First, transcripts with constant stability across samples were removed based on an unpaired t-test analysis. The mean of stability of a transcript between healthy and infected samples was compared and the transcripts with a p-value < 0.3 were kept for further analysis. It is important to note that the higher p-value than the statistical significance threshold at this step serves only as an initial filter and does not aim to provide statistical conclusions. This lenient threshold ($p < 0.3$) was selected to identify transcripts whose stability are changed during the disease development and are more likely to be associated with endometritis. Pairwise correlation coefficient was computed based on biweight midcorrelation method, as it is robust and resistant to outliers. Then, distance-based adjacency matrix of stability was constructed based on the correlation matrix and was transformed into a topological overlap matrix (TOM) using the TOM similarity function, thereby constructing the gene connectivity network. To ensure the reliability of network construction, the outlier samples were excluded (standardized connectivity less than − 2.5). Furthermore, to determine whether the sample data was complete as well as exclude the samples and genes with excessive missing entries and genes with zero variance, goodSamplesGenes function in WGCNA package was applied. To convert the correlation matrix to adjacency matrix, the soft threshold power (β) was set by plotting the R^2 value (scale-free topology fitting index) against soft thresholds, to be sure the resulting adjacency matrix follows the scale-free topology [102–104]. The approximate scale-free topology in the samples was achieved at a soft threshold of $\beta = 16$. Next, the TOM matrix was hierarchically clustered to identify gene modules, exhibiting similar stability patterns. Finally, co-expression gene modules were refined by the DynamicTreecut algorithm. The following major parameters were set in blockwiseModules function of WGCNA package: power = 16, corType= "bicor", networkType = "signed", TOMType = "signed", maxBlockSize = 17000, minModuleSize = 30, reassignThreshold = 0, mergeCutHeight = 0.25.

Based on our primary hypothesis, cells can alter the stability of genes as a post-transcription regulation to activate defense pathways and response to infection. Hence, modules containing genes with higher stability in infected samples (vs healthy samples) can be considered as functional modules involved in

endometritis development. It is worth noting that modules containing genes with higher stability in healthy samples are expected to be involved in general cellular processes such as cell cycle, division and metabolism. In other words, alterations in stability of genes can be a method by which the cell redirects its main programs to activate immune response, during infection. Thus, cell use post-transcription regulatory mechanisms to increase the stability of genes related to immune response and decrease the stability of genes associated with general functions. According to above explanations, it is expected that RNA stability in infected and healthy samples follow a distinct trend. To visualize this trend, stability of genes in all modules were visualized using heatmap plot. Except ambiguous stability trend across the samples, two distinct trends can be observed including 1) higher stability of genes in healthy samples than infected ones and 2) higher stability of genes in infected samples than healthy ones. Of these, group 2 is of great interest and we focused on this group, as it may contain key genes involved in immune system as well as endometritis development.

Network Propagation Analysis

Network propagation is based on the idea that genes contributing to similar phenotypes/biological processes, such as a specific disease, often interact with each other within a molecular network. Using this concept, known information about certain disease-related genes can be spread through the network to uncover additional genes that may not have been directly linked before, but are connected through these interactions. In network propagation analysis, a group of genes is first chosen as seed nodes. These seeds are given an initial score, which is then gradually passed on to their neighboring nodes across the network. This spreading process continues until the scores stabilize and each gene has a final value that shows how strongly it connects back to the seeds. Finally, the connected genes to the seeds are identified and can be considered as functionally related genes to them [105, 106]. Based on this idea, we recently developed a novel network propagation analysis to investigate the extent to which module genes are functionally related and may be involved in the similar biological processes. This method test weather genes within a module of interest are topologically proximal in a protein-protein interaction (PPI) network, as described in details in [14]. To do this, a given module was compared against two reference groups including 1) grey module from WGCNA output (non-co-expressed genes that are not assigned to any module) and 2) all mRNAs annotated in bovine GTF file (named random set). First, a subset of module genes (25% of total genes, randomly selected) was considered as seed and propagated across the PCNet gene network (Parsimonious Composite Network including18,820 nodes, 2,693,109 edges)[107] using the random walk with restart (RWR) algorithm implemented in a modified version of NetColoc (v3.9.0) [108]. Propagation scores (w' for seed genes and w" for non-seed genes) were standardized into Z-scores to distinguish biologically meaningful associations from random background interactions, considering $Z > 3$ as threshold for significant proximity. Notably, a higher Z-score shows that the two genes are associated more frequently than would be expected by chance, indicating a non-random and potentially biologically relevant association. Next, the number of propagated genes ($Z > 3$) overlapping with the module's genes obtained, which indicates module functionality. In other words, a higher degree of intersection between the number of propagated genes and module's genes provides evidence for functional connectivity of genes within the module. For a

