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FULL LENGTH ARTICLE

Anti-endothelial cell antibody rich sera from rheumatic heart disease patients induces proinflammatory phenotype and methylation alteration in endothelial cells

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DNA methylation;
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Abstract Rheumatic heart disease (RHD) is a major cause of cardiovascular morbidity and mortality in developing nations like India. RHD commonly affects the mitral valve which is lined by a single layer of endothelial cells (ECs). The role of ECs in mitral valve damage during RHD is not well elucidated. In here, anti-endothelial cell antibody from RHD patients has been used to stimulate the ECs (HUVECs and HMVECs). ECs proinflammatory phenotype with increased expression of TNFα, IL-6, IL-8, IFNγ, IL-1β, ICAM1, VCAM1, E-selectin, laminin B, and vimentin was documented in both ECs. The promoter hypomethylation of various key inflammatory cytokines (TNFα, IL-6, and IL-8), integrin (ICAM1) associated with leukocyte transendothelial migration, and extracellular matrix genes (vimentin, and laminin) were also observed. Further, the *in-vitro* data was in accordance with *ex-vivo* observations which correlated significantly with the etiological factors such as smoking, socioeconomic status, and housing. Thus, the

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study sheds light on the role of ECs in RHD which is a step forward in the elucidation of disease pathogenesis.

Introduction

Rheumatic heart disease (RHD) is one of the most common valvular disease affecting scores of patients in developing countries with few sporadic cases in developed countries. In 2015, RHD accounted for 33 million cases and 319,000 deaths worldwide out of which 13 million cases and 119,000 deaths were reported from India.[1] Although, various molecular and pathogenic factors have been attributed including genetic predisposition,[2-4] molecular mimicry with heart and endothelial proteins and GAS (Group A streptococcus or *Streptococcus pyogenes*) antigens,[5] yet the exact mechanism of RHD pathogenesis remains undeciphered.

In RHD, the mitral valve is predominantly affected. It is lined by a single layer of endothelial cells (ECs) which is believed to have a pertinent role in RHD development.[6] Additionally, RHD is a multifactorial disease which involves the environment, host, and pathogen. These three factors determine the disease causation and the degree of severity.[7] Environment and the pathogen are believed to be altering the host epigenetic signatures to establish the disease as evident in various autoimmune and infectious diseases.[8] The nonheritable epigenetic alterations are dynamic in nature due to their frequent interaction with the environmental factors such as nutritional status, living conditions etc.[9,10] One of the epigenetic changes i.e. methylation of DNA occurs predominantly at the CpG island of the gene promoter. Promoter DNA methylation might have an important role in RHD pathogenesis; wherein change in methylation pattern may regulate the various genes detrimental in RHD. Further, few studies are available which explore the methylation pattern in RHD.[11,12] Endothelial cells line the cardiovascular system and are among the firsts in response to the stimuli such as flow alteration, anti-endothelial cell antibodies or environmental factors e.g. smoke by secretion of cytokines such as TNFα and interleukins. TNFα, IL-6, IL-1β, IFNγ, and IL-8 have been well characterized in inflammation and autoimmune processes. Investigations have also revealed the role of epigenetic regulation of endothelial gene expression.[13,14] Endothelial cells are believed to play crucial role in RHD as well, however lack of information regarding the regulation of gene expression from epigenetic perspective in endothelium dysfunction have hindered the understanding of RHD. Thus, we hypothesize that the mitral valve lining of endothelial cells may play a central role in RHD development by the alteration in physiological genomic and epigenomic signatures. The unveiling of the study may shed light on the current understanding and could append further insight into cryptic etiology of RHD.

Materials and methods

Ethical statement

The study was conducted after the ethical permission from the Institute Ethics Committee, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (NK/653/Ph.D./14198). Written informed consent was collected prior to sample collection from the patient or the legal guardian.

Samples

Mitral valve tissue (n = 28) from chronic RHD patients undergoing valve replacement surgery were collected from Department of Cardiothoracic and Vascular Surgery, PGIMER. Age and sex matched control mitral valve (n = 22) were collected from autopsy cases with no sign of morphological or pathological heart disease. Mitral valve tissues (1–2 cm^2, 100–150 mg) were collected and immediately transported to the laboratory. Tissues were finely minced and kept overnight in RNA later™ (Ambion, USA) at 4 °C, and then stored at −80 °C till further use.

Blood samples (n = 37) were collected from the RHD patients visiting the Department of Cardiothoracic and Vascular Surgery, PGIMER, Chandigarh, India between 2015 and 2016 subjected to availability and consent of the patients. ARF (n = 14) blood samples were previously collected in Jai Vigyan mission mode (2000–2010, Indian Council of Medical Research, New Delhi) and stored at −80 °C. Umbilical cord (n = 10) was collected from the placental side of full term healthy mother immediately after their delivery from Department of Obstetrics and Gynaecology, PGIMER, Chandigarh, India. Age and sex matched control blood samples (n = 26) were collected from healthy volunteers without any history of autoimmune diseases, GAS infections, diabetes, or drugs consumption. For sera separation, blood was allowed to clot at room temperature followed by centrifugation at 1500 rpm for 5 min. Further, with the addition of 1x protease inhibitor (Sigma–Aldrich, USA) sera were stored at −80 °C till further use.

Culture of human micro vascular endothelial cells (HMVECs)

HMVECs of cardiac origin were obtained from Lonza (Switzerland). They were grown in endothelial cell basal medium (EBM)-2 supplemented with 5% fetal bovine serum (FBS), human recombinant epidermal growth factor

(hEGF), hydrocortisone, vascular endothelial growth factor (VEGF), human recombinant insulin-like growth factor (R3-IGF)-1, human recombinant epidermal growth factor (hFGF)-B, ascorbic acid, and gentamicin/amphotericin-B (GA)-1000. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere (Thermo Heraeus HERAcell® 240) and experiments were performed in cells grown to 60—70% confluency. Cell culture was routinely monitored under Nikon Eclipse Ti-S microscope (Japan) at various magnifications (10x, 20x, and 40x).

Isolation and culture of human umbilical vein endothelial cells (HUVECs)

HUVEC isolation was done as per Baudin et al[15] with minor modifications. The umbilical cord was transported to the laboratory in phosphate buffered saline with penicillin (1million unit/50 ml) immediately after the delivery. Cells were collected after the 0.2% collagenase (Gibco, USA) treatment and pelleted at 1500 rpm for 3 min and were cultured on 6 well plate (Corning, USA) at 37 °C in 5% CO_2 (Supplementary figure 1).

Enzyme-linked immunosorbent assay (ELISA)

RHD patient (n = 37) and healthy control (n = 26) sera were used for anti-endothelial cell antibody (AECA) estimation as per Conti et al[16] with a cell-surface ELISA on HMVECs through minor modifications. HMVECs were cultured on 96-well plate (Corning, USA) till 70—80% confluency and fixed with 0.1% glutaraldehyde. The nonspecific binding sites were blocked with 10% fetal calf serum (FCS) to avoid the false positive reaction. Further, the cells were incubated with 100 μl of the 1:200 diluted sera in 10% FCS for 1h. Unbound primary antibodies were removed by washing and the bound antibodies were detected with 1:6000 diluted horseradish peroxidase-conjugated goat anti-human IgG (Bangalore Genei, India), using *o*-phenylenediamine dihydrochloride (Sigma—Aldrich, USA). AECA binding index (%) was calculated using the following formula as described previously.[17] Binding Index (%) = (S-A)/(B-A) x 100 where S = Optical density (OD) of test, A = OD of negative control, and B = OD of positive control (pooled vasculitis sera, n = 5). AECA positivity was defined as a binding index higher than the mean ± 2 standard deviations of values observed in healthy controls.

