A variety of infectious agents have been investigated as the underlying pathogenesis of atherosclerosis contributed to the chronic inflammatory process. The most investigated agents have been *Chlamydia* (*C.* pneumoniae), *Mycoplasma pneumoniae*, *Helicobacter* (*H.* pylori) and viral factors. However, the role of infections in the pathogenesis of atherosclerosis is still unclear. The microbial spectrum is complex and the inflammatory process involved in atherosclerosis is low-grade. There is 1 study of fungal DNA in coronary plaques obtained from catheter-based atherectomy, and the antifungal action of statins and of the chemotherapeutic agent, paclitaxel, which coats stents, is also known. Filler et al describe the mechanism of the proinflammatory response of endothelial cells to *Candida* (*C.* albicans). Candidal induction of intercellular adhesion molecule (ICAM)-1 expression is independent of other cytokines, such as interleukin (IL)-1α and -1β and tumor necrosis factor (TNF)-α, so in the present study we investigated the presence of mycotic DNA in the non-atherosclerotic aortic wall of patients with advanced coronary artery disease (CAD) referred for direct myocardial revascularization, and we assessed a possible relationship between fungal DNA and ICAM-1 expression.

**Background:** Atherosclerosis is currently being investigated as a chronic inflammatory process and the role of infectious agents is unclear. The presence of mycotic DNA in the wall of the non-atherosclerotic aorta of patients with coronary artery disease (CAD) and its association with levels of soluble intercellular adhesion molecule (sICAM-1) expression was examined in the present study.

**Methods and Results:** In 40 patients with CAD and a comparative group of 20 patients with aortic valve stenosis (AS) without CAD, specimens of the aortic wall were obtained during cardiac surgery. Mycotic DNA was analyzed by polymerase chain reaction (PCR) using a fungus-specific universal primer pair, ITS3 and ITS4, to amplify a portion of the 5.8S rDNA region, the entire ITS2 region and a portion of the 28S rDNA region, and using a species-specific primer pair, CALB1 and CALB2, to specifically amplify *Candida* (*C.* albicans). The nested PCR method was performed to amplify the intergenic transcribed spacer regions of the rRNAs of *Candida* species. Before surgery the serum level of sICAM-1 was estimated. Mycotic DNA was detected in 48% of the CAD patients and in 40% of the AS patients, with *C. albicans* DNA in 58% and 100%, respectively (P>0.05). In CAD patients with a high level of sICAM-1, *C. albicans* DNA was found more frequently than in patients without elevated levels of sICAM-1 (P<0.05).

**Conclusions:** Mycotic DNA was found in the non-atherosclerotic aortic wall of CAD patients as well as in patients with AS. In the CAD patients *C. albicans* DNA was related to sICAM-1 expression. (Circ J 2010; 74: 749–753)

**Key Words:** Atherosclerosis; Coronary artery disease; Fungi; Intercellular adhesion molecule
9.5 years) with aortic valve stenosis (AS) without CAD (AS group) on coronary angiography, specimens were taken from macroscopically non-atherosclerotic aorta during cardiac surgery procedures. The baseline characteristics of the study groups are presented in Table 1. The severity of CAD was estimated by Gensini score,\(^\text{13}\) which was high and amounted to 100.3±38.6 (median 93.5).

From blood samples of all the patients taken before the cardiac surgery levels of soluble intercellular adhesion molecule (sICAM), total cholesterol, HDL-C, LDL-C, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and ejection fraction were added according to their level of sICAM: >297 ng/ml or ≤ 297 ng/ml.

The exclusion criteria were presence of infectious disease or acute coronary event within the past 3 months, current fever, use of immunosuppressant drugs, or immunological and/or neoplastic disease.

All patients gave written informed consent for the molecular detection of fungal DNA. The investigation conformed with the principles outlined in the Declaration of Helsinki and the study protocol was approved by the university ethics review board (approval reference number RNN/53/02/KE).

### Molecular Analysis

#### DNA Extraction

DNA from 25–30 mg tissue samples was extracted using commercially available DNeasy Tissue Kit (Qiagen, Syngen, Poland). The quality of the DNA isolated was estimated by Gensini score,\(^\text{13}\) which was high and amounted to 100.3±38.6 (median 93.5).

