CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Atrial Fibrillation Coincides with the Advanced Glycation End Product N^ε-(Carboxymethyl)Lysine in the Atrium

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Accepted for publication
April 7, 2015.

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Presence of advanced glycation end products (AGEs) in the heart induces a proinflammatory phenotype. However, the presence of AGEs within atrial tissue of atrial fibrillation (AF) patients is unknown and was analyzed here. Left atrial appendage tissue from 33 AF patients and 9 controls was analyzed for the presence of the major AGEs N^ε-(carboxymethyl)lysine (CML), VCAM-1, neutrophilic granulocytes, lymphocytes, and macrophages in both the fat tissue and myocardium separately. The total amount of fibrosis was also analyzed. Presence of CML was significantly higher in blood vessels of the left atrial appendage in AF patients as compared to controls, independent of diabetes mellitus. In AF patients, VCAM-1 expression in blood vessels and the numbers of infiltrated neutrophilic granulocytes, lymphocytes, and macrophages significantly increased compared to controls, and were highest in the fat tissue; there was no significant difference in fibrosis compared to controls. Interestingly, total amount of CML and fibrosis in AF and control patients correlated positively. Finally, there was no difference between AF patients based on AF type or surgical indication in the presence of CML, VCAM-1 expression, inflammatory cells, and fibrosis. Our results indicate that in AF the intramyocardial blood vessels of the left atrial appendage have an increased CML presence and proinflammatory status coinciding with a local increase in the number of inflammatory cells. (*Am J Pathol* 2015, 185: 2096–2104; <http://dx.doi.org/10.1016/j.ajpath.2015.04.018>)

Atrial fibrillation (AF) is the most common sustained cardiac rhythm disturbance in humans.¹ Recent studies indicate that inflammation contributes to the pathogenesis of AF.² The relationship between AF and systemic inflammation has indirectly been demonstrated in AF patients because they have an increase in serum levels of C-reactive protein and proinflammatory cytokines.³

Important initiators of inflammatory responses are advanced glycation end products (AGEs). AGEs accumulate during aging and at a faster rate in diabetes mellitus (DM).⁴ AGE depositions were also found in atherosclerotic plaques and cardiomyocytes of patients with and without DM,^{5,6} and are increased in the plasma of patients with heart failure.⁷ In a previous study, we showed that the AGE product N^ε-(carboxymethyl)lysine (CML) is increased in intramyocardial blood vessels in both the infarcted and noninfarcted areas in acute myocardial infarction

patients.⁸ As such, AGEs might cause endothelial dysfunction, but are also believed to be associated with impaired heart function.⁹ It is hypothesized that the impaired heart function in part coincides with an increase of endothelial adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1).¹⁰ Interestingly, fluorescent AGE plasma levels were also found to be significantly higher in persistent AF patients, independent of DM.¹¹ In addition, it is known that AF is associated with increased fibrosis.^{12,13} Importantly, in diabetic rats, inhibition of AGE formation by OPB-9195 resulted in reduced DM-induced atrial fibrosis, suggesting that AGEs facilitate atrial fibrosis.¹⁴ However, it is unknown whether AGEs are formed in the atria of AF patients.

Supported by the Netherlands Forensic Institute project 34.
Disclosures: None declared.

Table 1 Patient Characteristics

Characteristics	Controls (<i>n</i> = 9)	Atrial fibrillation (<i>n</i> = 33)	Significance
Age, years, mean (range)	61 (35–87)	68 (52–85)	
Male/female	6/3	22/11	
Primary cause of death	Pulmonary insufficiency (<i>n</i> = 3) Kidney insufficiency (<i>n</i> = 1) Pancreas carcinoma (<i>n</i> = 1) Suicide (<i>n</i> = 1) Acute aorta dissection (<i>n</i> = 3)	None	
Operation indication	None	CAD ± VS/VI (<i>n</i> = 13) VS/VI only (<i>n</i> = 17) Idiopathic AF (<i>n</i> = 3)	
Valve insufficiencies	None	MI (<i>n</i> = 19) TI (<i>n</i> = 10) AI (<i>n</i> = 2) AS (<i>n</i> = 7)	
Type of AF	None	Persistent (<i>n</i> = 6) Paroxysmal (<i>n</i> = 27)	
Thrombi inhibitors	None	Acetylsalicylic acid VKA Heparin	
Risk factors	Hypertension (<i>n</i> = 3)	AMI (<i>n</i> = 4) Obesity (<i>n</i> = 6) Hypertension (<i>n</i> = 21) Sleep apnea (<i>n</i> = 3)	
Degree of stenosis of coronary arteries	<25% (<i>n</i> = 1) 25%–50% (<i>n</i> = 3) 50%–75% (<i>n</i> = 3) >75% (<i>n</i> = 1) Unknown (<i>n</i> = 1)		
LA size	NA	Normal (<i>n</i> = 10) Dilated (<i>n</i> = 23)	
LV function	NA	Normal (<i>n</i> = 27) Reduced (<i>n</i> = 6)	
Diabetes mellitus	0	6	<i>P</i> < 0.05
Statin use	0	14	<i>P</i> < 0.001