Stimulation of HUVECs and HMVECs with sera

Cells were grown to 70—80% confluency and sera with high binding index (>75%) were added to the dilution of 1:50 for 3h, 6h, 12h, and 24h in 12 well cell culture plate. Cells were microscopically examined in Nikon Eclipse Ti-S microscope (Japan). Further, cells were lysed directly in the culture vessel for DNA and RNA isolation by TRIZOL reagent (Invitrogen, USA). Isolated RNA was dissolved in 20 μl DEPC water. RNAs purity was checked by spectrophotometer (260/280 nm) and was used for cDNA synthesis by Verso cDNA Synthesis Kit according to manufacturer's instruction (Thermo Fischer Scientific, USA). DNA was isolated by Sambrook et al[18] or by TRIZOL reagent and was assessed by running on an agarose gel (0.7—1%) to check its integrity. Further, DNA purity was checked by spectrophotometer (260/280 nm) and was stored at −20 °C till further use.

Real-Time PCR

Real-Time PCR was performed on Roche Lightcycler® 480 using SYBR Green I master (Roche, Germany) detection method using specific oligonucleotide primers. The reaction mixture contained 50 ng cDNA, 0.5 μM primer, 1X SYBR Mix (Roche, Germany) and nuclease free water for volume make up. The gene specific sequence of primers and their cycling conditions are shown in Supplementary Table 1. The gene expression was normalized by housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) expressions and relative mRNA levels were calculated by $2^{-\Delta\Delta Ct}$ method.[19]

DNA Methylation

Region specific promoter methylation was assessed by One Step qMethyl™ Kit (Zymoresearch, USA) as per

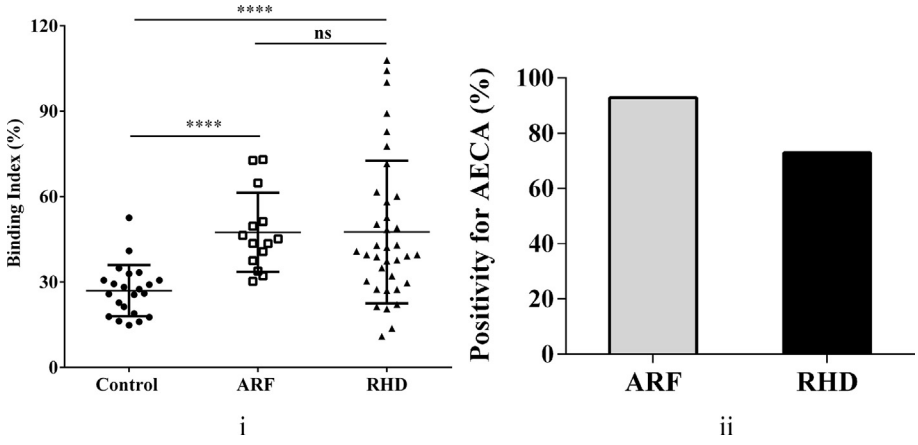


Figure 1 (i) Anti-Endothelial Cell Antibody (AECA) titers in Control, ARF, and RHD sera (ii) Percentage of patients positive for AECA after normalization with the controls. Experiment was performed in triplicate and data is represented as mean ± SD. Statistical significance was calculated by Student's t-tests. ns = non-significant, ****$p < 0.0001$.

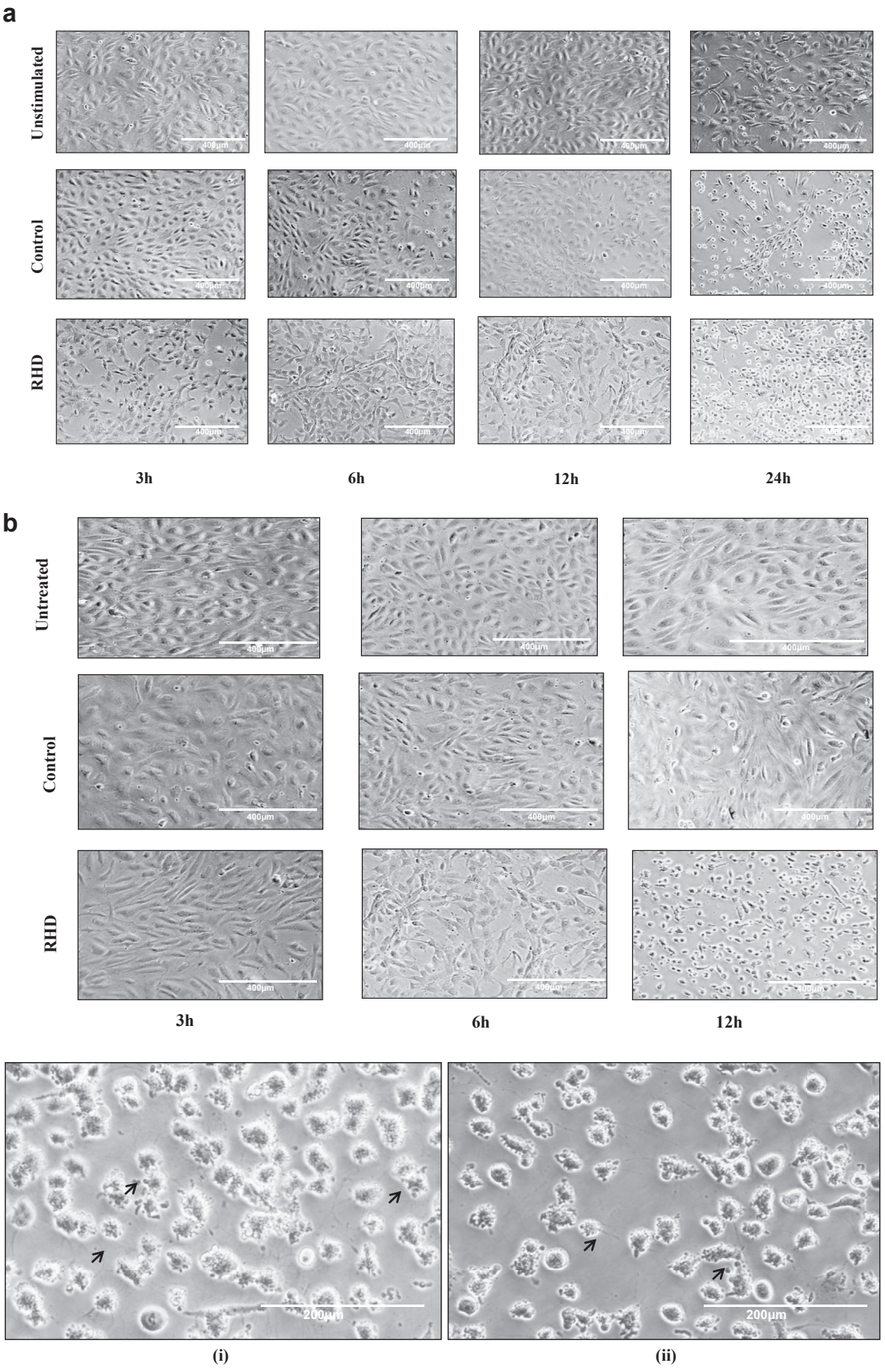


Figure 2 (A): Bright-field microscopic images of HUVECs stimulation with pooled RHD, control sera at 3h, 6h, 12h and 24h (10x). (B): Bright field microscopic images of HMVECs stimulation with pooled RHD and control sera at 3h, 6h, and 12h (10x). (C): Representative micrograph of ECs showing membrane blebbing and apoptotic bodies (arrow) upon RHD sera stimulation (i) HUVECs at 24 h, and (ii) HMVECs at 12 h (20x).