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#### Polymerase Chain Reaction (PCR)

We used a fungus-specific universal primer pair for the internal transcribed spacer 3 (ITS3) (5'-TCC CCT GCT TAT TGA TAT GC-3') and ITS4 (5'-TCC GTT CCT GTA TAT GC-3') to amplify a portion of the 5.8S ribosomal DNA (rDNA) region, the entire ITS2 region, and a portion of the 28S rDNA region for the fungal species, as previously described.\(^\text{14,15}\)

We also use a species-specific primer pair CAL1 (5'-TTC ATC AAC TTG TCA CAC CAG A-3') and CAL2 (5'-ATC CCG CCT TAC CAC TAC CG-3') to specifically amplify *C. albicans* in the DNA samples (reference GenBank accession number L47111, L2817). The forward primers were designed within the ITS1 region, and the reverse primers were designed from the ITS2 region.\(^\text{16}\)

For fungal DNA detection in the tissue specimens, PCR was performed according to the method of Luo and Mitchell.\(^\text{16}\) The PCR was carried out in a DNA Thermal Cycler (Biometra, Polgen, Poland) with the following thermal cycling conditions: 5 min initial denaturation step at 96°C, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 1.5 min.

The reaction mixture contained 2 μl (~100 ng) of diluted genomic DNA template, 0.2 μmol/L of each appropriate primer (Sigma-Genosys, Poland), 20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L KCl, 2 μmol/L MgCl₂, 2.5 μmol/L (each of the four) deoxyribonucleotide triphosphates (dNTP) and 5 U of Taq DNA polymerase (TaKaRa Shuzo Co, Ltd, Shiga, Japan) in a total volume of 20 μl.

PCR products were electrophoresed in a 1.5% agarose gel with ethidium bromide staining and 1× Tris-borate-EDTA buffer for 1–2 h. In addition, negative and positive controls were added to each amplification series. As a positive control *C. albicans* genomic DNA was used. DNA bands were visualized on a UV transilluminator and documented.

#### Nested PCR

The fungus-specific universal primers ITS1 (5'-TCCGTAGGGAAACGTGGG-3') and ITS4 (5'-TCCCGCTTTATATGATGC-3')\(^\text{17}\) were used as outer primers to amplify the intergenic transcribed spacer regions of rRNAs of *Candida* species. Specific inner primers were designed for *C. albicans* on the basis of the ITS1–ITS4 sequences derived from GenBank (reference accession number L47111) CAL1 (5'-AACHTCGTTTGTGGG-3') and CAL3 (5'-TGGACGTGTTACCGCGAACG-3').\(^\text{16}\) PCR amplification was performed in a final volume of 20 μl using a reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 100 mmol/L each deoxynucleoside triphosphate, and 1.25 U of Taq DNA polymerase (TaKaRa Shuzo Co Ltd). For the first PCR, 10 pmol of each outer primer was mixed with 2 μl of DNA. A Thermal Cycler (Biometra, Polgen, Poland) was used with the following temperature cycles: 95°C for 5 min; then 30 cycles of 20 s at 95°C, 15 s at 55°C, and 65 s at 72°C, and a final cycle of PCR extension at 72°C for 5 min. For the nested PCR, 1 μl of

**Table 1. Characteristics of the Study Participants**

<table>
<thead>
<tr>
<th></th>
<th>AS group (n=20)</th>
<th>CAD group (n=40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean±SD</td>
<td>49.9±9.50</td>
<td>54.2±7.61</td>
<td>NS</td>
</tr>
<tr>
<td>Sex: F/M</td>
<td>2 (10)/18 (90%)</td>
<td>8 (20)/32 (80%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking: yes/no</td>
<td>12 (60)/80 (20%)</td>
<td>38 (95)/5 (5%)</td>
<td>0.001</td>
</tr>
<tr>
<td>SI</td>
<td>395.17</td>
<td>617.08</td>
<td>0.04</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.8±4.24</td>
<td>27.5±3.62</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.6±1.29</td>
<td>4.23±1.10</td>
<td>0.05</td>
</tr>
<tr>
<td>WBC (x10⁹/mm³)</td>
<td>6.5±1.7</td>
<td>6.9±1.4</td>
<td>NS</td>
</tr>
<tr>
<td>sICAM (mean ng/ml)</td>
<td>336.7±84.04</td>
<td>286.6±130.5</td>
<td>NS</td>
</tr>
<tr>
<td>TC (mean mg/dl)</td>
<td>206.8±38.22</td>
<td>204.9±56.7</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mean mg/dl)</td>
<td>57.1±16.38</td>
<td>52.2±12.28</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mean mg/dl)</td>
<td>110.1±27.9</td>
<td>120.18±44.92</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mean mg/dl)</td>
<td>119.2±45.8</td>
<td>163.7±122.37</td>
<td>NS</td>
</tr>
<tr>
<td>EF (%)</td>
<td>58±8.0</td>
<td>55±9.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