AF, atrial fibrillation; AI, insufficiency of the aortic valve; AMI, acute myocardial infarction; AS, stenosis of the aortic valve; CAD, coronary artery disease; LA, left atrium; LV, left ventricle; MI, insufficiency of the mitral valve; NA, not available; TI, insufficiency of the tricuspid valve; VI, valve insufficiency; VKA, vitamin K antagonists; VS, valve stenosis.

Here, we studied CML presence in both the myocardium and fat tissue separately in the atria of AF patients, and further analyzed whether CML presence coincided with endothelial activation and infiltration of inflammatory cells.

Material and Methods

Human Heart Tissue

The left atrial (LA) appendage was dissected during cardiac surgery within 5 minutes after the start of surgery (coronary aortic bypass grafting and/or valve surgery; AF patients) and at autopsy (controls). Autopsies were performed between 2 to 57 hours (mean, 18 hours) after death. During the time between death and autopsy, bodies were preserved in a cooling chamber (4°C). Tissue samples were taken and then preserved in 4% formaldehyde within 3 hours after the start of the autopsy. In autopsied hearts, nitroblue tetrazolium staining was performed

to exclude acute myocardial infarction patients. The coronary arteries of control patients were analyzed for the degree of stenosis, indicative for putative coronary artery disease, except for 1 control patient for whom the coronary arteries were not collected (Table 1). Duration of hospital stay of control patients was between 0 and 21 days. During hospital stay, no surgical procedures were performed. AF patients had different surgical procedure indications. Namely, AF patients were operated on based on coronary artery disease with or without valve insufficiencies (*n* = 13), valve insufficiencies only (*n* = 17), or idiopathic AF (symptomatic despite adequate medication; *n* = 3). Thirty-three patients with either paroxysmal (*n* = 27) or persistent (*n* = 6) AF and 9 control patients (who died from a cause not related to cardiac disease) were included (Table 1). One control patient and six AF patients had DM. Left ventricular (LV) function of the AF patients was analyzed by echocardiography and described as being normal or reduced. LA size was also

Figure 1 Immunohistochemical staining of endothelial CML (score 3) (A), endothelial VCAM-1 (B), neutrophilic granulocytes (MPO) (C), lymphocytes (CD45) (D), and macrophages (CD68) (E) (all indicated by **arrows**) in the fat tissue in the left atrial appendage of atrial fibrillation patients. Original magnification: $\times 400$.

measured in the AF patients, a size of >40 mm was indicated as dilated.

This study was approved by and performed according to the guidelines of the ethics committee of the VU University Medical Center, Amsterdam, and conforms to the principles of the Declaration of Helsinki. Use of the leftover material after the pathological examination was completed was part of the patient's contract with the hospital.

