

ANGIOGENESIS AND LOCAL IMMUNITY TISSUE MARKERS IN PATIENTS WITH CORONARY ARTERY DISEASE AND AORTIC STENOSIS

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ABSTRACT

In the cases of coronary artery disease (CAD) and aortic stenosis (AS), effective treatment and prognosis can be achieved with analysis of different cardiac tissue markers, particularly looking at the blood vessel endothelium and local resistance of cardiomyocytes to the pathological process. Despite numerous tissue markers being considered for diagnosis, cardiomyopathies still occur at normal biomarker levels. This study aims to investigate additional biomarkers (CD34, galectin-10, ICAM-1 and VCAM-1) in the cardiac tissue of patients with CAD and AS. Tissue samples were obtained from the left auricula during ten cardiac valve surgeries; five control samples were from children – the archive material from the Institute of Anatomy and Anthropology at Rīga Stradiņš University. In addition to routine staining, VCAM-1, CD34, ICAM-1, and galectin-10 were detected immunohistochemically and evaluated semiquantitatively, followed by nonparametric statistical analysis. These findings suggest that the endocardial endothelium remains an active site of remodelling, as indicated by the upregulation of both CD34 and ICAM-1 expression. The decreased expression of CD34, ICAM-1, and VCAM-1 in the blood vessel endothelium may reflect endothelial dysfunction and a compromised vascular repair mechanism.

Keywords: CAD; AS; CD34; ICAM-1; VCAM-1; galectin-10; angiogenesis

INTRODUCTION

There is a high incidence of aortic stenosis (AS) and coronary artery disease (CAD) worldwide, contributing to cardiac morbidity and mortality [14]. Although progress is being made in understanding the molecular mechanisms

of these conditions, angiogenesis and the local immune response in the heart are some of the key processes that remain less explored, together with their respective biomarkers. Biomarkers play a vital role in medical diagnostics, and, in the case of AS and CAD, CD34 acts as an endotheliocyte progenitor cell marker [10]; intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) act as endotheliocyte quality markers [26]. Moreover, while galectin-10 is not currently associated with cardiomyopathies, its role in inflammation shows potential as a valuable addition alongside biomarkers already being explored.

CD34 is an endothelial progenitor and hematopoietic cell surface antigen that is most commonly expressed in capillaries, smaller arteries and veins [10]. Endothelial cells with CD34 tend to be more elongated and lack tight junctions [1], which could contribute to greater vascular permeability. With its glycosylated and sialylated domains, CD34 is considered a subfamily of sialomucins, which are also known to regulate cell-cell or cell-matrix adhesion [21]. With its presence on endothelial precursors, it plays a vital role in angiogenesis, either during development or after injury [21]. A study found that endothelial cells expressing CD34 show the ability to form new blood vessels after cardiac ischemia/reperfusion injury, compared with endothelial cells without CD34 expression [32]. However, the changes in CD34 in CAD and AS are not clear.

ICAM-1 (intercellular adhesion molecule) is a part of the immunoglobulin superfamily, together with antigen receptors, antigen-presenting molecules, cytokine receptors and others. As a cell surface glycoprotein receptor, it has an extracellular immunoglobulin domain, a transmembrane domain, and a short cytoplasmic domain [4]. It is expressed on vascular endothelial cells, epithelial cells and immune cells [4]; however, it also has a soluble secreted serum isoform [22]. ICAM-1 is known for the role of regulating the adherence of leukocytes to endothelial cells and promoting leukocyte migration during inflammation [26]. ICAM-1 regulates the levels of intracellular Ca^{2+} and leads to myosin contractility, thus maintaining endothelial structure [3, 4]. It is also important in regulating endothelial cell permeability [23]. By activating Akt (a protein kinase that promotes cell growth) and endothelial nitric synthase (a nitric oxide-producing enzyme), ICAM-1 regulates endothelial cell migration, which is crucial for areas where neovascularization or vascular repair is needed [4]. Moreover, it has been found that endothelial cell ICAM-1 recruited bone marrow-derived endothelial progenitor cells in ischemic hearts, contributing to angiogenesis [4, 16].