module, these steps were repeated 100 times and 100 numbers were obtained that used to generate a distribution. Finally, Welch's one-way ANOVA analysis was applied to statistically evaluate differences between the distributions of the module of interest and the reference groups. Thus, three pairwise comparisons performed including module of interest vs grey module, module of interest vs random set group and grey module vs random set group (p-values adjusted by FDR method).

Functional Enrichment Analysis

To better understand the functional mechanisms of each module and their corresponding hub genes, EnrichR tool (https://maayanlab.cloud/Enrichr/) was used. The genes of interest were subjected to gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. Functional terms with a padj < 0.05 (corrected using the FDR method) were considered as significant ones.

PPI Network Analysis

To explore the potential functional connections among the genes at the protein level, PPI network was constructed using the STRING database (https://string-db.org/) [109]. The constructed PPI network was then visualized and organized using Cytoscape software (version 3.10.2) to better show the interaction patterns among the hub genes [110]. Genes with the highest degree of connectivity in gene regulatory networks are considered as hub genes, which are key players in gene networks and are of great biological importance. To identify hub genes in the constructed PPI network, our previous method [111] based on cytoHubba (version 0.1), a Cytoscape plugin, was used [112]. Briefly, 12 different topological analysis methods are provided in this tool for ranking the important nodes in biological networks including density of maximum neighborhood component (DMNC), maximum click centrality (MCC), degree method, edge percolated component (EPC), maximum neighborhood component (MNC), bottleneck, EcCentricity, closeness, radiality, betweenness, stress and clustering coefficient. The top 50 hub genes in the PPI network were detected using each of the methods. Then, rankAggreg R package (version 0.6.6) applied to establish a consensus rank of the important genes [113], based on two methods including cross-entropy Monte Carlo algorithm and genetic algorithm. Overlapping genes between the two methods were defined as highly connected genes in the PPI network. On the other hand, kME index (module membership) was estimated to measures the strength of a gene's connection to the module. In other words, kME represents the degree of correlation between genes within the module [114, 115]. Finally, the highly connected genes (detected by cytoHubba and rankAggreg methods) with kME ≥ 0.5 were considered as hub genes, indicating their strong connection to the module and their importance in the PPI network.

Declarations

Competing interests

The authors declare that they have no known financial or personal conflicts of interest that could have influenced the work reported in this paper.

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Author Contribution

MZ conducted project administration, developed methodology, contributed to visualization, performed investigation, and drafted the manuscript. MRB supervised the study, conceptualized the research, developed methodology, contributed to visualization, drafted the manuscript, and performed formal analysis. AS assisted in methodology and reviewed the manuscript. All authors read and approved the final version of the manuscript.

Data Availability

The datasets analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository under accession number GSE66825 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66825). Additional supporting data are provided within the paper and its supplementary materials.