manufacturer's instruction. Promoter regions were identified for all the genes using standard human promoter databases such as Cold Spring Harbor Laboratory Mammalian Promoter Database (Version 2.0, May 2005) or Eukaryotic Promoter Database, Swiss Institute of Bioinformatics (epd.vital-it.ch/human/human_database.php). Nucleotide sequence −600bp to +100bp relative to transcription start site were selected for CpG islands identification using Methyl Express software (Applied Biosystems, USA) as per the standard criteria of CpG island identification.[20]

In here, we could identify promoter CpG island in TNFα, IL-6, IL-8, ICAM1, vimentin, and laminin B; thus methylation was analyzed in these genes only. Primers were designed by Primer 3 software (version 4.0.0) (http://bioinfo.ut.ee/primer3-0.4.0/) keeping in view that forward primer should have cut site for at least one methylation specific restriction enzyme present in the reaction mixture (Supplementary table 2). Methylation percentage was calculated for each region of gene spanned by specific primers. Average Ct values (ΔCt) were calculated by subtracting Ct value of test reaction to Ct value of reference reaction. Methylation percentage was represented as $100 \times 2^{-\Delta Ct}$.[21]

Statistical analysis

Statistical analysis was performed using SPSS software (version17.0) and GraphPad Prism 6 (Inc. CA, USA) software for windows. All the experiments were performed in triplicate and values were quantitatively expressed as the mean ± SD unless mentioned. The results were analyzed statistically following Student's *t*-test as well as chi-square test to compare the data between two groups and one-way analysis of variance (ANOVA) was used for comparison of more than two group means. If SD > mean within the groups, the nonparametric Mann−Whitney *U* test was used. A correlation study was also performed between two parameters by computing Pearson's correlation coefficient (r). *p*-value <0.05 was considered statistically significant for all the experiments.

Results

Anti-endothelial cell antibody (AECA) titer

We observed AECA titers were significantly ($p < 0.0001$) high in both ARF (BI 47.46 ± 13.88%) and RHD patients (BI 51.33 ± 33.73%) compared to the healthy control (BI 28.88 ± 9.78%). However, no significant difference was observed in AECA titers between the ARF and RHD groups [Fig. 1(i)]. Among the diseased groups, 92.85% ARF and 72.97% RHD samples showed positivity to AECAs [Fig. 1(ii)]. RHD patients (n = 28) admitted for the valve replacement surgery did not show any significant association between the AECA positivity and disease severity such as mitral stenosis or regurgitation (Supplementary table 3). Interestingly, when the AECAs titers were correlated with the environmental data, a significant ($p < 0.05$) association between the cooking style i.e. *chulha*/biomass fuel (use of wood/cow dung) was observed. Additionally, we also observed that positivity to AECA was significantly ($p < 0.01$) elevated in the lower socioeconomic group than the lower middle or upper middle socioeconomic strata indicating the propensity of the lower socioeconomic group in mounting the aberrant immune response. Patient sera with high AECA binding index were used for ECs stimulation.

Stimulation of HUVECs and HMVECs

The HUVECs and HMVECs were cultured till second passage with 70−80% confluency and thereafter stimulated with pooled RHD sera [(n = 5), BI>75%, and randomly selected pooled control sera (n = 5) with mean BI 27% for 3h, 6h, 12h, and 24h.

The unstimulated HUVECs and HMVECs had cobblestone morphology with oval nuclei and irregular cell shape throughout the experiment (Fig. 2). HMVECs didn't show any distinct feature at 3h post stimulation whereas; HUVECs showed irregular and flat morphology with crenated edges with clump formation at 3h. At 6h both ECs showed increased clumping and crenated edges [Fig. 2A]. ECs were flat, round, and clumped upon stimulation with RHD sera at 12h post stimulation, however, RHD sera stimulated HMVECs, in particular, showed membrane blebbing and couldn't be maintained thereafter [Fig. 2B and C(ii)]. At 24h post stimulation, the HUVECS were round in shape with visible membrane blebbing and apoptotic bodies [Fig. 2A and C(i)]. Interestingly, control sera stimulated cells showed normal pleomorphic, centrally bulged endothelial morphology till 12h but at 24h HUVECs showed round shape and membrane blebbing but to a meager extent than RHD sera stimulated cells. Further, DNA and RNA were from ECs for gene expression and methylation analysis.

Gene expression and DNA methylation in stimulated HUVECs and HMVECs

mRNA expression of cytokines (TNFα, IL-6, IL-8, IFNγ, IL-1β) integrins (ICAM1, VCAM1, E-selectin, and cytoskeletal genes, which also form the ECM (laminin B, vimentin) were studied. DNA methylation of promoter has been well known in the regulation of gene expression. Thus, the promoter methylation of TNFα, IL-6, IL-8, vimentin, laminin, and ICAM1 genes were also evaluated.

HUVECs were found to be activated upon stimulation as evidenced by an overall increased expression of cytokines and integrins [Fig. 3A]. The gene expression study of stimulated HUVECs revealed that all the cytokine genes were early responders i.e. they had a maximal expression at 3h followed by 6hr post stimulation except the IFNγ. IFNγ showed a gradual increase in expression from 3h till 24h. The increased expression of these cytokines might act in feed-forward mechanism required to establish an inflammatory state upon endothelial cell activation. The low levels of TNFα, IL-6, IL-1β, and IL-8 were also documented at 12h and 24h [Fig. 3A]. The integrins or the adhesion genes (ICAM1, VCAM1, and E-selectin) also appeared as early responders with high expression at 3h and 6h followed by a decline in their levels [Fig. 3A]. Apart from cytokines and integrins, the crucial role of ECM protein in RHD is well documented.[22] Herein, we observed a significant expression of vimentin in RHD sera stimulated cells than in control