VCAM-1 (vascular cellular adhesion molecule) is also a part of the immunoglobulin superfamily. As a transmembrane sialoglycoprotein, it has both cell

membrane and soluble isoforms [29, 30]. VCAM-1 interacts with circulating leukocytes via intracellular signalling leading to endothelial junction disruption and leukocyte migration to injured tissue [33]. Studies have shown that a high level of soluble VCAM-1 is strongly associated with a future fatal cardiovascular event [30] as well as with ongoing acute coronary syndrome and myocardial infarction [30], although there is limited research analysing the cardiovascular event prediction value of cell membrane VCAM-1 form.

Galectin-10 is a lectin and a cytoplasmic protein found in human eosinophils [28]. The presence of eosinophils and, therefore, galectin-10 indicates an immune response and inflammation of the tissue, as most organs do not possess resident eosinophils [6]. Current research does not seem to identify the presence of galectin-10 in the cardiac tissue of patients with cardiomyopathies.

Angiogenesis (the formation of new blood vessels from preexisting vasculature) is a fundamental process of physiological remodelling and adaptation of tissue under stress. In the case of ischemic heart disease, this response manifests as a limited compensatory process of collateral circulation [31], involving the coordinated activity of various angiogenic growth factors, smooth muscle cells, endothelial cells, pericytes, fibroblasts, and the extracellular matrix [9]. Inflammation, a hallmark of atherosclerosis, acts as a key trigger for angiogenesis in ischemic tissues by promoting the secretion of angiogenic growth factors and cytokines [25]. Therefore, examining tissue markers of angiogenesis in AS and CAD might reveal mechanisms of compensatory remodelling as well as potential therapeutic targets.

This study aims to investigate the appearance and distribution of CD34, ICAM-1, VCAM-1 and galectin-10 in the cardiac tissue of patients with AS and CAD, with a particular focus on their roles in angiogenesis and local immune responses involved in cardiac remodelling.

MATERIALS AND METHODS

The tissue samples used for this study were obtained during ten elective cardiac valve surgeries at Pauls Stradiņš University Hospital Surgery Centre from 2014 to 2017. Control samples from children were obtained from the archive of the Institute of Anatomy and Anthropology at Riga Stradiņš University. The study was conducted in accordance with the 1975 Helsinki Declaration and was independently approved by the Ethics Committee of Riga Stradiņš University, with approval numbers 14, dated 30.04.2015, and 29.05.2014.

A tissue sample (approximately 2 mm² in size) was obtained from the incision site for a venous cannula in the right atrium before establishing artificial blood circulation without creating any additional incision sites for tissue collection.

The patients

Six patients were hospitalized with CAD (all male), and four patients were hospitalized with degenerative aortic valve stenosis (one male, three females). CAD patients' age ranged from 52 to 80 years (with an average of 65 ± 8.6 years), and aortic valve stenosis patients' age ranged from 52 to 83 years (with an average of 69 ± 10.1 years). Written informed consent was obtained from all patients for participation in the study.

The control group

The control samples from children, five in total, were sourced from the archives of the Institute of Anatomy and Anthropology at Riga Stradiņš University. The samples were obtained from right atrial tissue during cardiac surgeries of children with congenital heart diseases (Table 1). The study was conducted in accordance with the 1975 Helsinki Declaration and independently approved by the Ethics Committee of Riga Stradiņš University (2015, No. 14). Parental consent was obtained in each case.

Tissue samples were collected from two girls and three boys aged from 16 days to 14 months.