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Figures

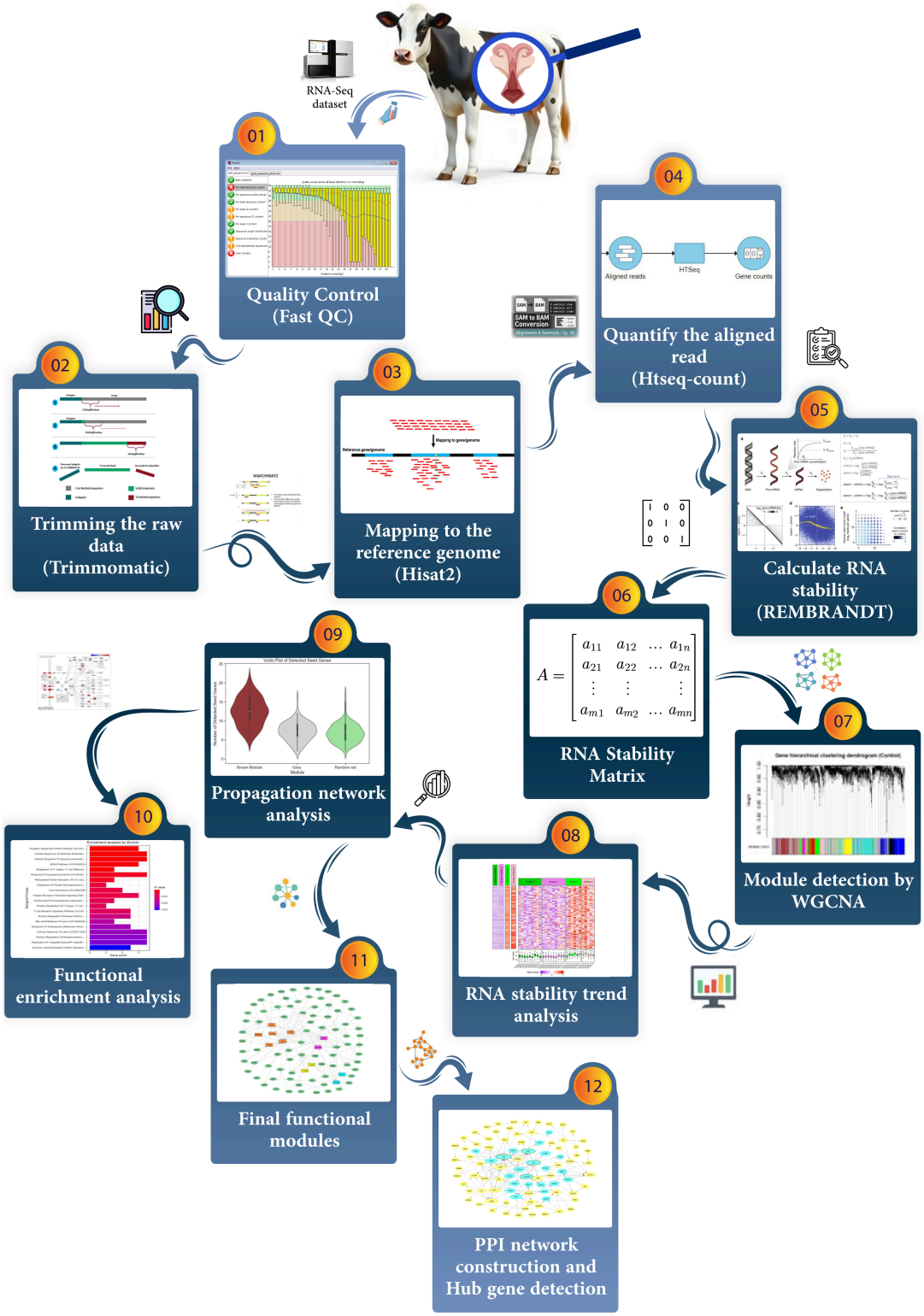


Figure 1

Overview of the used pipeline to investigate RNA stability in bovine endometritis based on RNA-Seq dataset.