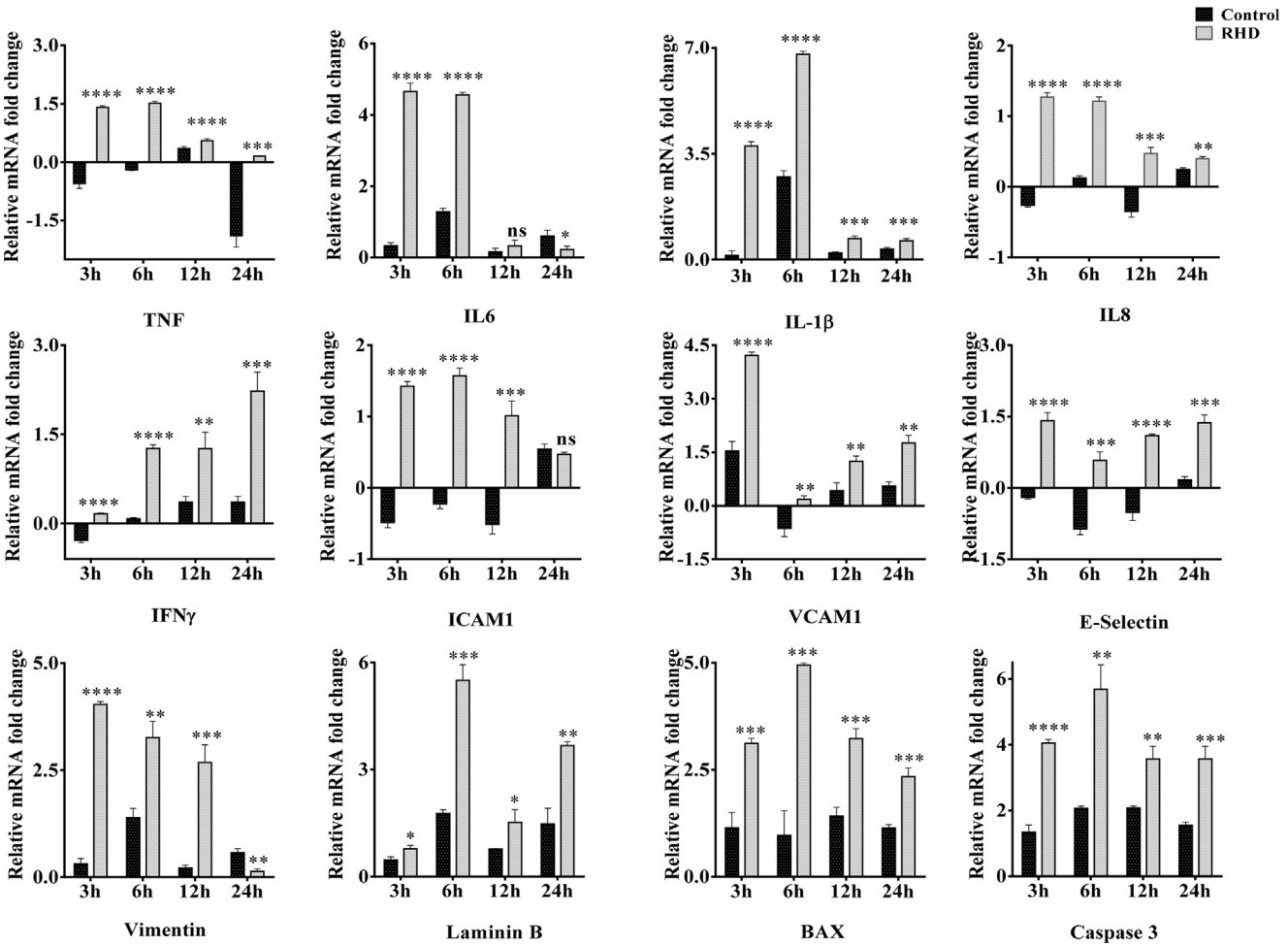
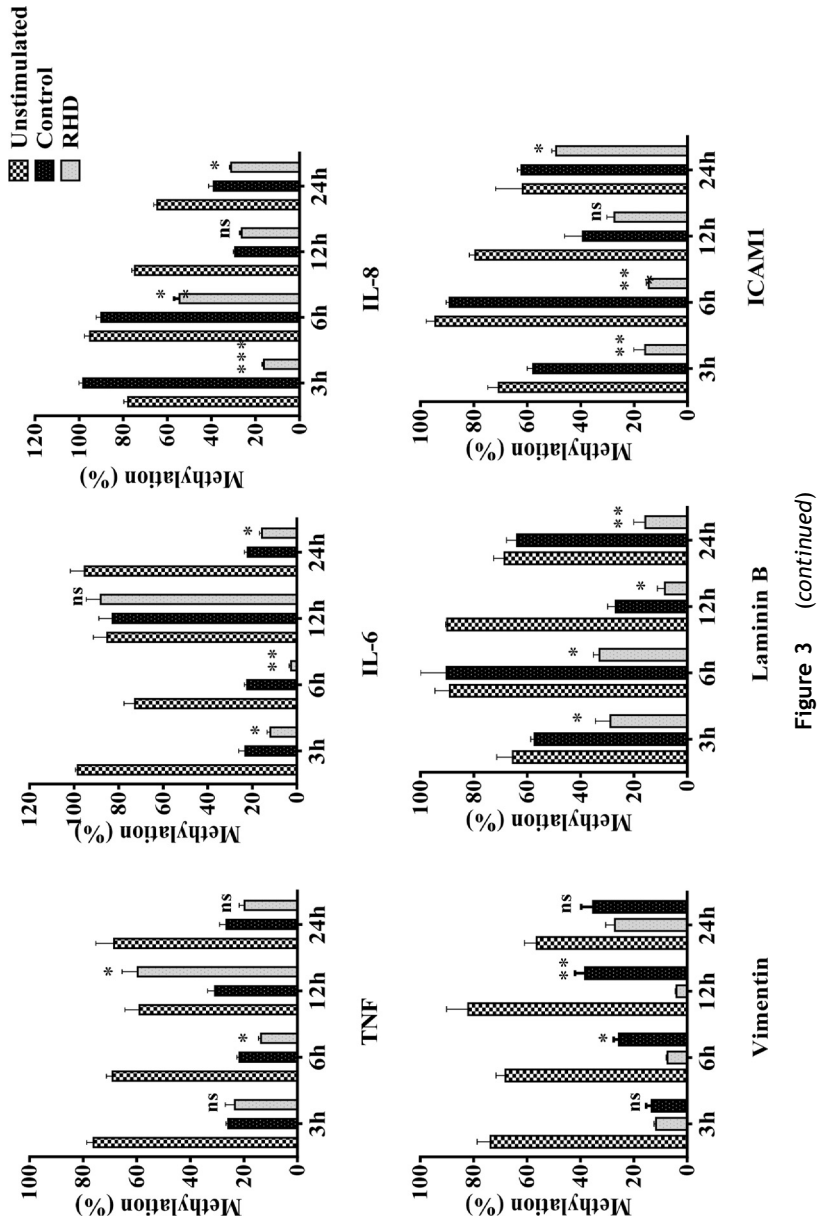


Figure 3 (A): Relative mRNA level of RHD and healthy control sera stimulated HUVEC at 3h, 6h, 12h and 24h. (B): DNA methylation (%) of promoter region in HUVECs stimulated by RHD and control sera. (C): Relative mRNA level of RHD and healthy control sera stimulated HMVEC at 3h, 6h, 12h and 24h. (D): DNA methylation (%) of promoter region in HMVECs stimulated by RHD and control sera. Experiments were performed in triplicate and data is represented as mean ± SD. Statistical significance was calculated by Student's t-tests or one way-ANOVA. ns = non significant,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$****$p < 0.0001$.



