Systemic sclerosis (SSc) is an inflammatory disease diagnosed by various symptoms manifesting in different body organs, resulting from either vascular damage or autoimmunity; on top of that, are fibrotic lesions that could be identified in almost all parts of the body. In fact, the most prominent characteristic of SSc is skin thickening due to fibrosis which results from excessive production of the extracellular matrix in connective tissue. Internal body organs are also affected by fibrosis which leads to organ failure and mortality. The exact etiology of this disease is not identified yet, although like other inflammatory diseases a combination of genetic predisposition and environmental factors, especially industrial pollutants are thought to be involved. SSc affects women 5 times more than men, and also is more prevalent among some ethnicities or populations. Subsequently, the prevalence of SSc shows a remarkably different distribution among various regions; it has a remarkable distribution among different ethnicities and they have been investigated in many ill-logic conditions and they have been investigated in many ill-logic conditions.13,14 As a result, researches are ongoing to elucidate all aspects of the SSc pathogenesis. Among the new fields of study, are the microRNAs; these small sequences of about 20 nucleotides have been found to contribute to many cellular functions such as metabolism, proliferation, senescence, and death. Therefore, they are implicated in various pathologic conditions and they have been investigated in many illnesses such as cancer, autoimmunity, impaired development, etc. Being able to target and regulate many genes involved in prominent signaling pathways progressing in hard-to-cure.
illnesses like cancer, miRNAs have been widely studied in those pathways contributing to major events in the pathogenesis of cancer.\textsuperscript{20,21} As we believe that fibrotic disorders like SSc utilize the same cellular key pathways employed by cancer,\textsuperscript{22-25} we sought to investigate the well-known components of cancer in our patients suffering from SSc. Of the most prominent shared pathways between cancer and fibrosis is the epithelial to mesenchymal transition (EMT)\textsuperscript{26-29}; this phenomenon not only underly the proliferation, invasion, and metastasis in cancer, but also is the main cause of fibroblast differentiation into myofibroblasts as the major event in fibrotic disorders, ultimately leading to organ dysfunction.\textsuperscript{30} One of the well-studied miRNAs in cancer is miR-138 which is capable of targeting and regulating the EMT pathway at many points.\textsuperscript{26,31-37} It has been found that this miRNA could target TGF-\(\beta\)-associated signaling molecules in cancer; which is also the key regulator in fibrosis and SSc.\textsuperscript{38-40} Accordingly, we proposed a possible role for miR-138 in SSc and decided to assess its gene expression in the blood of SSc patients to find out whether this miRNA is dysregulated in SSc or not. And to further evaluate any possible alterations to be employed as a diagnostic biomarker.

**Methods**

Patients: This was a cross-sectional randomized case-control study that was conducted between June 2017 and November 2017. The minimum sample size was calculated based on the prevalence of SSc, which is estimated to be 15 per 100,000 individuals,\textsuperscript{41} with a precision of 0.05 and type one error of 0.05.\textsuperscript{42} Equal numbers of patients either with limited or diffuse SSc (30 from each subtype, 60 in total) were randomly selected from the cohort of the Firuzgar Hospital, Tehran, Iran. All the patients filled out an informed consent. The diagnosis of SSc and categorization of the patients between its 2 subtypes was performed by an expert rheumatologist with years of experience in SSc, according to ACR/EULAR criteria. The history of the patients was thoroughly reviewed and their laboratory findings, as well as X-rays and high-resolution computed tomography (HRCT), were obtained (Figure 1). Individuals with a history of other complications and comorbidities such as malignancies and infections or those receiving certain drugs that would possibly affect the results were excluded. Due to the very few numbers of male SSc patients, they were omitted and the female patients only were enrolled. The mean age of the patients was 51 years and their average BMI was 24.4. The controls (30 individuals) were randomly chosen among the healthy female individuals who were referred to the laboratory for checkups, those matching the age of the patients with normal laboratory findings and no known history of any illnesses were enrolled. All the methods and procedures conducted in this study were confirmed by the ethics committee of the Iran University of Medical Sciences (ethics code: IR.IUMS.FMD.REC.1397.316).