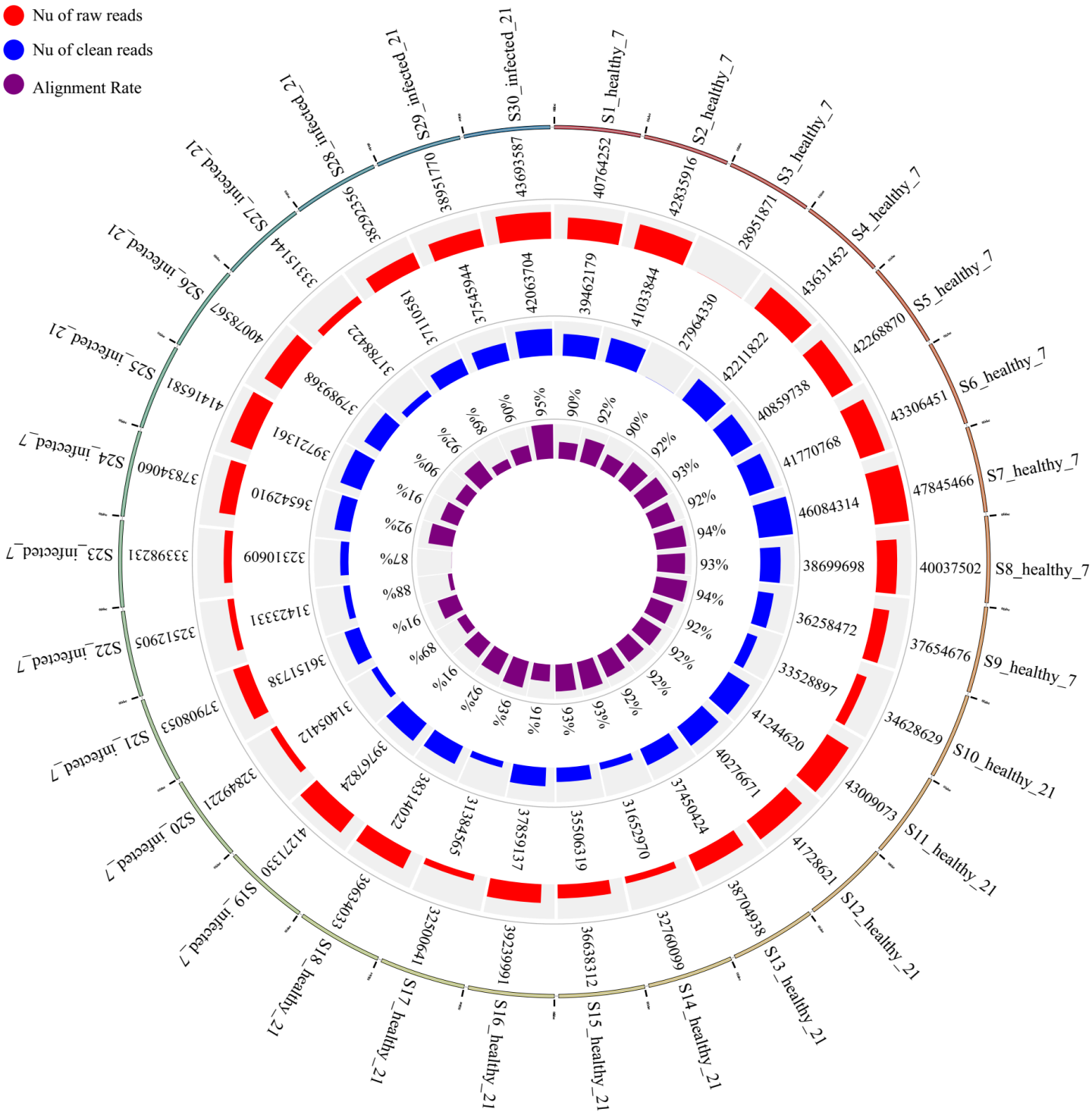


Figure 2

Circos plot indicating the number of raw reads (outer ring, red plot), clean reads (middle ring, blue plot), and alignment rate (inner ring, purple plot) per sample.

