

# Dysregulation of *TTN* Gene Expression, Titin Protein Levels, and Circulating ncRNAs (*miR-200a*, *lncRNA FOXD2-AS1*, and *snoRNA-42*) in Dilated Cardiomyopathy: A Pilot Study

Azhar Younus Dhannoon\*, Abeer Ataallah Ayyed Al-Hadidy, Owayes M. Hamed

Department of Biology, College of Science, University of Mosul, Mosul, Iraq

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**Abstract-** Dilated Cardiomyopathy is the major cause of congestive heart failure and sudden cardiac death. It is a type of cardiomyopathy characterized by ventricular dilatation, wall thinning and systolic dysfunction. *TTN* gene is implicated in DCM and encodes for giant titin protein. This study explores the association between *TTN* gene expression with some types of non-coding RNAs in DCM patients. The study comprised three age- and sex-matched groups (20 individuals each): healthy controls, male patients, and female patients. Relative expression levels of *TTN*, *miR-200a*, *lncRNA FOXD2-AS1*, and *snoRNA-42* were quantified using qRT-PCR, whereas serum titin protein levels were measured via ELISA. *TTN* gene expression was significantly elevated in both male and female DCM patients compared with controls ( $P < 0.001$ ). Furthermore, *miR-200a* and *snoRNA-42* levels were also significantly increased in DCM patients particularly in females. In contrast, *lncRNA FOXD2-AS1* was significantly decreased in both sexes of DCM patients. ROC curve analysis for *TTN* gene demonstrated high diagnostic performance, sensitivity and specificity with ( $P = 0.0022$ ), supporting *TTN* as a reliable molecular biomarker for DCM. Similarly, *miR-200a* showed strong diagnostic potential, particularly in females ( $P = 0.0275$  in males and  $P = 0.0022$  in females), underscoring its efficiency as a sex-specific biomarker. Furthermore, Titin protein level was significantly elevated in DCM patients, particularly in females. Pearson correlation analysis detected a negative correlation between *miR-200a* and *TTN* mRNA in both males and females, negative correlations were also observed between *miR-200a* and titin protein levels. In contrast, *TTN* gene expression and titin protein levels showed a positive correlation, indicating transcription-driven increases in titin level. To the best of our knowledge, this is the first study in Iraq, particularly in Mosul, to integrate Titin protein and *TTN* gene expression with multiple circulating ncRNA classes (*miR-200a*, *lncRNA FOXD2-AS1* and *snoRNA-42*) in patients with DCM. The combined analysis of *TTN* gene / Titin protein axis and ncRNAs within a clinical group from Mosul city provides a comprehensive view of post-transcriptional regulation and revealed sex-dependent molecular differences. Moreover, the strong diagnostic performance of *TTN* and *miR-200a* supports their potential as non-invasive biomarkers for DCM. By focusing on a Middle Eastern population, this study contributes novel region-specific molecular data and expands current understanding of DCM beyond previously studied populations. In DCM patients the expression level of *TTN* gene was significantly increased, demonstrating that transcriptional activation was correlated with myocardial remodeling. The dysregulated expression of ncRNAs (*miR-200a*, *lncRNA FOXD2-AS1* and *snoRNA-42*), suggested the existence of a complex post-transcriptional regulatory network affecting *TTN* expression. All of these findings highlight the potential of *TTN* and ncRNA signatures as promising non-invasive molecular biomarkers for DCM.