Table 1. Information about the control group

Control number	Age	Sex	Diagnosis
1	16 days	M	Patent ductus arteriosus Tricuspid atresia Coronary artery fistulas Hypoplastic right ventricle
6	4 months	M	Ventricular septal defect
2	6 months	F	Ventricular septal defect Patent ductus arteriosus
4	6 months	F	Tetralogy of Fallot
5	1 year 2 months	M	Ventricular septal defect

Abbreviations: male (M), female (F).

Routine staining

The extracted tissue was immediately deposited into Eppendorf tubes filled with picric acid solution. Tissue dehydration was performed at the Laboratory of the Institute of Anatomy and Anthropology at Riga Stradiņš University. Afterwards, the tissue was embedded in paraffin and cut with a microtome into 3 µm sections. The sections were placed in a warm water bath before being transferred to a glass slide, dried, and prepared for staining. Prepared tissue samples were stained with hematoxylin and eosin [5].

Immunohistochemical (IHC) analysis

Immunohistochemical analysis of tissue samples from the right atrium was used to detect CD34, ICAM-1, VCAM-1, and galectin-10, using the standard streptavidin and biotin method [2].

Following rehydration, the tissue samples were rinsed with TRIS buffer solution (15-M106, *Bio-Optica*, Italy) and boiled in EDTA buffer solution (T0103, *Diapath*, Italy). Afterwards, the tissue samples were blocked with 3% peroxide and repeatedly rinsed with TRIS buffer solution.

For antibody dilution, an antibody mixture was used (ab64211; *Abcam*, USA). The tissue samples were incubated with primary antibodies (Table 2), rinsed with TRIS buffer, subjected to HiDef Detection™ reaction amplification (954D-31, *Sigma-Aldrich*, USA), rinsed with TRIS buffer solution again, incubated with HiDef Detection™ HRP Polymer Detector (954D-32, *Sigma-Aldrich*, USA), and subsequently rinsed with the TRIS buffer once again. The samples were then stained with hematoxylin (ab143166; *Abcam*, USA).

After staining, the tissue samples were rinsed with running water, dehydrated with increasing concentrations of ethanol solution and clarified with carboxylic acid and xylol. Finally, the samples were sealed with coverslips and marked.

Table 2. Information about the antibodies used in IHC

Tissue factor	Product code	Working dilution	Company	Location
CD34	sc-19621	1:100	Santa Cruz Biotechnology Inc	Santa Cruz, CA, USA
ICAM-1	BBA17	1:300	R&D SYSTEMS	Germany
VCAM-1	BBA19	1:200	R&D SYSTEMS	Germany
Galectin-10	orb157023	1:200	Biorbyt	Cambridge, UK

Abbreviations: CD34 – cluster of differentiation 34, ICAM-1 – intercellular adhesion molecule 1, VCAM-1 – vascular cell adhesion molecule 1.

Assessment of biomarker quantity in cardiac tissue samples

All samples were assessed using light microscopy, and the relative quantity of CD34-, ICAM-1-, VCAM-1-, and galectin-10- positive structures in the cardiac tissue was determined, using the semiquantitative counting method [20].

The evaluation of positive structures in the visual field was performed using the following identifiers: 0 (no positive structure in the visual field), 0/+ (rare occurrence of positive structures in the visual field), + (few positive structures in the visual field), +/++ (few to moderate positive structures in the visual field), ++ (moderate number of positive structures in the visual field), ++/+++ (moderate to numerous positive structures in the visual field), +++ (numerous positive structures in the visual field), +++/++++ (numerous to abundant positive structures in the visual field), and ++++ (abundance of positive structures in the visual field).

Statistical analysis

The data were statistically analysed using *IBM SPSS Statistics 26.0*. The results were considered statistically significant at a p -value less than 0.05. As the data obtained from semiquantitative counting were nonnumerical, nonparametric tests and analytical statistics were applied. The Mann-Whitney U test was used to detect differences in the distribution of tissue marker quantity between the patient and control groups. Spearman's rank correlation, however, was used to determine correlations between biomarkers with the following interpretation of Spearman's ρ (r_s) values: r_s 0.00 – 0.19 (very weak positive correlation); r_s 0.20 – 0.39 (weak positive correlation); r_s 0.40 – 0.59 (moderate positive correlation); r_s 0.60 – 0.79 (strong positive correlation); and r_s 0.80 – 1.00 (very strong positive correlation).