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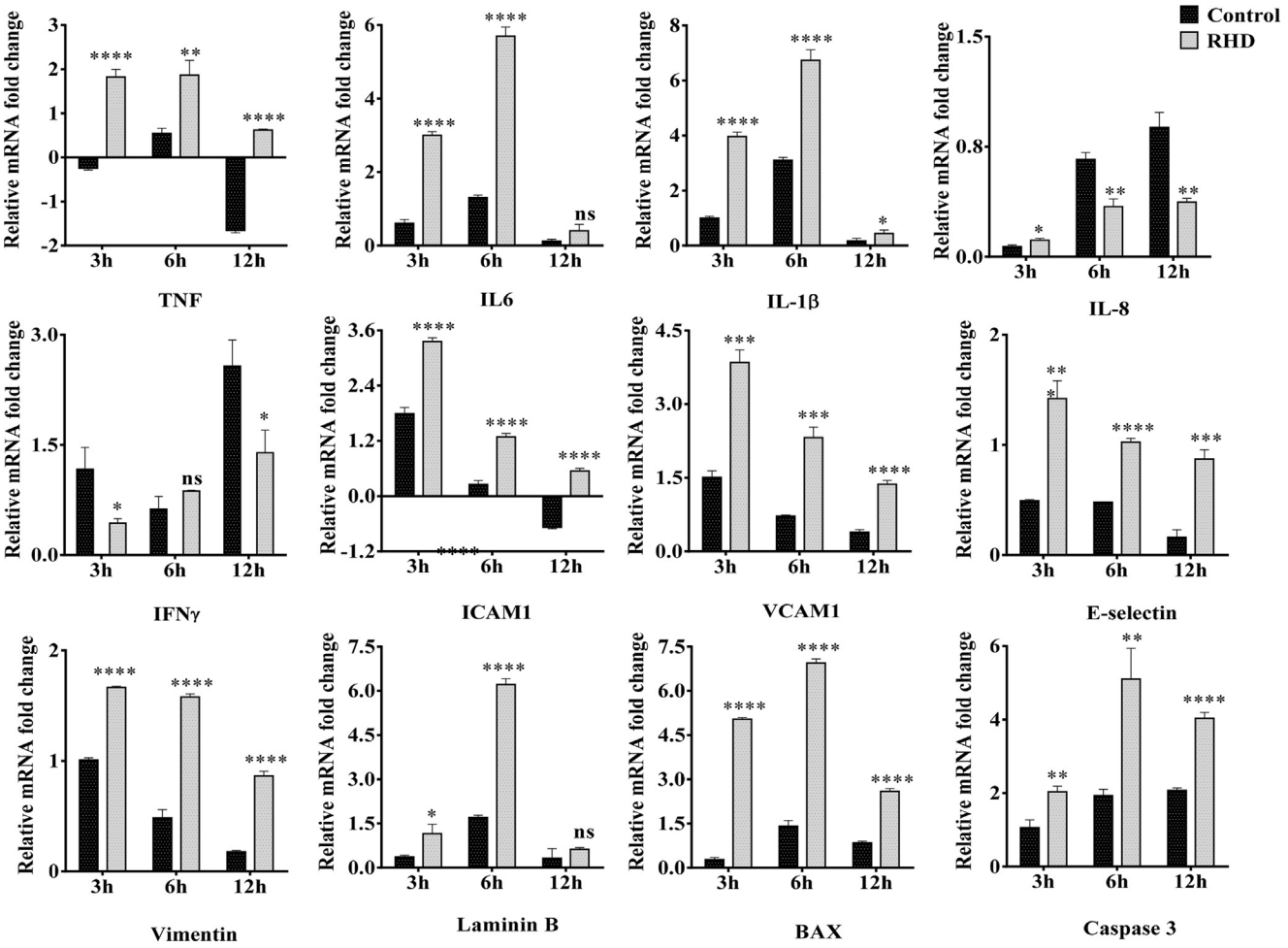

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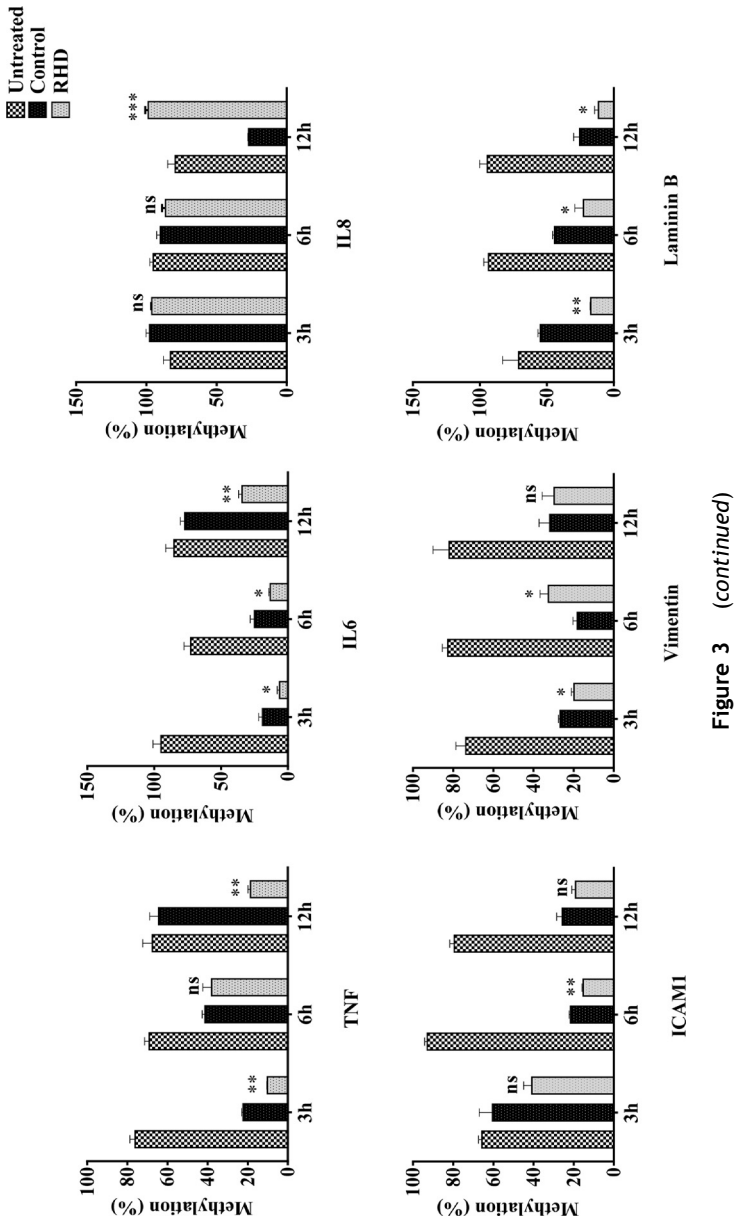

Figure 3 (*continued*)

Table 1 Relative mRNA level ($2^{-\Delta Cp}$) and fold change ($2^{-\Delta\Delta Cp}$) of proinflammatory, adhesion, and cytoskeletal genes in mitral valve of RHD patients and controls. p-value <0.05 was considered significant.

	Control	RHD	Fold Change	p-value
	Mean ± SD ($2^{-\Delta Cp}$)	Mean ± SD ($2^{-\Delta Cp}$)	($2^{-\Delta\Delta Cp}$)	
TNF	42.17 ± 163.93	4.43 ± 6.38	0.33	0.044
IL-6	3.14—4.28	0.63 ± 14.78	3.7	0.045
IFNγ	2.43 ± 3.30	0.36 ± 6.54	4.82	0.005
IL-8	0.81 ± 1.83	2.15 ± 4.29	5.87	0.004
IL-1β	0.98 ± 1.83	1.55 ± 3.07	1.22	0.86
ICAM1	3.51 ± 8.39	6.56 ± 7.30	6.7	0.001
VCAM1	0.52 ± 0.72	10.63 ± 21.77	14.56	0
E-selectin	2.64 ± 2.80	17.09 ± 16.80	3.13	0
Vimentin	417.26 ± 1267.93	991.29 ± 1345.4	9.14	0.001
Laminin B	2.50 ± 3.03	7.03 ± 7.58	4.58	0.005

mostly at all time points except at 24h whereas; laminin B, present on the basement membrane of ECs, was mostly upregulated all time intervals [Fig. 3A].