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**Corresponding Author:** A.Y. Dhannoon

Department of Biology, College of Science, University of Mosul, Mosul, Iraq  
Tel: +9647705212605, E-mail address: azhar.younis@uomosul.edu.iq

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## Introduction

Dilated cardiomyopathy (DCM) is characterized by dilation of the left ventricle or both ventricles, accompanied by systolic malfunction, occurring independently of coronary artery disease, valvular abnormalities or hypertension. DCM is defined by left ventricular (LV) or biventricular systolic dysfunction and dilatation not explained by abnormal loading conditions or coronary disease and affects approximately 1 in 250 individuals. Causes of DCM can be classified as being genetic or nongenetic (1). The titin (*TTN*) gene, consisting of 364 exons and located on chromosome 2q31, represents the most common genetic contributor to DCM. However, it is increasingly recognized that this genetic predisposition frequently interacts with extrinsic or environmental factors to drive disease progression. This gene encodes titin protein which is the largest human protein, it has structural, sensory, and signaling functions. Titin defects disrupt sarcomeric structural integrity and force transmission, thereby compromising cardiomyocyte contractility and ultimately driving the myocardial remodeling characteristic of DCM (2). Mutations that substantially disrupt the structure of full-length titin might cause dilated cardiomyopathy by means of several mechanisms. Change in titin levels could limit sarcomere formation and might cause cardiac dysfunction and remodeling (3).

Non-coding RNAs (ncRNAs) are a broad class of RNA molecules that are important regulators of gene expression even though they are not translated into proteins (4,5). The *miR-200* family has been implicated in the pathogenesis of both dilated cardiomyopathy and heart failure (6). *MIR-200a* is a short noncoding RNA and a member of the *miR-200* family, which consists of five members grouped and expressed as two distinct polycistronic pri-miRNA transcripts located on chromosome 1. *miR-200a* plays a regulatory role by targeting  $\beta$ -catenin, thereby influencing the Wnt/ $\beta$ -catenin signaling pathway (6,7).

Long non-coding RNAs (lncRNAs) are RNA transcripts longer than 200 nucleotides that lack the ability to encode proteins. They are key regulators of gene expression across multiple levels, including epigenetic, transcriptional, and post-transcriptional mechanism (8). They influence cardiovascular function through mechanisms such as chromatin remodeling and transcriptional or post-transcriptional regulation, particularly affecting cardiac remodeling, inflammation and apoptosis (9). According to increasing research in

heart failure (HF) patients, dysregulated lncRNAs are implicated in the development and progression of heart failure, by influencing cardiomyocyte hypertrophy, fibrosis, and contractile function through a variety of processes. snoRNAs are small non-coding RNAs mainly located in the nucleolus, that direct the chemical changes of rRNAs and snRNAs, primarily supporting the synthesis of scaffolds. Their dysregulation in DCM can affect protein synthesis and lead to fibrosis, remodeling, and functional deterioration in the heart (10). Although *TTN* mutations are well established as a major genetic contributor to dilated cardiomyopathy, most previous studies have focused primarily on DNA-level or truncations variants, with limited attention to *TTN* transcriptional regulation. In particular, the interaction between *TTN* gene expression and non-coding RNA-mediated regulatory mechanisms remains poorly understood. Moreover, few studies have simultaneously examined *TTN* gene and multiple classes of ncRNAs within the same clinical group. Furthermore, data exploring sex-specific molecular differences in this regulatory axis are scarce, and region-specific molecular profiles from Middle Eastern populations are largely lacking. Therefore, the combined investigation of *TTN* expression and circulating ncRNAs may provide novel insight into post-transcriptional regulation and biomarker potential in DCM.

Aim of the study: this study aimed to evaluate the level of gene expression for *TTN* gene, *Titin* protein, and detecting the relationship with ncRNA (*miRNA 200a*, *lncRNA FOXD2-AS1* and *snoRNA-42* genes) and to estimate their diagnostic value in DCM patients.

## Materials and Methods

### Patients selection and sample collection

This study included 60 adult participants (males and females) aged 30-45 years. Biological specimens were collected from the Mosul Center for Cardiac Medicine and Surgery, Al-Salam Teaching Hospital, and private laboratories in Mosul City. All patients with DCM were clinically diagnosed by a consultant cardiologist at these institutions. Since standardized reference ranges for serum titin levels are currently unavailable, healthy age- and sex-matched controls were recruited to establish a baseline for comparative analysis. Three milliliters (3 mL) of peripheral blood were drawn from both experimental and control groups and distributed into three specialized collection tubes: EDTA tubes for genomic DNA isolation, gel-separator tubes for serum extraction, and TRIZOL-

containing tubes for total RNA and ncRNA extraction. Serum titin protein levels were subsequently quantified using an ELISA kit (SUNLONG Biotech, China) according to the manufacturer's instructions. All measurements were performed in duplicate to ensure analytical reproducibility.