Immunohistochemistry

Sections (4 μ m) were dewaxed, dehydrated, and incubated in methanol/hydrogen peroxide (0.3%) for 30 minutes to block endogenous peroxidases. Next, antigen retrieval was performed either by heat inactivation in citrate buffer [pH 6.0; myeloperoxidase (MPO), CD31, and CD68] or Tris-EDTA buffer (pH 9.0; VCAM-1) or enzymatically (CML) at 37°C for 30 minutes using pepsin–hydrogen chloride 0.1% solution. CD45 required no antigen retrieval. Slides were then incubated with either rabbit anti-human VCAM-1 (1:100 dilution; Santa Cruz Biotechnology, Heidelberg, Germany) or MPO (1:700 dilution; Dako, Heverlee, Belgium) or mouse anti-human CD68 (1:400 dilution; Dako), CD31 (1:40 dilution; Dako), CML⁸ (1:500 dilution), or CD45 (1:50 dilution; Dako) for 1 hour at room temperature, followed by incubation with Envision (undiluted, anti-mouse/rabbit; Dako) for 30 minutes at room temperature. Sections were visualized using 3,3'-diaminobenzidine (0.1 mg/mL 0.02% H₂O₂) and counterstained with hematoxylin, dehydrated, and covered. With each staining, a phosphate-buffered saline control was included. All of the controls yielded negative results (data not shown).

Morphometric Analysis

In each LA tissue, the number of extravascular neutrophilic granulocytes (MPO-positive), lymphocytes (CD45-positive), macrophages (CD68-positive), and the number of VCAM-1–positive blood vessels were counted in both the myocardium and fat tissue separately. The score for the fat tissue is the score of epicardial fat and intramyocardial fat taken together that are both present in the LA samples. The total surface area of the myocardium and fat tissue of each sample was then measured using QPRODIT version 3.2 (Leica Microsystems, Cambridge, UK).¹⁵ The number of extravascular inflammatory cells or positive blood vessels per mm² were calculated as the total score for each specimen. In both the myocardium and fat tissue, CML scores were determined as described before,⁸ and also divided by the total surface area, resulting in a final score of CML intensity per mm². Two independent observers scored the tissue slides (M.P.V.B. and L.R.). The interobserver variation was $<10\%$. CD31 was used as a marker for identifying thrombi in the endocardium and/or intramyocardial blood vessels.

Quantification of Fibrosis

Fibrosis was visualized histochemically using a Picrosirius Red staining. Sections (4 μ m) were dewaxed, rehydrated, and incubated in a 0.2% Sirius Red solution (solution made in 1.2% picric acid; VWR International, Radnor, PA) for 60 minutes at room temperature. Subsequently, staining was developed in 0.01 N HCl for 2 minutes at room temperature. The sections were then washed in tap water, dehydrated, and covered. For quantitative analysis, six representative photographs were taken randomly throughout the epicardium,

Figure 2 CML positivity in the left atrial (LA) appendage of atrial fibrillation (AF) patients. **A:** CML score in the myocardium (circles) and fat tissue (squares) in the LA of AF patients and controls (C). **B:** CML score in the myocardium (circles) and fat tissue (squares) in the LA of AF patients with (DM+) and without (DM-) diabetes mellitus (DM). **C:** Number of blood vessels positive for the different intensity scores of CML (1, minor; 2, moderate; 3, strong) in the myocardium in AF patients and controls. **D:** Number of blood vessels positive for the different intensity scores of CML in the fat tissue in AF patients and controls. Data are medians and interquartile ranges. $n = 33$ (A, AF patients); $n = 9$ (A, controls); $n = 6$ (B, DM+); $n = 27$ (B, DM-). * $P < 0.05$, ** $P < 0.01$, Mann-Whitney U -test; † $P < 0.05$, †† $P < 0.001$, Wilcoxon test.

endocardium, myocardium, and fat tissue of each LA tissue section, using a Leica DM/RB microscope equipped with a polarization filter. ImageJ software version 1.63 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) was used to calculate the percentage of fibrosis in each image. The mean percentage of all six images was taken as the data value of an individual patient.

Statistical Analysis

Statistics were performed using the SPSS statistics program (Windows version 15.0: IBM, Armonk, NY). Data were analyzed using the Mann-Whitney U -test, Wilcoxon signed rank test, McNemar test, and Chi-square analysis. $P < 0.05$ was considered significant. Correlation analysis was performed using the Spearman correlation coefficient, with the following guidelines for determining the correlation strength: $r \pm 0.10$ to 0.29 indicates absence of correlation, $r \pm 0.30$ to 0.49 indicates a medium correlation, and $r \pm 0.50$ to 1.0 indicates a strong correlation. When using correlations, it is suggested that statistical significance should be reported, but ignored, and the focus should

be directed at the amount of shared variance.¹⁴ Therefore, we also calculated the coefficient of determination (CoD).¹⁶