The promoter methylation of all the genes under the study showed variation at time intervals. The promoter of TNFα, IL-6, IL-8 cytokines, ICAM1 and laminin B were largely hypomethylated upon RHD patient sera stimulation. To our surprise, the vimentin showed overall hypermethylation in contrast to its high mRNA expression [Fig. 3B].

The gene expression level of cytokines, integrins, ECM and proapoptotic genes in stimulated HMVECs were almost similar to stimulated HUVECs with minor variations. The exceptions were IL-8 and IFNγ which showed lower expression in HMVECs stimulated with RHD sera in comparison to control [Fig. 3C]. Promoter methylation of TNFα, IL-6 cytokines, ICAM1, and laminin B were largely hypomethylated and showed similar methylation pattern to HUVECs. IL-8 methylation was similar in RHD and control group except at 12h where its promoter was hypermethylated in RHD sera group contrastingly; IL-8 gene expression was significantly high at this time point indicating the role of other gene regulating mechanism. Adhesion molecule ICAM1 was found to be hypomethylated in RHD sera stimulated cells in comparison to control at all dilutions and time intervals [Fig. 3D]. Vimentin, on the other hand, showed inconclusive methylation pattern, wherein; it was hypomethylated at 3h followed by an increase in methylation at 6h and 12h. However, we did observe that vimentin transcripts were high in RHD sera stimulated cells in comparison to control sera stimulated cells. Thus it may indicate the role of another mechanism in the regulation of vimentin expression [Fig. 3D].

Gene expression and promoter methylation in RHD patients

Relative mRNA levels of proinflammatory cytokines, adhesion molecules, and ECM genes were assessed during the study (Table 1). All proinflammatory cytokines showed significant upregulation in RHD patients similar to stimulated ECs. Additionally, significant upregulation of integrins (ICAM1, VCAM1, and E-selectin), vimentin, and laminin were also documented in RHD group in comparison to controls (Fig. 4). Thus, it was evident that there is an ongoing localized inflammatory process in the mitral valve of RHD patients wherein increased expression of cytokines, adhesion molecules, and ECM protein playing a definite role is observed.

Gene expression was also assessed for their probable association with clinical parameters. The correlation analysis revealed that females displayed a higher expression of IL-8 ($p < 0.05$) and IFNγ ($p < 0.01$) compared to their male counterparts (Supplementary Table 4). We also noted a significant upregulation ($p < 0.05$) of IFNγ in RHD patients with left ventricle hypertrophy. Additionally, VCAM1 expression was significantly ($p < 0.05$) associated with a mild form of mitral stenosis whereas; TNFα ($p < 0.05$) and ICAM1 ($p < 0.05$) were significantly more expressed in RHD patients with moderate aortic regurgitation [Supplementary Table 5].

Environmental factors and other factors including alcohol consumption, smoking, housing (*kaccha* house: made of mud, *pukka* house: made up of bricks), cooking style at the time of disease diagnosis, socioeconomic status (Kuppuswamy scale)[23] are known have a definite role in RHD patients. Interestingly, we found a significant association between IL-1β and smoker in the family ($p < 0.01$) and also between the vimentin expression in patients staying in *kaccha* houses ($p < 0.01$) (Supplementary Table 6).

A significant difference in percentage methylation was observed between RHD and control group (Table 2). The promoter of IL-6 ($p < 0.01$) and IL-8 ($p < 0.01$) were hypomethylated in RHD patients as compared to control whereas, marked hypermethylation was documented in case of TNFα ($p < 0.01$) (Fig. 5). In addition, adhesion gene ICAM1 ($p < 0.01$) and cytoskeletal protein, laminin B ($p < 0.05$) were also hypomethylated in RHD patients. On the contrary, we observed significant hypermethylation of vimentin ($p < 0.01$) in RHD cases compared to control.

Correlating the data with clinical and environmental variables we observed a significant association between laminin hypermethylation in patients with left ventricle hypertrophy ($p < 0.05$), smokers ($p < 0.05$), and patients who used biomass fuel as a mode of cooking ($p < 0.05$) (Supplementary Table 6). Hypomethylation of the IL-8 promoter ($p = 0.046$) in the patients with NYHA 3 classification was also observed which signifies the limitation in ordinary activities (Supplementary Table 5).

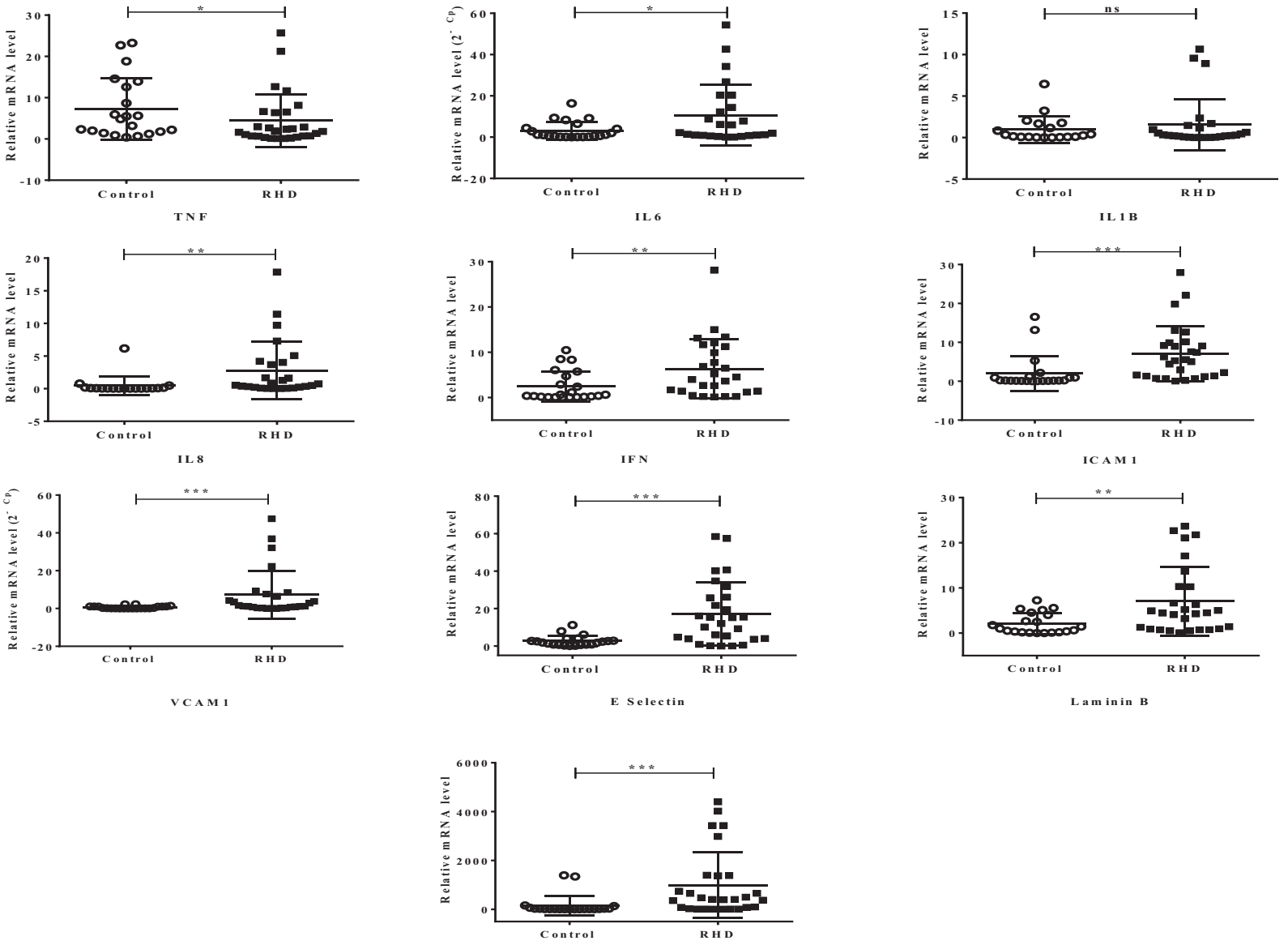

Figure 4 Relative gene expression of proinflammatory (TNFα, IL-6, IL-1β, IL-8, and IFNγ), adhesion (ICAM1, VCAM1, and E selectin), and matrix genes (Vimentin, laminin B) in RHD patients and controls ($2^{-\Delta Cp}$). Experiment was performed in triplicate and data was represented as mean $\pm$ SD. Statistical significance was calculated by Student's *t*-tests or one way-ANOVA. ns = non-significant, *p $<$ 0.05, **p $<$ 0.01, **p $<$ 0.001****p $<$ 0.0001.