### **Ethical Considerations**

The research protocol permission form from the Ministry of Health and Environment/ Iraq served as the basis for the study (Facilitation Letter Ref. No. 58908, dated December 9, 2024).

Extraction of RNA and non-coding RNA (miRNA, lncRNA and snoRNA)

RNA was extracted from blood using the Transgeneic Trizol kit, combining Trizol-phenol-chloroform lysis with silica membrane purification to obtain both mRNA and ncRNA in a single procedure.

### **Polyadenylation (Poly A polymerase)**

After RNA extraction, the Poly A process must be followed for ncRNA extraction.

Converted to cDNA: After extracting mRNA and non-coding RNA, reverse transcriptase was used in the RT-PCR process to synthesize cDNA from all RNA molecules.

### **Quantification of *TTN* gene expression levels by qPCR Technique**

The specific *TTN* and housekeeping gene primers (designed by primer 3 software for this study) used to determine the levels of gene expression by q-PCR. The primer sequences were as follows: *TTN* forward 5'-AGGAGCAAGTGACCATCAGTG -3' and reverse 5'-CAGCTGCACTTTTGTGTCAGCAT -3'; and housekeeping gene Ribosomal Protein S9 (*RPS9*) forward 5'-GACCCAGATCATGTTTGTAG-3' and reverse 5'-CGTACAGGGATAGCACAG-3'.

### **Detection the expression levels for ncRNA (mi-RNA, lncRNA and snoRNA) genes by used qPCR technique**

Specific primers (designed by primer 3 software for this study used to detect the levels of gene expression of miRNA, lncRNA and snoRNA molecules and housekeeping gene (*U6*) gene by qPCR. The primer sequences used were as follows: miR-200a forward 5'-AACAGAGGTGGGAGGTGGG -3' and reverse 5'-CAGTGCAGGGTCCGAGGT -3'; lncRNA forward 5'-TGGACCTAGCTGCAGCTCCA-3' and reverse 5'-AGTTGAAGGTGCACACACTG-3'; snoRNA-42 forward 5'-TGGATTTATGGTGGGTCCTT-3' and

reverse 5'-GCTCACAGCCCCACAGGTAAG-3'; and housekeeping gene *U6* forward 5'-GTGCTGCTTGGGCAGCA-3' and reverse 5'-GAAATATGGAACGGTTC-3'. The corresponding relative expression of ncRNAs was normalized to *U6* small nuclear RNA as an endogenous reference gene. Relative gene expression was calculated by  $2^{-\Delta\Delta Ct}$  method.

### **Quantitative qRT-PCR reaction setup**

To prepare a total reaction volume of 20  $\mu$ L, each reaction mixture contained 10  $\mu$ L of Ultra Syber q-PCR master mix, 0.5  $\mu$ L of forward primer, 0.5  $\mu$ L of reverse primer, 4  $\mu$ L of cDNA template, and 5  $\mu$ L of nuclease-free water.

### **qPCR reaction program**

The program of RT-PCR involve four steps for mRNA and ncRNA molecules: pre-denaturation at 95° C for 10 minutes, denaturation at 95° C for 15 seconds, annealing/extension at 60° C for 1 minute, and melting curve analysis at 95° C for 15 seconds, followed by 60° C for 1 minute, 95° C for 15 seconds, and 60° C for 15 seconds.

### **Statistical analysis**

Statistical analysis was conducted using GraphPad Prism, one-way ANOVA and applying t-tests for quantitative data, Pearson correlation for relationships between variables and Receiver operating characteristic (ROC) curve analysis to determine diagnostic thresholds (sensitivity, specificity, AUC), and the P-value<0.05 was considered significant.