RESULTS

Pyknotic nuclei, prominent vacuolization, fibrosis, and some infiltration of lymphocytes were noted in the tissue samples of patients. The control samples presented a practically unchanged tissue appearance: myocardium with cardiomyocytes, occasional fibroblasts and immune cells, endocardium with endothelial cells and subendothelial connective tissue and epicardium with mesothelial cells, blood vessels and connective tissue (Figure 1).

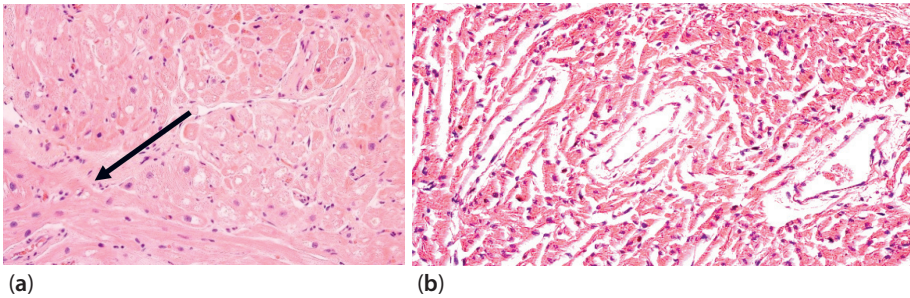


Figure 1. Hematoxylin and eosin staining of patient (a) and control (b) tissue samples. Note the vacuolization of cardiomyocytes (arrow), ingrowth of fibrous tissue (a), and unchanged tissue (b). 250x.

The distribution of CD34

The median quantity of CD34 positive structures in patient samples was few (+) positive structures in the endocardium endothelium only (Figure 2, Table 3). In the control samples, the median quantity of numerous (+++) CD34 positive structures in the endocardium endothelium was noted as well.

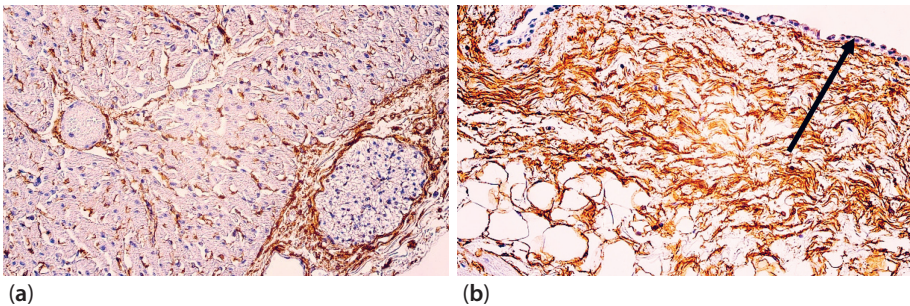


Figure 2. Immunohistochemistry of CD34-positive structures in control (a) and patient (b) samples. (a) Control sample with numerous positive structures in the endothelium, 250x. (b) Patient sample with few positive structures in the endothelium (arrow), 250x.

Table 3. Semiquantitative evaluation of CD34-positive structures in control and patient samples.

Sample group	CD34		
	BV	CM	E
Patient median	0	0	+
Control median	0	0	+++

Abbreviations: BV – blood vessel endothelium, CM – cardiomyocytes, E – endocardium endothelium.

Using the Mann-Whitney U test, a statistically significant difference ($U = 0$, $p = 0.002$) was detected in the endocardium endothelium between the control and patient groups. However, no statistically significant difference was found between the blood vessel endothelium ($U = 25$, $p = 1$) and cardiomyocytes ($U = 25$, $p = 1$).