**Figure 1.** Comorbidities (A) and autoantibodies (B) found in SSc patients enrolled in the current study, the bars represent the percent of the affected individuals with each comorbidity.
RNA isolation and cDNA synthesis

Whole blood was drawn from the patients into EDTA vials by an expert laboratory technician considering the skin and vascular problems of the patients. Immediately, the samples were transferred to the laboratory and underwent RNA isolation steps from the whole blood using RNAzol BD (MRC, USA); 500 µl of blood was added to 1 ml of the RNAzol reagent and the whole process was carried out according to the manufacturer protocols. The quality and concentration of the isolated RNA samples were evaluated using gel electrophoresis and the NANODROP instrument (Thermo Fischer, USA). Subsequently, fresh isolated RNA was used as the template for synthesizing cDNA using TaqMan advanced microRNA synthesis kit (Thermo Fischer, USA) following the manufacturer’s instructions, then the resulting cDNA was kept at −20°C to be later employed in the qPCR tests.

qPCR

The relative gene expression of miR-138 was assessed in the whole blood of the patients and the controls using TaqMan advanced microRNA assays specific for miR-138 and miR-199-5p as the internal control according to the manufacturer and previous studies. Also, cel-miR-39 (Qiagen, Germany) was used as the external control. All the qPCR tests were performed in duplicates along with no template controls and no primer controls using RotorGene Q real-time PCR system (QIAGEN, Germany).

Statistical analysis

The relative gene expression was calculated and compared between the study groups using the 2−ΔΔCT with the miR-199-5p as a normalizer. qPCR data were compared using one-way ANOVA and Tukey's post hoc, correlations were assessed using Pearson's correlation tests. All data were analyzed and plotted using GraphPad PRISM software; the data are represented as mean ± SD and the P values equal to or below .05 were considered statistically significant.

Results

It was observed that the relative expression of miR-138 was considerably decreased in both limited and diffuse patients in comparison to the controls (P values ⩽ .0001) (Figure 1). Also, there was a further slight reduction in the miR-138 gene expression in diffuse patients relative to the limited patients (P-value = .9184) (Figure 2).

Since we observed a remarkable downregulation of miR-138 in SSc patients, we sought to evaluate whether this miRNA could be utilized as a biomarker capable of differentiating the SSc patients from the healthy controls. Hence, ROC curve analysis was performed on the gene expression data; it was found that miR-138 gene expression is effectively useful in the diagnosis of the patients. Figure 2 illustrates the diagnostic value of miR-138 gene expression data using the ROC curve plots in different states; a good Area Under the Curve (AUC), as well as acceptable sensitivity and specificity, was obtained as shown in Figure 3 and Table 1.

In order to gain a better insight into the role of miR-138 in systemic sclerosis and how it could affect the disease symptoms and complications we conducted a global comparison of the miR-138 expression between individual subsets based on showing a specific symptom or having a specific auto-antibody; The results of this analysis are presented in Figure 4 in details. Altogether, it seems that having high levels of miR-138 could be associated with a lower incidence of PAH, Digital ulcers, and Myositis, as well as decreased autoantibodies against DNA topoisomerase, ribonucleoproteins, and RNA polymerase III; whereas upregulation of miR-138 correlates with increased incidence of ILD, Telangiectasia, and ACA autoantibody (Figure 4). Also, it seems that the expression of miR-138 in female cases is lower than that of the male cases, although the number of male cases was 7 compared to 60 females.