Table 2 Percent methylation of pro-inflammatory (TNFα, IL-6, IL-8), adhesion (ICAM1), cytoskeletal gene (Vimentin, Laminin B) in RHD patients and controls. Student t-test was used for normally distributed data whereas Man Whitney U test was used for skewed. p-value <0.05 was considered significant.

	Control	RHD	p-value
	Mean ± SD	Mean ± SD	
TNFα	17.60 ± 6.78	33.74 ± 14.83	0.0007
IL-6	29.48 ± 21.35	20.19 ± 23.10	0.0032
IL-8	12.80 ± 4.06	12.07 ± 9.94	0.0018
ICAM1	16.13 ± 15.28	8.19 ± 4.98	0.0002
Vimentin	44.55 ± 22.20	76.16 ± 25.43	0.0015
Laminin B	25.27 ± 26.39	8.45 ± 8.99	0.029

Discussion

RHD is associated with cardiovascular morbidity and mortality in young adults; however, the pathogenesis of RHD is inadequately understood. Recent studies have shed some light on its cryptic etiology,[24] though precise information regarding the exact role of endothelial cells (ECs) in valvular etiology remains elusive. The entire cardiovascular system is lined by ECs which regulates a diverse range of physiological processes for e.g. inflammation, angiogenesis, production of extracellular matrix (ECM) proteins, and matrix remodeling. ECs are known to have phenotypic and genotypic variability depending on their location. Thus, studying two ECs (HMVECs and HUVECs) from different origins will provide insight in ECs response towards AECA rich sera stimulation. Damage to ECs by AECAs has been associated with various pathologies.[25] In this study, we observed a significantly high level of AECA in ARF and RHD patients. The high levels of AECAs in patients with mitral stenosis and mitral regurgitation further suggests its role in mitral valve damage which is most commonly affected (>70% cases) in RHD.[26] The high AECAs level could be considered pathogenic in ARF and RHD, as they might play a role in causing valvulitis by binding to valvular endothelium. AECAs induced damage followed by an improper immune response in disease establishment may perhaps be subjective to underlying genetic predisposition and environmental factors.[27]

RHD has been associated with risk factors such as lower socioeconomic status, overcrowding, and other environmental factors which render an increased susceptibility to disease.[28] We found higher AECAs in RHD patients who belonged to lower socioeconomic group. Similarly, a previous study also showed that patients belonging to the low socioeconomic group had higher degree of immune activation.[29,30] The high AECAs were also observed in patients using biomass (wood/cow dung) as a mode of cooking. Biomass fuels produces toxic smoke which upon inhalation acts as an endogenous source of local inflammation[31] and further increases the circulatory neutrophils and monocytes.[32] It has also been reported that low-level autoantibodies are instigated upon exposure to smoke.[33] This makes

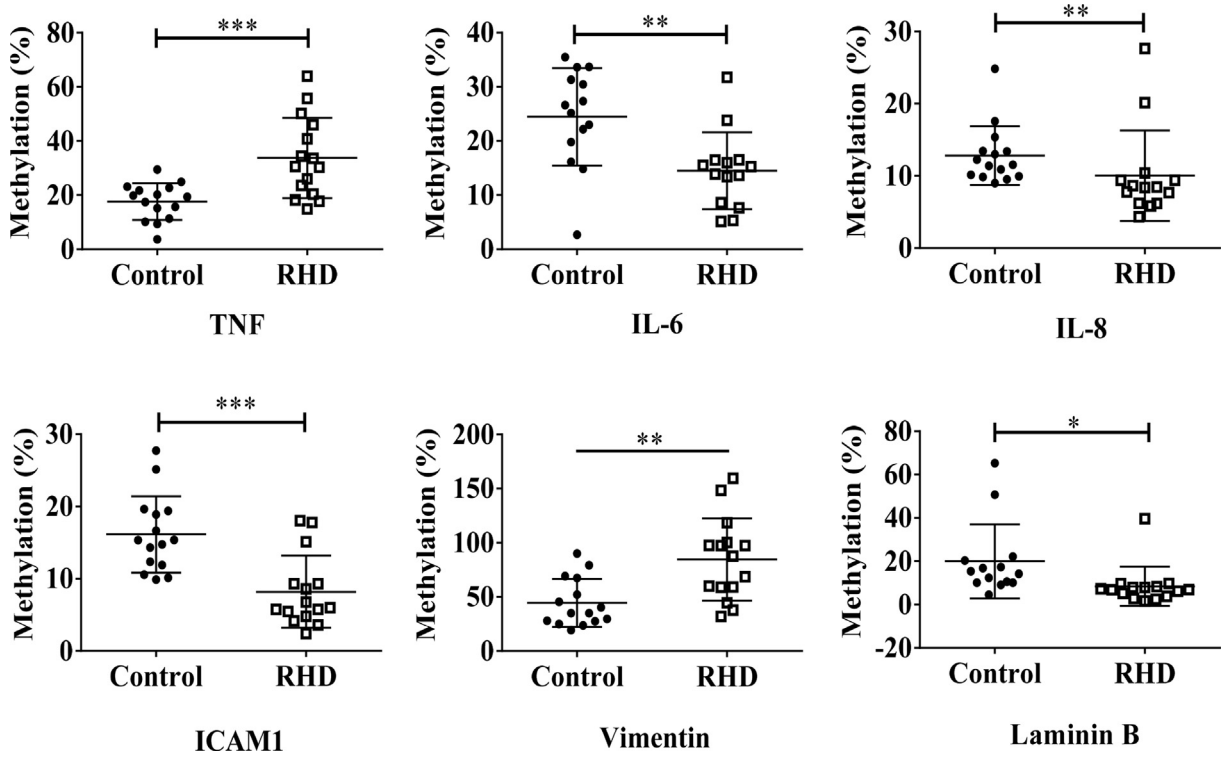

Figure 5 Promoter methylation (%) of proinflammatory, adhesion, and cytoskeletal gene from mitral valve tissues of RHD patients and control. Experiment was performed in triplicate and data is represented as mean ± SD. Statistical significance was calculated by Students t tests or one way-ANOVA. ns-non significant, *p < 0.05, **p < 0.01, ***p < 0.001****p < 0.0001.

us believe that in RHD, the initial exposure to biomass smoke may lead to a sensitization phase with a low level of cross reactive antibodies which upon streptococcal infection may increase multifold and aid in RHD development.