The distribution of galectin-10

The median quantity of galectin-10 positive structures in patient samples was none (0) positive structures in the endocardium endothelium, few (+) positive structures in the blood vessel endothelium, and moderate (++) in cardiomyocytes (Figure 3, Table 4). In control samples, a median quantity of few (+) galectin-10 positive structures in the blood vessel endothelium and numerous to abundant (+++/+++++) positive structures in cardiomyocytes were noted.

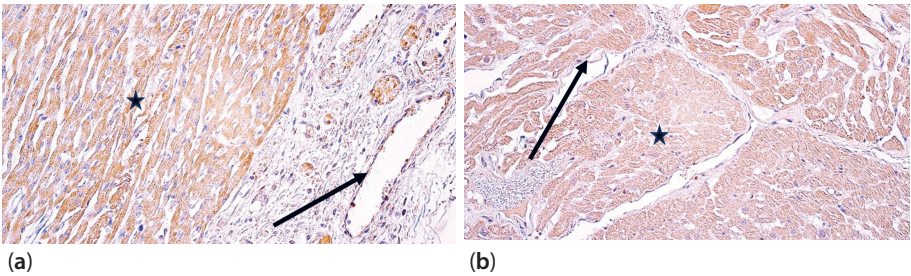


Figure 3. Immunohistochemistry of **Gal-10** positive structures in control (a) and patient (b) samples. (a) Control sample with few positive structures in the blood vessel endothelium (arrow) and numerous cardiomyocytes (star), 250x. (b) Patient sample with few (arrow) positive structures in the blood vessel endothelium and numerous (star) positive cardiomyocytes, 250x.

Table 4. Semiquantitative evaluation of Gal-10 positive structures in control and patient samples.

Sample group	Gal-10		
	BV	CM	E
Patient median	+	++	0
Control median	+	+++ /+++++	0

Abbreviations: BV – blood vessel endothelium, CM – cardiomyocytes, E – endocardium endothelium.

Using the Mann-Whitney U test, a statistically significant difference ($U = 0$, $p = 0.002$) was revealed in cardiomyocytes between the control and patient group. However, no statistically significant difference was found between the blood vessel endothelium ($U = 22.5$, $p = 0.738$) and the endocardium endothelium ($U = 25$, $p = 1$).

The distribution of ICAM-1

The median number of ICAM-1 positive structures in patient samples was none (0) positive structures in blood vessel endothelium or cardiomyocytes, but moderate (++) positive structures occurred in the endocardial endothelium (Figure 4, Table 5). In the control samples, a median quantity of moderate (++) ICAM-1 positive structures in the blood vessel endothelium and moderate (++) positive structures in the endocardial endothelium were noted.

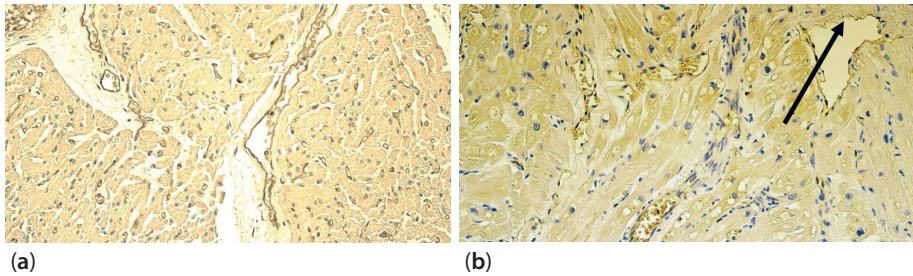


Figure 4. Immunohistochemistry of **ICAM-1** positive structures in control (a) and patient (b) samples. (a) Control sample with moderate positive structures in the blood vessel endothelium, 250x. (b) Patient sample with few positive structures in the blood vessel endothelium (arrow), 250x.

Table 5. Semiquantitative evaluation of ICAM-1 positive structures in control and patient samples.