Discussion

In this study, the relative expression of miR-138 was assessed in the whole blood of SSc patients with either limited or diffuse subtypes of the disease for the first time. It was observed that this miRNA is downregulated in the whole blood of the SSc patients in comparison to the healthy controls; it was even further decreased in patients with the diffuse subtype. subsequently, it was demonstrated in ROC curve analyses that the
The relative expression of miR-138 could be utilized as a reliable diagnostic biomarker for effectively diagnosing both subtypes of SSc. It could not be served for discriminating the limited subtype from its diffuse counterpart. The selection of this miRNA in the current study was based on previous research regarding the various possible functions of miR-138 in several cancers. We aimed to evaluate miR-138 expression since we had focused on the TGF-β signaling pathways, especially the non-canonical (non-SMAD) pathways, such as the PI3K-AKT-mTOR pathway which contributes to the EMT process. EMT is the main underlying mechanism responsible for the activation of fibroblasts in fibrotic disorders as well as cancer cell survival, proliferation, and invasion. In this regard here we summarize some of the main findings achieved so far, especially in the field of cancer research, in order to propose a role for miR-138 in fibrotic disorders such as SSc. Although the reports are not consistent; altogether, they provide an insight into different roles that miR-138 may play in the context of fibrotic disorders. Some studies demonstrated that miR-138 is a negative regulator of the EMT pathway which serves an anti-tumor role, while some other studies consider it as a pro-tumor factor by inducing the EMT. In support of our theory, Wu et al have demonstrated that miR-138 inhibits EMT in mice models of idiopathic pulmonary fibrosis (IPF) through direct targeting of the ZEB2 transcription factor.
which is responsible for activating the transcription of mesenchymal markers and repressing the epithelial markers; therefore, it is possible that the downregulation of miR-138 in our study somehow has resulted in the overactivation of the ZEB2. Also, it was revealed that the lncRNA PFAR promotes fibroblast activation through the downregulation of miR-138.51 Interestingly, it has been shown that the expression of miR-138 is downregulated by the TGF-β1 in the process of EMT in the primary lung cancer cells38; TGF-β on the other hand is recognized as the master inducer of fibrosis which could explain the downregulation of miR-138 in SSc.52 Besides, miR-138 was demonstrated to be able to reduce the proliferation and invasion of the prostate cancer cell lines through inhibition of the Wnt/β-catenin pathway which is also involved in fibrogenesis and SSc.53,54 Also, in renal and lung carcinoma cell lines and patient samples, it was observed that the expression of miR-138 is reduced which results in the overexpression of SOX4 in the EMT pathway.32,55 Interestingly, miR-138 was shown to be upregulated in chondrocytes from mice models of rheumatoid arthritis, which propose a role for this miRNA in rheumatologic diseases like SSc; this finding is consistent with our data as well.56 Additionally, it was demonstrated that miR-138 could directly bind to and regulate HIF1-α which subse-

Figure 4. The differences in miR-138 expression between pairs of different subsets. Each pair of bars represents the expression of miR-138 in a specific subset based on the comorbidities or autoantibodies found in SSc patients.
which is a key contributing factor in the pathogenesis of both cancer and systemic sclerosis (Figure 5). Our study was conducted in a single cohort and the number of patients enrolled was quite small; indeed, increasing the sample size and gathering data from other cohorts in the country would enhance the robustness of our results and help with drawing a comprehensive conclusion. In the current study, only gene expression of miR-138 was evaluated in whole blood and no functional analysis was performed so as to elucidate the exact role of miR-138 in the pathogenesis of SSc, nor the putative targets were identified which is a major limitation in our study. It is suggested to assess the expression of miR-138 in clinical samples such as skin or lung fibrotic lesions to further gain insight into the local expression of this miRNA in SSc. Also, investigating the epigenetic factors affecting miR-138 expression, such as long non-coding RNAs (e.g., HOTAIR and PFAR) would be beneficial in understanding the etiology of SSc.\textsuperscript{51,56} Further evaluation of miR-138 role in the pathogenesis of SSc could also pave the way for introducing this miRNA as a novel therapeutic target too.\textsuperscript{27}

**Conclusion**

miR-138 is significantly downregulated in patients with SSc, especially in those with diffuse subtypes, suggesting a role for
miR-138 in SSc which definitely need further evaluations and confirmation in larger populations. On the other hand, Given the appropriate AUC of the ROC curves, we suggest that miR-138 expression could be included in the diagnosis of the SSc patients along with other criteria or biomarkers.

**Declarations**

**Ethics approval and consent to participate**

All patients filled out a consent form confirmed by the ethics committee of the Iran University of Medical sciences (ethics code: IR.IUMS.FMD.REC.1397.316).

**Consent for publication**

Not applicable.

**Authors’ contributions**

PB performed all lab procedures from sample preparations to qPCR tests, also a major contributor to statistical/bioinformatics analyses and writing of the manuscript. HP helped with the patients’ enrollment and diagnosis and also edited the manuscript. NM has designed the study and provided the study materials, also she has guided all the processes of the study from the first idea to the preparation of the manuscript. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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