## **Results**

### **Analysis of *TTN* gene expression level**

The results of this study showed a significant elevation in the expression levels of the *TTN* gene in DCM patients compared with healthy controls. The mean and standard deviation (Mean $\pm$ SD) in male patients were 4.131 $\pm$ 1.775, whereas in female patients they were 2.505 $\pm$ 0.591, relative to the control group (1.059 $\pm$ 0.402;  $P$ <0.001) (Table 1). *TTN* expression was significantly higher in both patient groups than in controls ( $P$ <0.001), and the difference between male and female patients approached statistical significance ( $P$ =0.053) (Figure 1). ROC curve analysis for both male and female DCM patients demonstrated remarkable discrimination (Table 2), with 100% sensitivity and 100% specificity. Furthermore, *TTN* expression yielded an Area Under the Curve (AUC) of

1.000 ( $P=0.0022$ ), confirming its high diagnostic accuracy in identifying DCM.

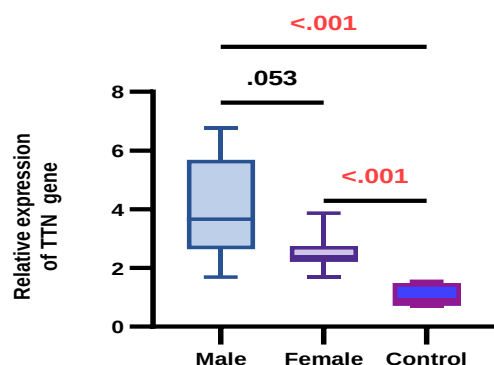


**Figure 1.** Relative *TTN* gene expression comparison among study groups (male, female and control group)

#### Analysis of *miR-200a* gene expression level

The data in table (1) shows a clear increase in *miR-200a* expression in both male and female with DCM. The mean expression of *miRNA* in females show a significant elevation  $7.784 \pm 0.5550$  compared to control  $1.114 \pm 0.2737$  which representing more than a six-fold increase. In contrast, an increase was also observed in males, the mean expression was  $2.758 \pm 0.4222$ , while controls exhibited  $1.283 \pm 0.2845$ .

Figure (2) illustrates the relative expression levels of *miR-200a* in patients compared with controls, the relative expression of *miR-200a* was markedly elevated in female patients compared with female controls ( $P<0.0001$ ), showing the strongest upregulation among the studied groups. Male patients also exhibited higher expression than their respective controls ( $P=0.0125$ ). When comparing the two patient groups directly, females demonstrated significantly higher levels than males ( $P<0.0001$ ). ROC analysis in table (2) showed perfect discrimination in both males and females' patient, notably in the females with a perfect AUC of 1.000 ( $P=0.0022$ ), exceeding the male group (AUC=0.860,  $P=0.0275$ ).

#### Analysis of *snoRNA-42* gene expression level

The current results in table (1) show a numerical elevation in the level of *snoRNA-42* among the patient group (female and male) compared to the control groups, with a noticeable increase in females; the mean was  $3.661 \pm 0.4738$  relative to the male group mean, which was  $2.576 \pm 0.5583$ . In contrast, in the control group, both male and female exhibited similar expression levels; the mean was  $1.797 \pm 0.7668$ .

Furthermore, the  $P$  between the female's patients with control was statistically significant was 0.049. the  $P$

between male patients with control was 0.42 figure (3).

#### Analysis of *lncRNA FOXD2-AS1* gene expression level

The patients with DCM had lower levels of *lncRNA FOXD2-AS1* expression than the control group figure (4). Compared to male controls ( $1.451 \pm 0.5288$ ), the mean value for men was  $1.099 \pm 0.3146$ , which is slightly lower. However, a greater significant decrease was seen in female patients, with a mean of  $0.5379 \pm 0.09434$  compared to  $1.451 \pm 0.5288$  in female controls table (1). In addition, the  $P$  between female patients with control was statistically significant was 0.033.