Sample group	ICAM-1		
	BV	CM	E
Patient median	0	0	++
Control median	++	0	++

Abbreviations: BV – blood vessel endothelium, CM – cardiomyocytes, E – endocardium endothelium.

Using the Mann-Whitney U test, a statistically significant difference ($U = 0$, $p = 0.001$) was found in the blood vessel endothelium between the control and patient groups. However, no statistically significant difference was found between cardiomyocytes ($U = 25$, $p = 1$) and the endocardium endothelium ($U = 22.5$, $p = 0.738$).

The distribution of VCAM-1

The median number of VCAM-1 positive structures in patient samples was rare (0/+) positive structures in the blood vessel endothelium, and none (0) positive cardiomyocytes or endocardium endotheliocytes were detected (Figure 5, Table 6). In control samples, the median quantity of rare (0/+) VCAM-1 positive structures in the blood vessel endothelium was noted.

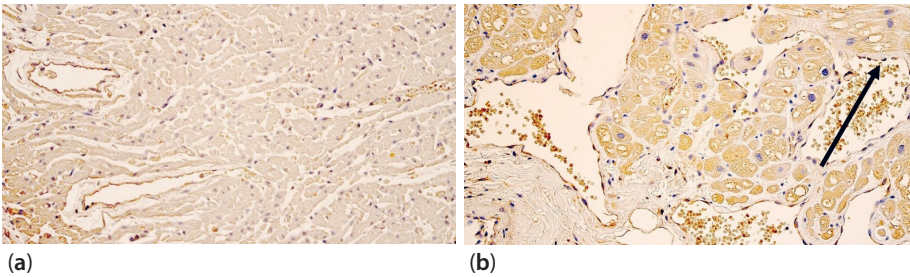


Figure 5. Immunohistochemistry of **VCAM-1** positive structures in control **(a)** and patient **(b)** samples. **(a)** Control sample with few positive cells in the blood vessel endothelium, 250x. **(b)** Patient sample with few positive structures in the blood vessel endothelium (arrow), 250x.

Table 6. Semiquantitative evaluation of VCAM-1 positive structures in control and patient samples.

Sample group	VCAM-1		
	BV	CM	E
Patient median	0/+	0	0
Control median	0/+	0	0

Abbreviations: BV – blood vessel endothelium, CM – cardiomyocytes, E – endocardium endothelium.

Using the Mann-Whitney U test, no statistically significant differences were found between cardiomyocytes ($U = 25, p = 1$), endocardium endothelium ($U = 25, p = 1$), or blood vessel endothelium ($U = 15.5, p = 0.21$) between the control and patient group.

With the use of Spearman’s rank correlation coefficient, a statistically significant (Spearman’s $\rho = 0.915, p < 0.005$) strong positive correlation was found between the CD34-positive endocardium endothelium and the ICAM-1 positive endocardium endothelium in patient samples. However, no correlations were found between biomarkers in controls.

DISCUSSION

In patient tissue with hematoxylin and eosin staining, pyknotic nuclei were noted, indicating irreversible cell damage, as well as vacuolization, which indicates cellular damage and degenerative changes. Ingrowth of fibrosis shows tissue remodelling and scarring. Together with inflammatory infiltrate, these are nonspecific changes characteristic of cardiovascular disease [8, 24].

A strong, statistically significant positive correlation between CD34 and ICAM-1 expression in the endocardial endothelium was observed in our patients. CD34, a marker of endothelial progenitor cells and vascular integrity [12] and ICAM-1, a key adhesion molecule upregulated during endothelial activation and inflammation [11], appearing together suggest that the endocardium may undergo active remodelling via regeneration and inflammatory activation. This correlation may indicate that the same endothelial populations involved in repair are simultaneously engaged in inflammatory signalling.