Results

CML Deposition in Blood Vessels

In our initial analysis, we found a difference in the amount of CML deposition between the atrial myocardium and atrial fat tissue. Therefore, we analyzed the amount of CML deposition in these tissues separately. CML deposition was predominantly found in the blood vessels and was significantly increased in the fat tissue (Figure 1A) and the myocardium of AF patients compared to controls (fat tissue, $P = 0.005$; and myocardium, $P = 0.006$) (Figure 2A). In both controls and AF patients, CML levels in the fat tissue were significantly increased compared with the myocardium (controls, $P = 0.025$; and AF, $P < 0.001$) (Figure 2A). Moreover in AF patients, a strong positive correlation was found between CML deposition in the fat tissue and in the myocardium ($r = 0.585$, $P < 0.001$, CoD = 34.2%). As a control, we used autopsy material from patients without any clinically identifiable heart disease. Although these patients did have age-related atherosclerosis of the coronary arteries, no related heart disease was detected.

In both the myocardium and fat tissue, no significant differences were found between the amount of CML in AF patients with and without DM (Figure 2B) and with age (data not shown).

To evaluate the distribution of different CML intensities in the blood vessels, the intensity scores were compared between controls and AF patients (Figure 2, C and D). The pattern of CML intensity scores found in AF patients and controls was similar in both the myocardium and fat tissue.

Figure 3 VCAM-1 expression in the left atrial (LA) appendage of atrial fibrillation (AF) patients. Mean number of VCAM-1-positive blood vessels per mm^2 in the myocardium (circles) and fat tissue (squares) in the LA of AF patients and controls (C). Data are medians and interquartile ranges. $n = 33$ (AF patients); $n = 9$ (controls). * $P < 0.05$, Mann-Whitney U -test; † $P < 0.05$, †† $P < 0.001$, Wilcoxon test.

Figure 4 Analysis of the number of inflammatory cells in the left atrial (LA) appendage in atrial fibrillation (AF) patients. Mean number of extravascular neutrophilic granulocytes (PMNs) (A), lymphocytes (B), and macrophages (C) per mm² in the myocardium (circles) and fat tissue (squares) in the LA of AF patients and controls (C). Data are medians and interquartile ranges. $n = 33$ (AF patients); $n = 9$ (controls). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Mann-Whitney U -test; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, Wilcoxon test.

Albeit, in AF patients, a larger number of blood vessels with intensity scores 1 and 2 were found in the myocardium, compared with controls, and was significant for score 2 ($P = 0.005$) (Figure 2C). In the fat tissue, an increase in the number of blood vessels with all three intensity scores was found in AF patients, compared with controls, in which scores 1 and 2 were significant (score 1, $P = 0.015$; and score 2, $P = 0.028$) (Figure 2D).

In addition, we did not find differences in the amount of CML deposition, VCAM-1 expression, and infiltrating inflammatory cells in both the myocardium and fat tissue and total amount of fibrosis, between AF patients treated with ($n = 14$) or without ($n = 19$) statins, between persistent AF ($n = 6$) and paroxysmal AF ($n = 27$) patients, and between AF patients based on their surgical indication (data not shown).

Endothelial Cell Activation

CML can induce a proinflammatory status of the blood vessels via up-regulation of adhesion molecules⁹; we therefore subsequently analyzed whether CML deposition coincided with VCAM-1 expression.

In AF patients, increased numbers of VCAM-1–positive blood vessels were found in both the myocardium and the fat tissue (Figure 1B) compared to controls (Figure 3), which was significant in the myocardium ($P = 0.028$). In AF and control patients, significantly more VCAM-1–positive blood vessels were found in the fat tissue compared to the myocardium (controls, $P = 0.018$; and AF, $P < 0.001$) (Figure 3). Moreover, a strong positive correlation was found between the number of VCAM-1–positive blood vessels in the fat tissue and the myocardium of AF patients ($r = 0.673$, $P < 0.001$, CoD = 45.3%).

Thrombi formation is often found in AF patients and is preceded by endothelial activation and/or damage.¹⁷ Therefore, we also analyzed whether thrombi, as identified by CD31

staining, were present in the endocardium and/or intramyocardial blood vessels in these patients. However, endocardial thrombi were found in only one AF patient.