AS, aortic valve stenosis; CAD, coronary artery disease; SI, smoking index calculated according to daily cigarette consumption and duration of smoking; BMI, body mass index; WBC, white blood cells; sICAM, soluble intercellular adhesion molecule; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; EF, ejection fraction.
the product obtained from the first amplification and 10 pmol of each inner primer was mixed in fresh reaction mixture. The second amplification was performed under the conditions described, except for the annealing temperature of 66°C, which was specific for the pair of inner primers. The nested PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide. Appropriate negative controls, the DNA extraction and reaction mixture controls, were tested for each amplification reaction.

### Statistical Analysis

Student’s t-test, the χ² test or Fisher’s exact test was performed as appropriate. All calculations were performed with SPSS for Windows (Chicago, IL, USA). P<0.05 was statistically significant.

### Results

Fungal DNA was found in 19 of 40 (48%) samples from the CAD patients and in 8 from 20 (40%) AS patients (P>0.05). The DNA of C. albicans was detected by nested PCR in 11 (58%) and 8 (100%) specimens, respectively, of fungal DNA positive subjects (P>0.05). The mean level of sICAM-1 did not differentiate CAD and AS patients (P>0.05).

In 15 patients (40%) with CAD the sICAM-1 level was significant elevated (423.4±112.6 ng/ml, P<0.001). These patients, in comparison with those without elevated sICAM-1 (mean of 204.6±39.5 ng/ml), had a history of more episodes of acute coronary syndrome (P<0.05) and peptic ulcer (P<0.01) (Table 2). C. albicans DNA was found more frequently in aortic wall specimens from CAD patients in the high-sICAM group than in those from the not elevated-sICAM-1 group: 9 (60%) vs 5 (20%), respectively (P<0.05).

Among the AS patients, 6 (30%) had an elevated level of sICAM (mean, 397.7±72.1 ng/ml). The presence of C. albicans DNA did not differentiate patients with valve disease related to the level of sICAM-1 (43% and 33%, respectively, P>0.05).

### Discussion

The classical coronary risk factors do not fully explain the pathogenesis of CAD. Increasing evidence has been presented that a low-grade inflammatory process and infection may be involved in the development of atherosclerosis.

Numerous studies have reported associations between CAD and bacterial or viral pathogens. To date the presence of fungal components in atherosclerotic plaque has been demonstrated in 1 study only.8 In the present study we frequently detected DNA of C. albicans in specimens of aortic wall from patients with CAD and high levels of sICAM-1. From the study by Filler et al.11 it is known that the mechanism of cytokine production in response to C. albicans is mediated by synthesis of TNF-α, which in turn induces endothelial cells to secrete IL-8 and express E-selectin by an autocrine mechanism. Candidal induction of vascular cell adhesion molecule 1 (VCAM-1) expression is mediated not only by TNF-α but also by IL-1α and IL-1β, and ICAM-1 expression is independent of these 3 cytokines. The last finding was background to our study of sICAM-1 as the choice for a potential link between C. albicans and CAD.

The mediators of inflammation can be found at all stages of atherosclerotic plaque formation. Cellular adhesion molecules are critical in the adhesion of circulating leukocytes to the endothelial cells, their endothelial transmigration and atherogenesis.18 Moreover, it has been revealed in an experimental model of atherosclerosis in apoE-deficient mice that antioxidant treatment with N-acetylcysteine suppressed superoxide production and macrophage accumulation as assessed by ICAM-1 expression in the atherosclerotic lesions.19

It has been reported that the plasma concentration of sICAM-1 is elevated many years in advance of the first myocardial infarction.18 In the present study patients with high levels of ICAM-1 had more episodes of acute coronary syndrome in their life.