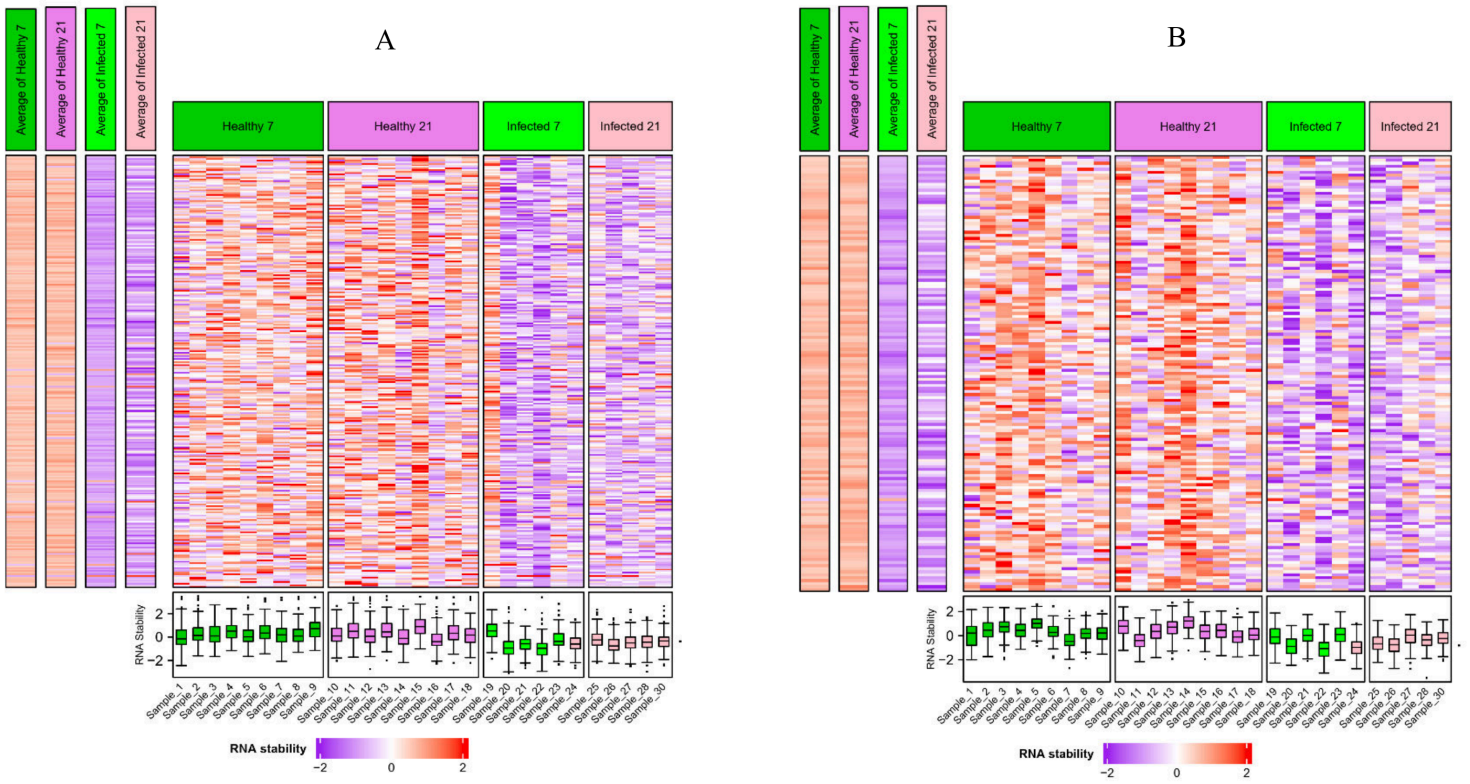


Figure 3

Heatmap plot related to RNA stability trend across the samples in healthy and infected samples at 7 and 21 days postpartum for A) turquoise and B) yellow modules. Healthy and infected samples for 7-day postpartum are shown in green, and for 21-day postpartum are shown in pink. A box plot representing RNA stability is provided below each heatmap. The left side heatmap plots show the average of RNA stability in healthy and infected conditions for an overall stability assessment. Red color indicates higher RNA stability.