#### Analysis of titin protein level

Due to the absence of established standardized reference ranges for serum titin levels in the general population, we utilized healthy controls as a baseline for comparison. The current study's quantitative analysis of serum titin levels showed that DCM patients had higher levels than healthy controls. particularly among the female group the Mean $\pm$ SD was  $1909 \pm 679.3$  pg/mL, with statistically significant  $P=0.0022$ ). While, male exhibit  $1329 \pm 444.7$  pg/mL with non-statistically significant  $P$  figure (5).

#### Analysis the correlation between *miR-200a*, mRNA *TTN* gene and titin protein

According to Pearson correlation figure (6) male patients had negative correlation ( $r= -0.43$ ) between *miR-200a* and *TTN* mRNA, suggesting that *miR-200a* is active in the injured cardiac muscle and act as a molecular "brake" to control gene production despite general up-regulation. Furthermore, a negative correlation ( $r= -0.12$ ) was observed between *miR-200a* and titin protein, suggesting that higher *miR-200a* expression may contribute more directly to reduce the protein levels, potentially via post-transcriptional repression or promotion of mRNA degradation.

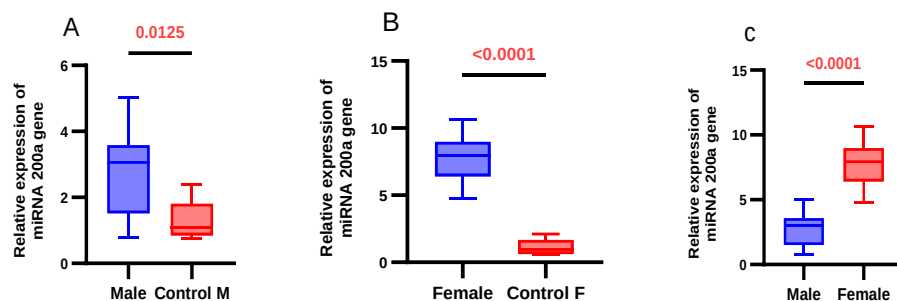
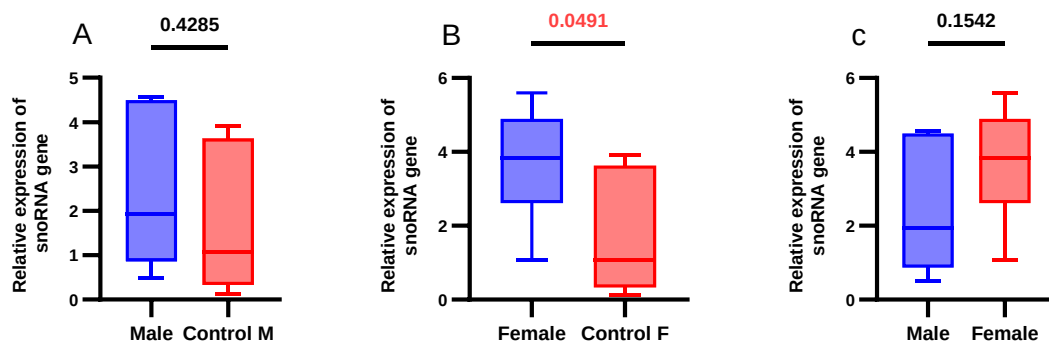
Concurrently, the positive correlation between *TTN* mRNA and titin protein ( $r=+0.33$ ) highlights the continued effectiveness of transcription-to-protein coupling in DCM. Female DCM patients exhibited negative correlation was observed between *miR-200a* and *TTN* mRNA ( $r= -0.44$ ) and a negative correlation was found between *miR-200a* and TITIN protein levels ( $r= -0.29$ ). At the same time, *TTN* mRNA and titin protein showed a more robust positive correlation ( $r= +0.67$ ), suggesting more efficient transcription-to-protein coupling in females.