Extravascular Inflammatory Cells

Infiltration of lymphocytes and macrophages in the myocardium of the atria has been shown in AF patients.^{18–20} However, to the best of our knowledge, this has not been studied yet in the fat tissue, where we found the highest proinflammatory status of the blood vessels. Therefore, infiltration of these inflammatory cells, including neutrophilic granulocytes, was studied both in the fat tissue and myocardium of AF and control patients.

In AF patients, a large, significant increase in extravascular neutrophilic granulocytes was found in the fat tissue (Figure 1C) compared with controls ($P = 0.001$) (Figure 4A). However, in the myocardium, the number of neutrophilic granulocytes was significantly lower compared to controls ($P = 0.003$) (Figure 4A). In controls, the number of neutrophilic granulocytes in the fat tissue was significantly lower compared with the myocardium ($P = 0.028$) (Figure 4A). By contrast, in AF patients, the number of neutrophilic granulocytes in the myocardium was significantly lower compared with the fat tissue ($P < 0.001$) (Figure 4A). Furthermore, in AF patients, a strong positive correlation was found between the number of neutrophilic granulocytes in the fat tissue and the myocardium ($r = 0.502$, $P = 0.003$, CoD = 25.2%).

The most abundant increase of inflammatory cells in the fat tissue of AF patients was found for lymphocytes (Figure 1D), which did focally aggregate and were increased significantly compared to controls ($P < 0.001$) (Figure 4B). Also, significantly more lymphocytes were found in the fat tissue of AF patients compared to the myocardium ($P < 0.001$) (Figure 4B). Moreover, a medium positive correlation was found between the number of lymphocytes in the fat tissue and the myocardium of AF patients ($r = 0.330$, $P = 0.061$, CoD = 10.9%).

Figure 5 **A** and **B**: Analysis of fibrosis. Picrosirius Red staining of the left atrial (LA) appendage of a control patient (**A**) and atrial fibrillation (AF) patient (**B**). **C**: Mean percentage of fibrosis in the LA of AF patients and controls. **Arrows** indicate fibrosis. Data are medians and interquartile ranges. $n = 33$ (C, AF patients); $n = 9$ (C, controls). Original magnification: $\times 250$. F, fat tissue; M, myocardium.

In AF patients, no increase in the number of macrophages was found in the fat tissue (Figure 1E) compared to controls ($P = 0.915$) (Figure 4C). By contrast, in the myocardium of AF patients, a small, significant decrease in the number of macrophages was found compared to controls ($P = 0.008$) (Figure 4C). In AF patients, the number of macrophages was significantly higher in the fat tissue compared to the myocardium ($P < 0.001$) (Figure 4C). In addition, a strong positive correlation was found between the number of macrophages in the fat tissue and the myocardium in AF patients ($r = 0.748$, $P < 0.001$, CoD = 55.9%). Furthermore, medium positive correlations were found between CML deposition and the number of extravascular macrophages in both the fat tissue and myocardium in AF patients (fat tissue, $r = 0.400$, $P = 0.021$, CoD = 16.0%; and myocardium, $r = 0.512$, $P = 0.002$, CoD = 26.2%).

Finally, there seems to be a disconnect between the presence of CML and the extravascular inflammatory cells in the myocardium of AF patients. We, however, do not have an explanation for this finding.

Fibrosis

AF has been associated with increased fibrosis; therefore, we also analyzed whether the increased presence of AGEs coincided with increased fibrosis. Most fibrosis was found in the epicardium and endocardium of the LA; it was also found within the myocardium and fat tissue in equal amounts in both control (Figure 5A) and AF (Figure 5B) patients. No significant difference ($P = 0.154$) in the amount of fibrosis was found in the LA of AF patients compared to controls (Figure 5C). Interestingly, a medium positive correlation was found between total CML presence (both fat tissue and myocardium CML scores taken together) and total amount of fibrosis in both AF patients ($r = 0.340$, $P = 0.053$, CoD = 11.6%) and controls ($r = 0.450$, $P = 0.224$, CoD = 20.0%).