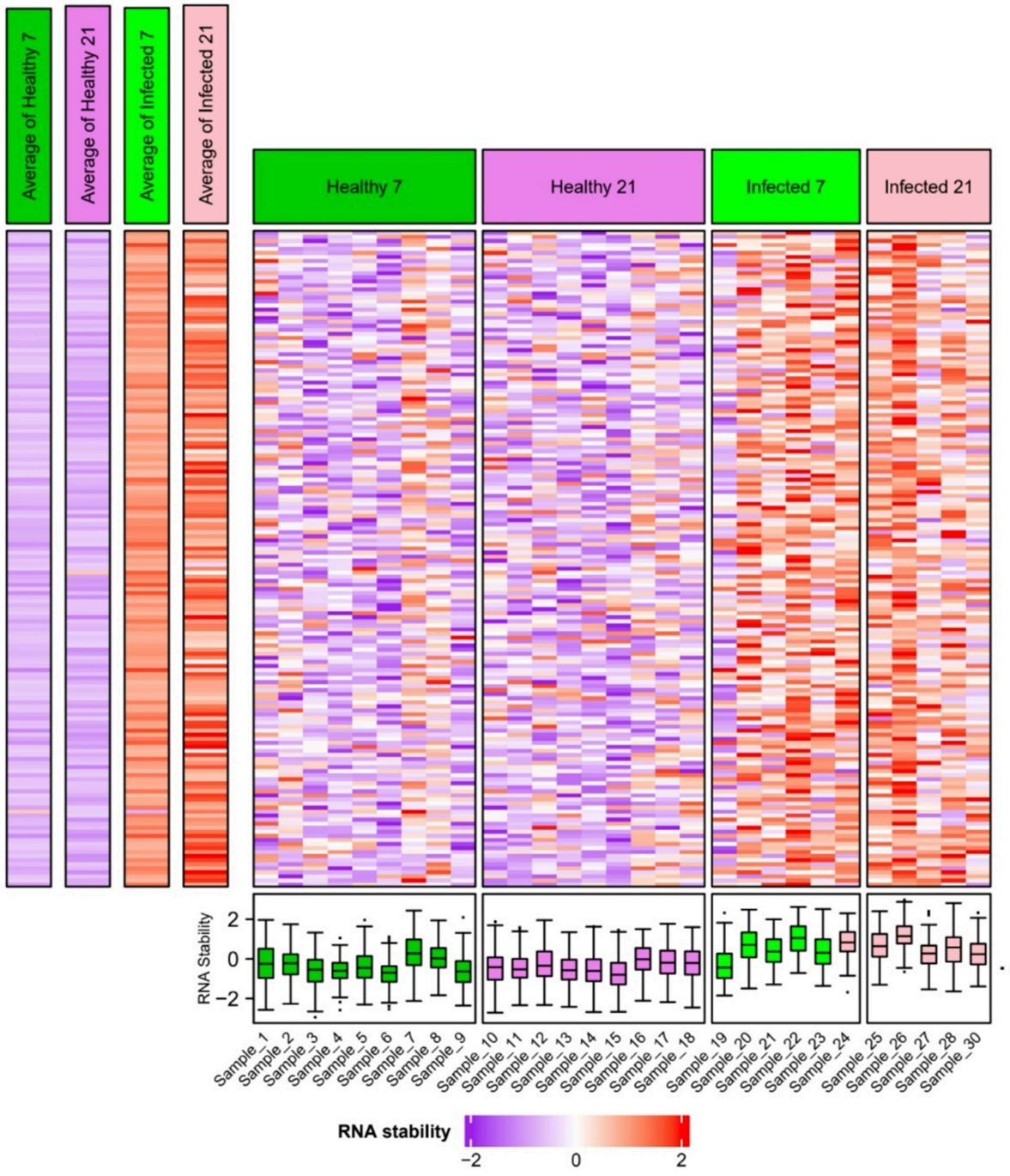


Figure 4

Heatmap plot related to RNA stability trend across the samples in healthy and infected samples at 7 and 21 days postpartum for brown module. Healthy and infected samples for 7-day postpartum are shown in green, and for 21-day postpartum are shown in pink. A box plot representing RNA stability is provided below each heatmap. The left side heatmap plots show the average of RNA stability in healthy and infected conditions for an overall stability assessment. Red color indicates higher RNA stability.