No other statistically significant correlations between biomarkers were found in either patients or controls. This could indicate that specific relationships between regenerative and inflammatory markers occur mostly directly in disease-affected areas, such as the endocardium endothelium but not in the myocardium or blood vessel endothelium. A previous study has also shown that endothelial activation markers, including ICAM-1 and VCAM-1, may have localized expression in cardiac tissue [26].

In the endocardium endothelium, numerous CD34 positive cells were found in controls compared to few in patients. This may suggest that younger hearts have a greater capacity for endothelial repair, whereas adult hearts with chronic ischemia lose this regenerative potential. It has been suggested that aging and cardiovascular risk factors impair the function of CD34+ endothelial progenitor cells [17, 19]. The depletion of circulating CD34+ endothelial progenitor cells [27] and their impaired mobilization have also been observed in AS and CAD patients, which could contribute to the reduced CD34 positive cell presence in diseased tissues.

In the blood vessel endothelium, few galectin-10 positive cells were found in both controls and patients; in cardiomyocytes, their number was moderate in patients and numerous to abundant in controls. As galectin-10 is associated with eosinophil activity and immune regulation [13], its abundant presence in controls may indicate a stronger immune environment in children's myocardium. A reduced number of galectin-10 positive cardiomyocytes in patients could suggest altered immune function in adult hearts.

A moderate amount of ICAM-1 positive cells was found in the blood vessel endothelium of the controls but not in the patients, whereas a moderate amount of ICAM-1 positive cells was observed in the endocardium endothelium in both groups. The absence of ICAM-1 positive cells in the blood vessel endothelium of patients could reflect advanced endothelial damage with decreased inflammatory marker expression, which is consistent with the contribution of endothelial cell dysfunction to atherosclerotic cardiovascular disease described in the literature [7].

The number of VCAM-1 positive cells was low in both patients and controls, with few positive cells observed in the blood vessel endothelium and none in cardiomyocytes or the endocardium endothelium. Although VCAM-1 is associated with vascular inflammation [30], its low expression could be related to its activation during the early inflammatory response [15] rather than in the chronic disease stage. This could be explained by VCAM-1 being the first adhesion molecule expressed before atherosclerotic plaque development [30] where it mediates monocyte adhesion to the intima [18].

This study has several limitations that must be considered. The relatively small sample size limited the statistical power of the findings as well as their generalizability to a broader population. While the observed correlations were consistent, a larger cohort would be necessary to confirm the reproducibility of the results in different subpopulations.

The age range of the patients varied, introducing a variety of confounding factors. Endothelial and immune marker expression is influenced by aging independently of disease, which complicates the interpretation of the observed differences, even though the age groups were matched as closely as possible.

Due to ethical constraints, it was not possible to obtain healthy human heart tissue. The control samples used in this study were from individuals with congenital heart disease who lacked signs of inflammation. These samples may not fully reflect baseline physiological conditions, and the possibility of sub-clinical changes influencing marker expression cannot be ruled out.

The study was conducted using immunohistochemical staining and qualitative or semiquantitative methods of assessment. Quantitative data from biochemical analyses (such as ELISA) could provide valuable insight into the exact concentrations of the markers in tissue. Additionally, genetic analysis to identify gene expression patterns associated with altered marker expression or disease progression could also prove valuable, given the complex and multifactorial nature of AS and CAD.

Despite these limitations, these findings offer novel insight into endothelial and inflammatory alterations in the cardiac tissue of patients with AS and CAD, providing a foundation for further study.

CONCLUSIONS

The findings of this study suggest that in patients with AS and CAD, the endocardial endothelium remains an active site of remodelling, as indicated by the upregulation of both CD34 and ICAM-1 expression. In contrast, the decreased expression of CD34, ICAM-1, and VCAM-1 in the blood vessel endothelium may reflect endothelial dysfunction and compromised vascular repair mechanisms. These contrasting expression patterns highlight region-specific endothelial responses in diseased hearts. Further research is needed to clarify the molecular mechanisms behind these changes and to determine their usefulness as potential targets for therapeutic intervention or early disease markers.

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