LV Function and LA Size

Finally, we have compared LV function with LA size in AF patients only. For this, we gave patients with a normal LV

function a score of 0 ($n = 27$), and with a reduced LV function, a score of 1 ($n = 6$). The same was done for LA size, the patients with a normal LA size ($n = 10$) were given a score of 0, whereas patients with a dilated LA ($n = 23$) were given a score of 1. After comparison, significantly more AF patients had dilated LA in comparison to decreased LV function ($P < 0.001$). Albeit, five of six patients with reduced LV function also had a dilated LA. In addition, we analyzed whether differences existed in the presence of CML, amount of fibrosis, number of VCAM-1–positive blood vessels, and number of inflammatory cells between patients with decreased LV function and normal LV function, and between patients with LA dilation and normal LA size. Patients with LA dilation were found to have significantly higher numbers of neutrophilic granulocytes in the myocardium compared to patients with normal LA size ($P = 0.003$). Furthermore, in patients with reduced LV function, significantly more fibrosis was found compared to patients with normal LV function ($P = 0.036$). All other parameters did not significantly differ between patients with decreased LV function and normal LV function or between patients with LA dilation and normal LA size.

Discussion

Atrial inflammation plays an important role in the induction and progression of AF. Accumulation of AGEs in the ventricular microvasculature of the heart has been shown to be involved in the inflammatory processes underlying cardiac disease.^{8,21,22} However, whether AGEs are involved in atrial inflammation underlying AF is unknown. We found significantly increased endothelial deposition of CML in the LA of AF patients, especially in the fat tissue and the myocardium, which coincided with significantly increased endothelial expression of VCAM-1 both in the fat tissue and myocardium. We also report a significant increase of neutrophilic granulocytes, lymphocytes, and macrophages in the fat tissue. Also, we observed that the number of macrophages correlated positively with CML deposition in blood vessels of the LA in AF patients.

Because AGEs facilitate endothelial dysfunction, impaired contraction of the heart, and vascular and myocardial stiffness,

they play an important role in cardiovascular dysfunction.⁹ A recent study showed increased plasma levels of AGEs in AF patients, independently of DM.¹¹ We have now shown significantly increased CML in the endothelium in both fat tissue and the myocardium in the LA of AF patients, compared to controls, distributed among more blood vessels, suggesting a role for AGEs in atrial inflammation underlying AF. It is known that AGEs accumulate in DM and aging in the heart.^{21,23} However, we found no difference between patients with or without DM, suggesting that DM is not a major contributor to the CML presence in our AF patient group. Moreover, no significant difference was observed between the mean age of controls and AF patients. In addition, no linear relation was found between age and formation of CML depositions, independently of AF, indicating that the increase in CML expression found in AF patients is not related to the patient's age.

CML can activate endothelial cells to up-regulate endothelial adhesion molecules such as VCAM-1.⁹ We now found significant increased expression of VCAM-1 in the blood vessels in the LA of AF patients, coinciding with CML expression. Yamashita et al²⁰ have also shown a nonsignificant increase of VCAM-1 expression in atrial tissue in AF patients, although they analyzed tissue homogenates, whereas we quantified VCAM-1 expression in blood vessels using immunohistochemical analysis. It is known that neutrophils, lymphocytes, and monocytes can bind to VCAM-1, via the integrin VLA-4 that is present on all these cells, to transmigrate to the extravascular tissues.²⁴ Therefore, the endothelial expression of VCAM-1 in the atria of AF patients creates a feasible state for the transmigration of these immune cells across the endothelium. Indeed, coinciding with VCAM-1 and CML depositions, we found increased infiltration of neutrophilic granulocytes, lymphocytes, and macrophages in the fat tissue, although not the myocardium of the LA, in AF patients.

A few studies have described inflammatory cells in the atria of AF patients, namely, lymphocytes in the right and left atria and also macrophages in the LA, both in the myocardium and endocardium.^{18–20,25} However, it is unclear whether the fat tissue, which in our study is where we found the majority of inflammatory cells, was also analyzed in these studies.