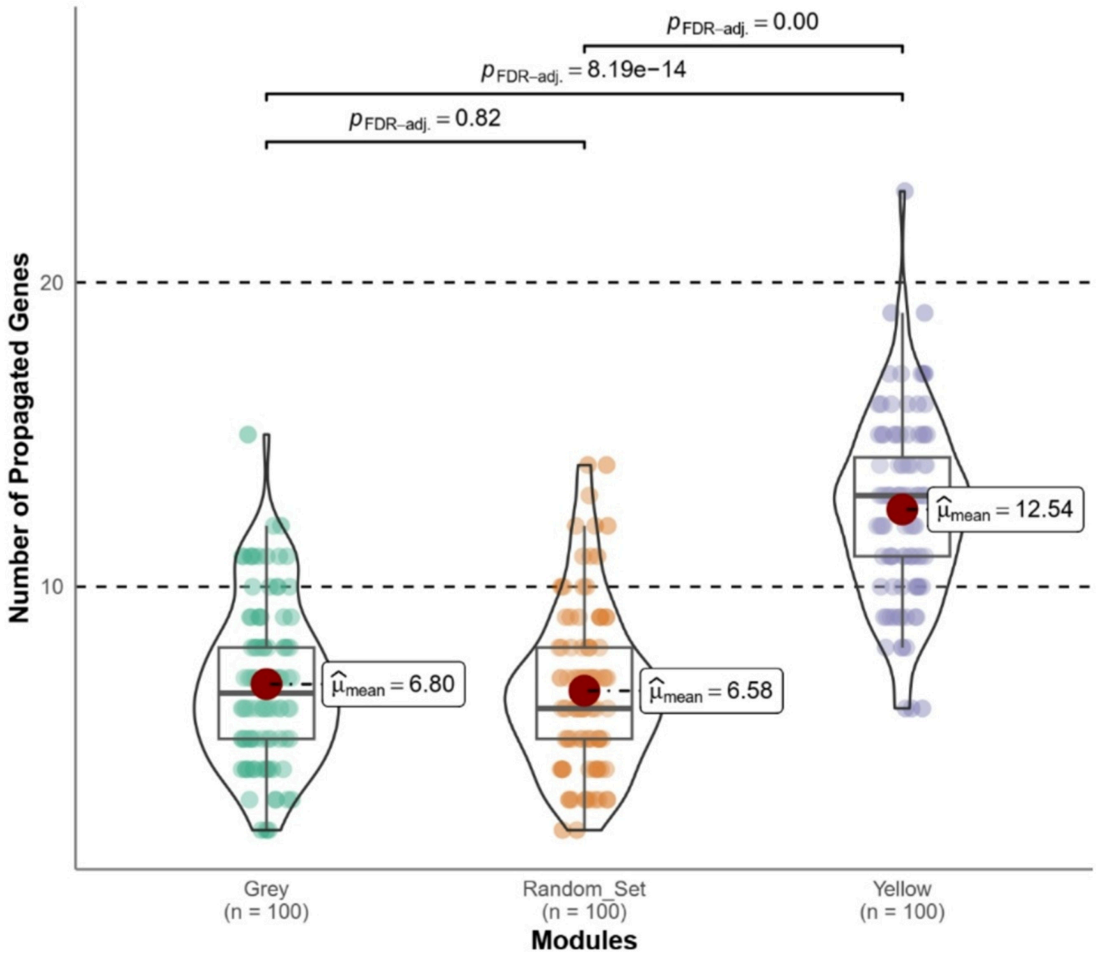


Figure 5

Distribution of the propagated genes during 100 iterations for brown module. Grey and random set are the two reference groups.