To unravel the underlying pathobiology of AECAs and the role of ECs we carried out an *in-vitro* study by culturing, human microvascular endothelial cells of cardiac origin (HMVECs) and human umbilical vein endothelial cells (HUVECs, a macrovascular ECs) from the umbilicus. The ECs upon AECA stimulation depicted apoptotic features such as membrane blebbing and formation of apoptotic bodies,[34] with upregulation of proapoptotic genes BAX and caspase-3. This suggests that AECAs have the ability to induce apoptosis which could be the triggering event in valvular dysfunction post RHD. It is known that alteration in DNA methylation patterns results in altered endothelial gene expression, which lead to the pathology of endothelial cells. DNA methylation in ECs has also been attributed to endothelial apoptosis, pulmonary hypertension and atherosclerosis.[14,15] Thus, to assess the ECs response to AECA rich sera, inflammation associated genes' expression and their methylation pattern were analyzed.

The gene expression analysis of RHD and control sera stimulated ECs revealed that most of the genes were early responders and had a correlation with their promoter methylation. TNFα, IL-6, and IL-1β are potent immunomodulatory cytokines which increases the secretion of other cytokines, adhesion molecules and also plays important role in chronic as well acute inflammation. In RHD sera stimulated ECs we found elevated expression of TNFα and significant ($p < 0.05$) but low expression of TNFα in mitral valve tissues of RHD patients. The hypomethylation of TNFα promoter in RHD sera stimulated ECs might imply toward AECA ability in inducing an alteration in chromatin structure and making DNA accessible to transcription factors. In contrast, hypermethylation of TNFα in mitral valve of RHD patients was in line with its low TNFα expression. Thus, low TNFα expression could be time dependent decrease or due to hypermethylated promoter or its inhibition by IL-6 or IFNγ.[35] The increased expression of IL-6 in ECs and RHD patients may have a critical role in RHD pathogenesis due to its capability of creating a proinflammatory environment, neovascularization and fibrosis.[36-38] Similarly, high expression of IL-1β in RHD sera stimulated ECs were in line with the previous reports which also showed increased expression of IL-1β upon AECAs binding.[39] Interestingly, cytotoxic effect of AECA is observed on activated ECs in conjunction with IL-1β but not in non-activated ECs upon AECA binding.[40] RHD patients also showed high IL-1β thus implying towards AECA-IL-1β mediated mitral valve damage. IL-8, a chemoattractant, plays an imperative role in autoimmune disorders, was found to be downregulated in HMVECs whereas upregulated in HUVECs stimulated with RHD sera and in RHD patients. The difference in response by ECs might be due to their origin as HUVECs are derived from immune-naive fetal tissue which probably showed functional differences from adult vascular endothelium i.e. HMVECs.[41] IFNγ is a proinflammatory cytokine and a key cytokine of Th1 mediated autoimmunity. The high levels of IFNγ in ECs and RHD patients were documented and further analysis revealed a significant association between IFNγ and left ventricle hypertrophy in RHD patients. High expression of IL-8 ($p < 0.05$) and IFNγ ($p < 0.05$) in mitral valve of female RHD patients than in males further confirms the high disease severity in the females[42] probably due to severe Th1 immune response with increase IFNγ.[43] The elevated expression of IFNγ also plays a vital role in vascular remodeling, fibrosis,[44] and heart failure.[45]

We also found high IL-1β in RHD patients who were passively exposed to cigarette smoke. Similar to biomass fuel, cigarette smoke also causes increase in the circulatory neutrophils and activated cytotoxic T cells.[46] The cigarette smoke also induces different T-cell responses to vimentin in transgenic mice expressing the HLA-DRB1*0401 allele, further linking the autoimmune response to an environmental factor.[47] Thus, the production of other pro inflammatory cytokines including IL-1β may be augmented in autoimmune diseases.[48]

Thus, high expression and promoter hypomethylation of proinflammatory cytokines in ECs and chronic RHD might point towards the induction of inflammatory state in ECs upon initial AECA interaction which persists in individuals eliciting aberrant immune response probably due to underlying genetic susceptibility and environmental factors. This may, in turn, result in apoptosis of ECs consequentially causing damage to mitral valve by ECM remodeling and fibrosis. Apart from proinflammatory cytokines, integrins have been actively implicated in various diseases including autoimmune disorders.[49] There increased expression during inflammation helps in transendothelial migration of immune cells from circulation to the site of injury.[50] In our study, we report the high expression of ICAM1, VCAM1, and E-selectin in ECs and RHD patients with hypomethylated promoter ICAM1. A similar study too showed global hypomethylation and a negative correlation between ICAM1 promoter methylation and its mRNA level.[12] Thus, promoter hypomethylation and increased mRNA level expression of ICAM1 in RHD may be correlated with of valvular lesion, cardiac muscle cell death and heart failure.[51] ECM proteins also interact with the cells to alter its physiological and pathological properties. Vimentin is produced by ECs and mesenchymal cells as a constituent of ECM.[52] We found upregulation of vimentin in both types of ECs. Similarly, RHD mitral valve also displayed significantly higher expression of vimentin ($p = 0.001$) in contrast to control. Vimentin promoter showed variable methylation pattern in ECs probably due to their difference in origin. On the other hand, we observed significant hypermethylation ($p < 0.01$) of vimentin promoter in RHD mitral valve. The higher gene expression and high promoter methylation of vimentin in mitral valve tissue of RHD patient suggests that it may be regulated by another epigenetic mechanism independent of DNA methylation such as histone modifications or the micro RNAs. The increased expression of vimentin might be due to physiological response in case of ECs enhanced by TNFα and/or IL-1β stimulation that are produced in response to RHD sera. Apart from vimentin, laminin along with collagen IV forms a network to provide structural stability and biological activity to the basement membrane of the blood vessels.[53] We found high expression of laminin B in both types of ECs. In line with the *in vitro* findings, we report a significant upregulation of laminin ($p < 0.01$) in RHD group compared to control group. Laminin B promoter was hypomethylated in both ECs stimulated with RHD sera as

well as in RHD mitral valve. There was significant hypomethylation ($p < 0.05$) of laminin and its upregulation in its transcript level ($p = 0.005$) in RHD patients having left ventricle hypertrophy. Thus, high expression of laminin highlights an additional role in ECM deregulation and cardiac hypertrophy.[54]

DNA methylation of endothelial cells has been found to be associated with various diseases such as cancer, autoimmune diseases including rheumatic heart disease.[12,13] Our data provide new insights into epigenetic mechanism i.e. DNA methylation that may be associated to development of RHD. The hypomethylation of various key inflammatory genes suggest the crucial events in development of chronic inflammatory environment which ultimately result in loss of valvular function.

Conclusion

This study highlights the interaction between autoantibodies and the ECs. Two ECs (HUVECs and HMVECs) showed an almost similar response to AECA rich sera which suggests the conservation of endothelial response, where cross reactive antibodies bind and cause endothelial pathology. However, we also observed chronic inflammation in mitral valve of RHD patient similar to ECs response to AECA rich sera with promoter hypomethylation of inflammatory, adhesion and ECM genes. Thus, it can be speculated that continuous stimulation of ECs may result in constant upregulation of these genes which are known to maintain the chronic localized inflammatory state by activation of the downstream inflammatory pathway. These observations also emphasize the role of environmental factors which might be instrumental in the pathogenesis of RHD. Hence, a complex mechanism with an interdependent role is responsible inflammation and disease severity in chronic RHD.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.gendis.2018.02.002.

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