Importantly, in the present study, a strong and positive linear relation between the CML deposition in the fat tissue and the myocardium of the LA was found in AF patients. Remarkably, the increased CML deposition was significantly higher in the fat tissue compared to the myocardium, coinciding with a more extensive VCAM-1 expression and inflammatory cell infiltration, suggesting a more prominent role for the fat tissue. Indeed, strong CML accumulation has been shown in human fat tissue in obesity²⁶ and was found to result in increased inflammation in an obese mouse model.²⁶ More interestingly, the inflammatory activity of epicardial fat tissue, adjacent to the LA, atrioventricular groove, and left main artery, increased in AF patients.²⁷ Proinflammatory markers, such as IL-6, IL-1 β , tumor necrosis factor- α , and

MCP-1, are secreted by human epicardial fat tissue in patients with coronary artery disease.²⁸ Therefore, these proinflammatory properties of epicardial fat tissue and the subsequent release of proinflammatory cytokines could explain the infiltration of immune cells in the fat tissue in the atria.

Recently, it has also been shown that the secretome from human epicardial fat tissue of patients undergoing routine cardiac bypass surgery is able to induce fibrosis through the release of the adipo-fibrokinase activin A in an organo culture of rat atria.²⁹ Furthermore, it has been shown that myeloperoxidase, which is abundantly expressed by neutrophilic granulocytes, can act as a profibrotic mediator of AF.³⁰ Although we did find increased inflammation, including increased numbers of neutrophilic granulocytes in the fat tissue of AF patients, we did not find an increase in fibrosis in these patients compared to controls. It is possible that the coronary artery disease in the control patients might have affected the amount of fibrosis in these patients. In addition, only the LA appendage was analyzed; more changes might be present in other parts of the LA. Importantly, AF patients with decreased LV function did have a significantly higher amount of fibrosis compared to AF patients with normal LV function, suggesting that failure of the LV is related to the fibrosis in the LA in these patients. Interestingly, we also observed a positive correlation between the presence of CML and fibrosis in the LA of AF patients and controls, suggesting that the presence of CML is in part associated with fibrosis. In the present study, 82% (27 of 33 patients) of the AF patients had paroxysmal AF, and our results of fibrosis were comparable with the study of Geuzebroek et al,¹³ who analyzed fibrosis in lone AF [consisting mostly of paroxysmal AF patients (72%)] and AF patients with mitral valve disease [consisting mostly of more chronic forms of AF, especially permanent AF (46%)]. They found increased fibrosis in the AF patients with mitral valve disease and no increase in the lone AF group, compared to controls. These findings suggest that fibrosis may be more related to the chronic forms of AF, albeit fibrosis might well be a consequence of risk factors, such as mitral valve disease, heart failure, and hypertension.

Statins have an anti-inflammatory effect, but have been described in AF patients with mixed results.^{31,32} In the present study, no differences were found in the numbers of inflammatory cells nor the amount of VCAM-1—positive blood vessels between statin-treated and nontreated patients. This suggests that statin treatment, at least in our study, did not have an inhibiting effect on inflammation in the atria of AF patients. It has also been shown that statins are able to reduce serum levels of AGEs in type 2 DM patients,³³ although in the present study, statins did not reduce the formation of CML depositions in the atrial tissue of AF patients.

A prothrombotic or hypercoagulable state is typical in AF and is thought to be caused by blood flow abnormalities, disturbances in the vessel wall, and abnormal blood constituents.³⁴ Thrombi, then, are often found in the LA appendage in AF patients, wherein endothelial activation and/or damage

plays an important role.¹⁷ In the present study, thrombi were found in the LA in only one patient. It has to be noted that all AF patients used anticoagulant agents, which may explain the low amount of thrombus formation.

Some limitations need to be discussed. Although most control patients also had cardiac comorbidities, namely coronary artery disease, this study does not prove that a causative relationship exists between AF and the presence of AGEs. It can be questioned whether surgery of AF patients caused and/or contributed to the inflammation and presence of AGEs. However, it is important to realize that the formation of AGEs under pathological conditions, as well as the formation of the inflammatory infiltrate, takes hours up to days. In our study, the LA appendages were excised within 5 minutes after the start of the surgical procedure, making it very unlikely that the inflammatory reaction and presence of AGEs as found in these patients are related to the surgical procedure.

In conclusion, our data show that presence of CML deposition coincides with AF and increased inflammation, suggesting, but not proving, an important role of CML in the process of AF, independent of DM and age.

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