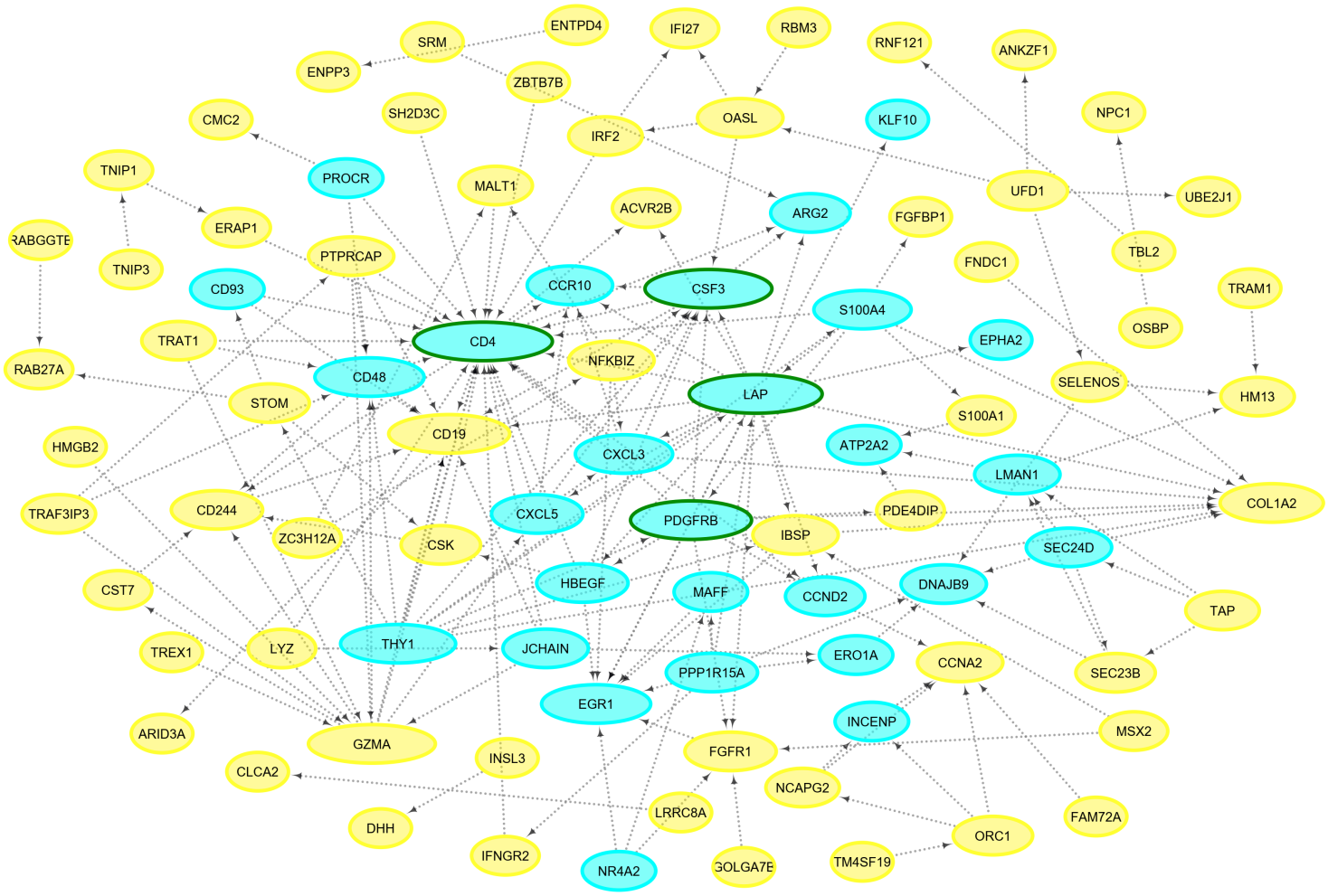


Figure 6

PPI network of the genes within brown module. Size of the nodes are based on their connection numbers. Blue nodes indicate the final hub genes. The hub genes with more than 10 connections (*CD4*, *LAP*, *CSF3* and *PDGFRB*) are bolded (green color around the circles).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiles.xlsx
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