**Table 1. Expression profiles of *TTN*, selected circulating ncRNAs, and serum titin protein levels in DCM patients and healthy control**

| Gene                     | Sample | Mean±Std. deviation patient | Median patient | Mean±Std. deviation control | Median control |
|--------------------------|--------|-----------------------------|----------------|-----------------------------|----------------|
| <i>TTN</i>               | Male   | 4.131±1.775                 | 3.661          | 1.059±0.4018                | 0.8913         |
|                          | Female | 2.505±0.5908                | 2.364          | 1.059±0.4018                | 0.8913         |
| <i>miR- 200a</i>         | Male   | 2.758±1.335                 | 3.059          | 1.283±0.6361                | 1.102          |
|                          | Female | 7.784±1.755                 | 7.933          | 1.114±0.6120                | 0.9862         |
| <i>snoRNA-42</i>         | Male   | 2.576±1.751                 | 1.932          | 1.797±1.717                 | 1.064          |
|                          | Female | 3.661±1.498                 | 3.825          | 1.797±1.717                 | 1.064          |
| <i>lnc-RNA FOXD2-AS1</i> | Male   | 1.099±0.9950                | 0.5664         | 1.451±1.182                 | 1.149          |
|                          | Female | 0.5379±0.2983               | 0.5498         | 1.451±1.182                 | 1.149          |
| Titin protein            | Male   | 1329±444.7 pg/ml            | 1300           | 1146±229.8                  | 1146           |
|                          | Female | 1909±679.3 pg/ml            | 1858           | 1118±192.9                  | 1079           |

**Table 2. ROC analysis for *TTN* and *miR-200a* genes expression levels**

| Gene            | Sample            | AUC    | 95% confidence interval | Sensitivity % | Specificity% | P-value |
|-----------------|-------------------|--------|-------------------------|---------------|--------------|---------|
| <i>TTN</i>      | Male vs control   | 1.000  | 1.000 to 1.000          | 100%          | 100%         | 0.0022  |
|                 | Female vs control | 1.000  | 1.000 to 1.000          | 100%          | 100%         | 0.0022  |
| <i>MiR-200a</i> | Male vs control   | 0.8600 | 0.6617 to 1.000         | 90%           | 80%          | 0.0275  |
|                 | Female vs control | 1.000  | 1.000 to 1.000          | 100%          | 100%         | 0.0022  |

**Figure 2. Relative *miR-200a* gene expression level comparison among study groups (A. Males, B. Females, C. Control)****Figure 3. Relative *snoRNA-42* gene expression level comparison among study groups (A. Males, B. Females, C. Control)**

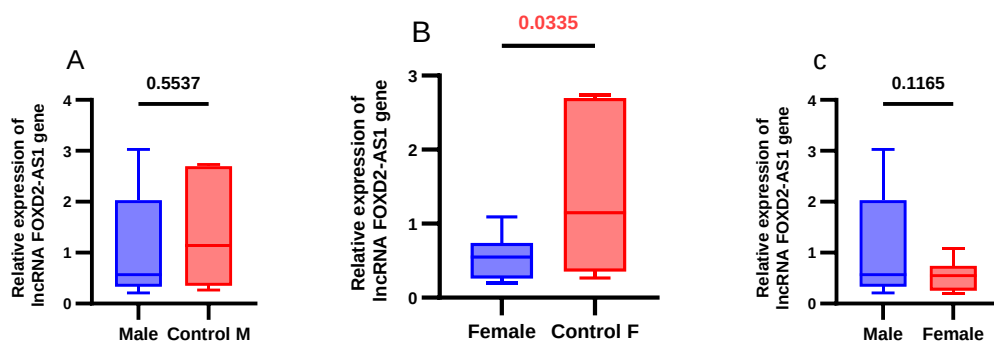


**Figure 4.** Relative *lncRNA FOXD2-AS1* gene expression level comparison among study groups (A. Males, B. Females, C. Control)

