

## B Lymphocytes and Macrophages in the Perivascular Adipose Tissue Are Associated With Coronary Atherosclerosis: An Autopsy Study

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**Background**—Macrophages and T lymphocytes in the perivascular adipose tissue (PvAT) were previously linked to coronary artery disease. However, the role of these cells and B lymphocytes in the human PvAT adjacent to unstable atherosclerotic plaques has not been investigated. Moreover, previous studies were inconclusive on whether PvAT inflammation was restricted to the surroundings of the atheroma plaque.

**Methods and Results**—Coronary arteries were freshly dissected with the surrounding PvAT. Atherosclerotic plaques were classified according to the internationally accepted anatomopathological criteria. Immune cells in the PvAT were detected using immunohistochemistry and then quantified. We used linear and logistic regressions with robust standard errors, adjusted for possible confounding factors. In 246 atherosclerotic plaques (205 stable and 41 unstable plaques) from 82 participants (mean age=69.0±14.4 years; 50% men), the percentage of arterial obstruction was positively correlated with the densities of CD68<sup>+</sup> macrophages ( $P=0.003$ ) and CD20<sup>+</sup> B lymphocytes ( $P=0.03$ ) in the periplateau PvAT. The number of cells was greater in the periplateau PvAT than in the distal PvAT (macrophages,  $P<0.001$ ; B lymphocytes,  $P=0.04$ ). In addition, the density of macrophages in the periplateau PvAT was greater in the presence of unstable plaques ( $P=0.03$ ) and was also greater near unstable plaques than in the distal PvAT ( $P=0.001$ ). CD3<sup>+</sup> T lymphocytes were not associated with percentage of obstruction and stable/unstable plaque composition.

**Conclusions**—The density of CD20<sup>+</sup> B lymphocytes and CD68<sup>+</sup> macrophages in periplateau PvAT was increased with plaque size, and the CD68<sup>+</sup> macrophages were greater near unstable atherosclerotic plaques than near stable lesions. This inflammation was more intense in the periplateau PvAT than in the PvAT distal to the atherosclerotic plaques. (*J Am Heart Assoc.* 2019;8:e013793. DOI: 10.1161/JAHA.119.013793.)

**Key Words:** atherosclerosis • B cell • coronary artery disease • inflammation • macrophages • pericoronary adipose tissue

Although the frequency of coronary heart disease has declined recently, it remains the leading cause of mortality<sup>1</sup> and disability worldwide.<sup>2</sup> The main cause of coronary heart disease is atherosclerosis.<sup>3</sup> The immune system has a central role in the development of coronary artery disease (CAD).<sup>4</sup> Therefore, some clinical trials have investigated the role of anti-inflammatory agents to treat patients with CAD. In JUPITER (Justification for the Use of Statins in Prevention: an

Intervention Trial Evaluating Rosuvastatin), statin therapy was highly effective in decreasing major adverse cardiovascular events in individuals with low-density lipoproteins and high levels of high-sensitivity CRP (C-reactive protein), suggesting an anti-inflammatory action of rosuvastatin.<sup>5</sup> In CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study), therapy with monoclonal antibody target at interleukin-1 $\beta$  (canakinumab), which is involved in the interleukin-6 signaling,

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Accompanying Tables S1 through S5 and Figures S1 through S5 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.013793>

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## Clinical Perspective

### What Is New?

- Our study demonstrated that inflammation mediated by CD68<sup>+</sup> macrophages and CD20<sup>+</sup> B lymphocytes in the perivascular adipose tissue (PvAT) adjacent to the most important atherosclerotic plaque increased with the percentage of arterial obstruction in the coronary arteries.
- We are the first to demonstrate that the density of C68<sup>+</sup> macrophages in the PvAT adjacent to unstable plaques was greater than in the PvAT adjacent to stable plaques.
- Inflammation was more intense in the PvAT adjacent to stenotic and acute lesions than in the adipose tissue distal of lesions and without atherosclerosis.

### What Are the Clinical Implications?

- Basic research using human samples of PvAT adjacent to coronary arteries with and without atherosclerosis can expand the findings from animal models on the associations between specific immune cells and atherosclerotic plaque size and composition.
- These studies are important to better determine potential targets for the development of new drugs in the different stages of the atherosclerotic process.

significantly reduced major adverse cardiovascular events independent of lipid levels. However, patients who used canakinumab had more deaths secondary to infections, especially in older adults and patients with diabetes mellitus.<sup>6</sup>

In addition, the perivascular adipose tissue (PvAT) has been reported as a potential trigger for atherosclerosis, independent of traditional approaches, where CAD initiates from endothelial cells and the intima layer.<sup>7</sup> In experimental studies, PvAT transplanted in low-density lipoprotein-deficient knockout mice promoted endothelial dysfunction and atherosclerosis in aortic and thoracic aorta.<sup>8</sup> PvAT also showed increased MCP-1 (monocyte chemoattractant protein-1) levels after transplantation of perivascular visceral adipose tissue adjacent to carotid arteries, causing large lipid-rich atherosclerotic lesions, high macrophage density, and fibrin deposits compared with mice transplanted with subcutaneous adipose tissue.<sup>9</sup> However, extrapolation of these results for humans is difficult because of differences between human and animal models, including characteristics of atherosclerotic plaque.<sup>10</sup> Translational studies from experimental to human have reported that inflammation in the epicardial adipose tissue (EAT) and in the PvAT was associated with CAD evaluated by biopsy,<sup>11–13</sup> autopsy,<sup>14–17</sup> and cardiac imaging.<sup>14,18,19</sup> Most studies investigated the role of CD68<sup>+</sup> macrophages in the EAT<sup>11–13</sup> and PvAT<sup>15–17</sup> on stable CAD. The number of CD68<sup>+</sup> macrophages in the PvAT was also correlated with the number of immune cells in the atherosclerotic plaques.<sup>15–17</sup>