Conflicting results concerning the prevalence of the DNA of different infectious agents in atherosclerotic and non-atherosclerotic specimens were recently reported.20 In 10 atherosclerotic aortic specimens obtained from non-atherosclerotic regions of the ascending aorta wall, Myocardial revascularization with saphenous grafts requires proximal anastomoses to the ascending aorta and usually the cardiac surgeon uses a specific tool to produce a circular opening in the non-atherosclerotic aortic wall. The standard procedure of aortic valve replacement comprises aortotomy, so it is possible to obtain a very small sample of the aortic wall without detriment to the patient. This method of aortic sample collection was accepted by the local bioethics committee.

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In the present study we investigated the frequency of fungal DNA in specimens obtained from non-atherosclerotic regions of the ascending aorta wall. Myocardial revascularization with saphenous grafts requires proximal anastomoses to the ascending aorta and usually the cardiac surgeon uses a specific tool to produce a circular opening in the non-atherosclerotic aortic wall. The standard procedure of aortic valve replacement comprises aortotomy, so it is possible to obtain a very small sample of the aortic wall without detriment to the patient. This method of aortic sample collection was accepted by the local bioethics committee.

Conflicting results concerning the prevalence of the DNA of different infectious agents in atherosclerotic and non-atherosclerotic specimens were recently reported.20 In 10 atherosclerotic aortic specimens obtained from autopsies in 80%, herpes simplex virus (HSV)-1 and cytomegalovirus (CMV) were detected in 80% and 40%, respectively, while in 23 non-atherosclerotic tissue samples HSV-1 and CMV were found in 13% and 4%, respectively.20 In 52 specimens from macroscopically healthy regions of the wall of the ascending aorta, there was 1 case (1.9%) of C. pneumoniae and none of the samples obtained from the same patients were positive for H. pylori.21 Other investigations have not confirmed this difference in the frequency of pathogens DNA in the atheromatous and healthy aorta. In our recent study of samples of non-atherosclerotic aorta from CAD patients, we found

### Table 2. Relationship Between Coronary Artery Disease and Level of sICAM-1

<table>
<thead>
<tr>
<th>CAD group</th>
<th>ACS (%)</th>
<th>Peptic ulcer (%)</th>
<th>TC (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>Fibrinogen (g/L)</th>
<th>HT (%)</th>
<th>DM (%)</th>
<th>Smokers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>112.8±43.7</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>4.5±1.3</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>151±15.3</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>4.08±0.9</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>105±45.7</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>4.08±0.9</td>
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<td>&gt;0.05</td>
<td>4.08±0.9</td>
<td>60</td>
<td>20</td>
</tr>
</tbody>
</table>

Data are mean±SD or percent.

ACS, acute coronary syndrome; HT, hypertension; DM, diabetes mellitus. Other abbreviations see in Table 1.
a large frequency of *H. pylori* (80%). In the same patients *C. pneumoniae* was also detected in macroscopically healthy aortic wall with a frequency of 27.5%, which is similar to other data for atherosclerotic plaques. Moreover, I studied to detect *H. pylori* DNA even in specimens of atherosclerotic artery.

The results of several studies indicate a lower frequency of microorganisms in non-atherosclerotic tissue. On the other hand, it is well known that the DNA of common microorganisms, such as CMV or *C. pneumoniae*, is widely distributed in the organs of healthy subjects, which suggests that the DNA of infectious agents can also be detected in healthy tissues. These observations agree with the results of the present study, that *C. albicans* DNA can be found in the non-atherosclerotic aortic wall of AS patients without CAD. Ott et al reported 92% of patients with fungal DNA coronary atherectomy samples and none in any control samples.

The possible interpretation of our results is that the common features of the pathogenesis of CAD and AS are associated with *sICAM-1* and *C. albicans*, but by different signal pathways as proposed by Orozco et al. There are studies about calcific aortic stenosis and *ICAM-1* expression in aortic valve intimal cells, but not in serum. Furthermore, *C. albicans* stimulates the synthesis of *ICAM-1* by a discrete mechanism, different from those mediating similar responses to other microbial pathogens.

This study has limitations, the main one being the small number of subjects.

Conclusions

Fungal DNA, especially that of *C. albicans*, can be detected in the non-atherosclerotic aortic wall of patients with CAD, as well as in AS patients. In patients with advanced CAD, the presence of *C. albicans* DNA is related to the expression of *sICAM-1*. However, these findings do not allow determination of the role of fungi, and *C. albicans* in particular, in the pathogenesis of atherosclerosis.

Acknowledgment

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Disclosure

Conflict of Interest: non declared.

References