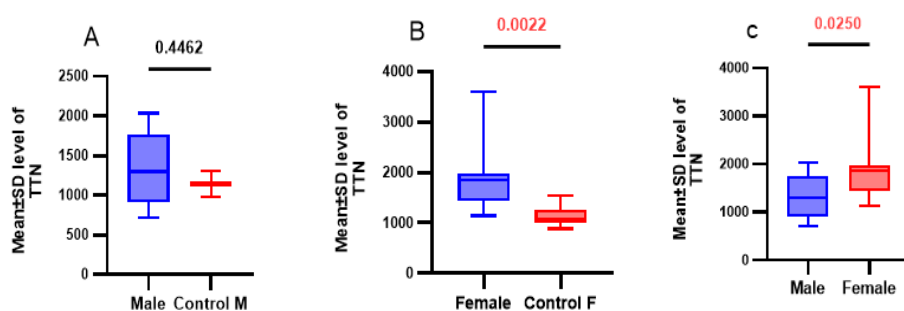


**Figure 5.** Serum titin protein level comparison among study groups (A. Males, B. Females, C. Control)

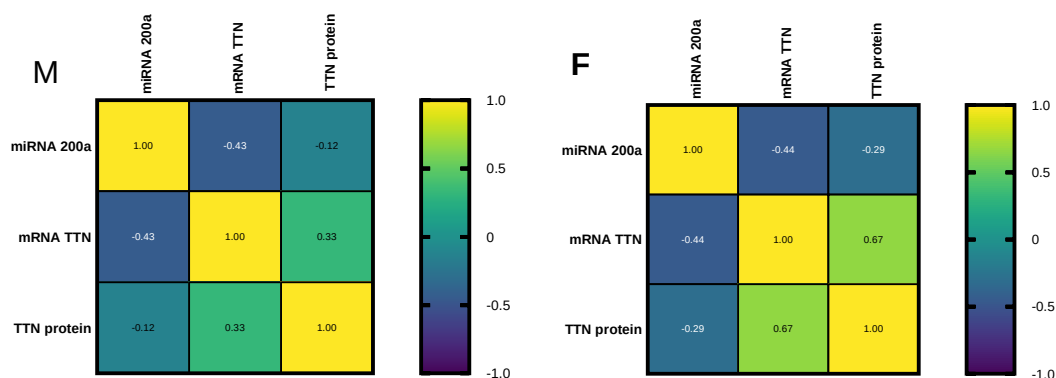


**Figure 6.** Heatmaps for correlation between *miR-200a*, *TTN* mRNA and Titin Protein among patients' groups, M=patients male and F=patients female

## Discussion

Dilated cardiomyopathy (DCM) is a major contributor to heart failure and sudden cardiac death, characterized by adverse left ventricular remodeling and progressive contractile dysfunction. Although structural variants and truncating mutations in the *TTN* gene are well established

in DCM pathogenesis, the transcriptional regulation of *TTN* and its post-transcriptional modulation by circulating non-coding RNAs (ncRNAs) remain incompletely understood. In this study, we investigated the integrated molecular profile of *TTN* mRNA, serum titin protein, and specific circulating ncRNAs (*miR-200a*, *snoRNA-42*, and *lncRNA FOXD2-AS1*) in DCM patients,

uncovering pronounced sex-dependent expression signatures and diagnostic biomarker utility.

Our findings demonstrate a significant upregulation of *TTN* transcript levels in DCM patients, which positively correlated with circulating titin protein concentrations. Under chronic biomechanical stress, the myocardium activates compensatory transcriptional programs. However, this upregulation often involves an isoform shift toward the larger, more compliant N2BA titin isoform relative to the stiffer N2B isoform (11,12). Although increased expression of compliant titin temporarily preserves diastolic filling under elevated wall stress, it compromises active force generation and accelerates progressive ventricular dilatation (10,13). Furthermore, elevated circulating titin protein levels, particularly in female patients, likely reflect ongoing myofibrillar damage and oxidative cleavage mediated by upregulated calpains and the ubiquitin-proteasome pathway in response to myocardial oxidative stress (14,15).

The marked difference observed in females could further be attributed to sex-related differences in susceptibility to myofibrillar lysis or the modulatory effects of hormonal variables on sarcomeric integrity (16).

The pronounced upregulation of circulating miR-200a in DCM patients, along with its strong negative correlation with *TTN* mRNA and protein levels, highlights a critical post-transcriptional regulatory loop. MicroRNAs typically downregulate target transcripts via 3'-UTR binding, translational repression, and mRNA destabilization (17).

While the initial upregulation of *miR-200a* and activation of the Keap1/Nrf2 antioxidant axis may serve as a cytoprotective mechanism against oxidative stress, chronic elevation may trigger pathological hypertrophy and ventricular remodeling via PI3K/Akt, Sirt3/AMPK, and Wnt/ $\beta$ -catenin pathways (18,19), simultaneously functioning as a molecular brake on *TTN* transcription. Significantly higher expression in females suggests sex-specific genetic plasticity, warranting further investigation into sex-biased therapeutic targets. These outcomes contrast with several previous publications that demonstrated decreased expression of *miR-200a* in animal models of diabetic cardiomyopathy and chronic heart failure, where its restoration enhanced cardiac remodeling (20).

Regarding small nucleolar RNAs, both male and female groups showed higher levels of *snoRNA-42* expression than controls, with a notably higher increase in females, suggesting that snoRNAs participate in

compensatory processes in response to myocardial injury (21). SnoRNA overexpression indicates that nucleolar stress programs are activated and that the failing myocardium has higher needs for rRNA/snRNA modification. Such upregulation may influence nuclear RNA processing or mRNA stabilization in large structural transcripts such as *TTN*, corresponding with the elevated *TTN* gene expression and circulating titin fragments (22). Additionally, although *lncRNA FOXD2-AS1* has not been previously described in DCM, its prominent downregulation in female patients (23,24) highlights potential biological, hormonal, or sex-related differences in regulating cardiac remodeling, inflammatory signaling, and apoptosis.

Overall, the simultaneous global upregulation of *TTN* mRNA and *miR-200a* in DCM patients does not contradict the inverse correlations observed within individual profiles (25). The global elevation of *TTN* reflects a disease-driven surge in sarcomeric activation, whereas increased miR-200a acts as a post-transcriptional regulatory response aimed at limiting excessive transcription or accelerating transcript turnover (26). Importantly, ROC curve analyses revealed outstanding diagnostic performance for both *TTN* mRNA and *miR-200a* (AUC up to 1.000 in females), indicating their viability as minimally invasive molecular biomarkers for DCM.

The study reveals that the expression levels of the *TTN* gene are significantly increased in DCM patients and exhibit measurable transcription-protein coupling. This is further supported by the increase in serum titin levels, a measurable clinical sign of active sarcomeric injury and myocardial remodeling particularly in the female group. Meanwhile, sex-specific regulation patterns of dysregulated ncRNAs (e.g. *miR-200a*, *snoRNA-42* and *FOXD2-AS1*) contribute to molecular remodeling in the diseased myocardium. Furthermore, inverse intra-patient correlations between *miR-200a* and *TTN* are indicative of a post-transcriptional regulatory relationship. In summary, these findings demonstrate the diagnostic potential of the genomic, proteomic and ncRNA signatures in DCM and may indicate that sex-specific molecular responses significantly affect biomarker behavior and disease progression.

## Limitations

There are limitations of our study. The modest sample size (n=60) limits the statistical power and ability to control for potential confounding variables, such as specific medication use (e.g., beta-blockers, ACE

inhibitors and Clopidogrel), comorbidities and disease severity. Despite the fact that the participants were clinically diagnosed with DCM, these factors could theoretically influence ncRNA and titin expression levels. Our findings should therefore be seen as exploratory. Additionally, larger prospective, longitudinal studies are needed to validate these signatures and control for these clinical variables.

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