CD3<sup>+</sup> T lymphocytes in the PvAT were identified only in studies using biopsy samples.<sup>11</sup> The subtype CD8<sup>+</sup> T lymphocytes in the EAT were more frequent in patients with CAD than in controls, whereas the density of the CD4<sup>+</sup> T lymphocytes present in the EAT was similar between CAD and control groups.<sup>12</sup> The role of B lymphocytes in the PvAT adjacent to the atherosclerotic plaques remains unknown.<sup>20</sup> CD20<sup>+</sup> B lymphocytes secreted cytokines, which increased the vulnerability of the atherosclerotic plaques by the recruitment of macrophages and increased the necrotic core in advanced lesions.<sup>21</sup> Moreover, CD20<sup>+</sup> B lymphocytes produced and secreted antibodies against antigens in the atherosclerosis plaque, such as  $\beta$ -2 glycoprotein I, heat shock proteins (HSP),<sup>22</sup> and self-modified antigens.<sup>23</sup> The CD20<sup>+</sup> B lymphocytes can also present antigens to T lymphocytes, which secrete cytokines and promote a proinflammatory response.<sup>24</sup> Indeed, the association of these immune cells with unstable atherosclerotic plaques has not been investigated. In addition, it is unknown whether these immune cells are restricted to the region surrounding the atherosclerotic plaques. A greater inflammation density was detected around culprit lesions compared with nonculprit lesions by imaging methods,<sup>14,18</sup> whereas previous autopsy studies were inconclusive.<sup>16,17</sup> Autopsy studies investigated only macrophages in the PvAT.<sup>15–17</sup> Although biopsy studies explored other types of immune cells,<sup>11,13</sup> samples obtained during cardiac surgery could initiate an inflammatory response, with an increase in monocytes and heat shock proteins, mainly with the use of extracorporeal circulation.<sup>25</sup> Moreover, no studies to date have accounted for confounders in statistical analyses.<sup>12–17</sup> Therefore, we investigated the association of the density of CD68<sup>+</sup> macrophages, CD3<sup>+</sup> T lymphocytes, and CD20<sup>+</sup> B lymphocytes in the periplaque PvAT with plaque composition and with the percentage of arterial obstruction. In addition, we compared the density of these immune cells in the periplaque PvAT and in the distal PvAT without atherosclerosis. We hypothesized that the density of CD68<sup>+</sup> macrophages, CD3<sup>+</sup> T lymphocytes, and CD20<sup>+</sup> B lymphocytes in the PvAT adjacent to coronary artery lesions would be greater in the presence of larger arterial obstruction and that the density of these immune cells would be greater in PvAT adjacent to coronary arteries with unstable lesions in comparison with stable lesions. In addition, we supposed that the inflammation in periplaque PvAT would be greater than in distal PvAT.

## Methods

This study was approved by the local ethics committee, and the next of kin (NOK) signed the informed consent before the sample collection. The data that support the findings of this study are available from the corresponding author on request.

## Subjects

This cross-sectional study included cadavers of subjects who had died from nontraumatic causes.<sup>26</sup> The cadavers underwent full-body autopsy at the São Paulo Autopsy Service from 2013 to 2015. Inclusion criteria were as follows: aged  $\geq 30$  years; postmortem interval  $< 24$  hours; and a NOK with at least weekly interaction with the deceased in the 6 months before death. We excluded participants with cardiovascular conditions that could induce inflammation (ie, presence of a stent, chronic or acute pericarditis, pericardial effusion, myocarditis, endocarditis, Chagas disease, hemopericardium, cardiac tamponade, and previous cardiac surgery, including transplant). We also excluded participants with signs of systemic inflammation: autoimmune diseases, use of corticosteroids, use of immunosuppressive drugs, treatment with chemotherapy or/and radiotherapy, and diagnosis of sepsis before death. Moreover, we evaluated the presence of sepsis signs in the postmortem examination. Although the sepsis diagnosis is clinical, previous studies showed that it is possible to infer its presence by postmortem anatomopathological examination.<sup>27,28</sup> A pathologist (L.F.F.S.) examined slides stained with hematoxylin-eosin from participants' lung, heart, liver, kidney, and spleen. The presence of at least 2 organs with signs of sepsis combined with focus of infection was necessary for the diagnosis of sepsis.<sup>28</sup> For example, we excluded participants with the following: (1) neutrophil infiltration in the lung associated with pleuritis caused by bronchopneumonia (Figure S1A and S1B); (2) acute tubular necrosis in the kidney secondary to shock (Figure S1C); (3) acute splenitis (Figure S1D); and (4) bronchopneumonia with hepatic congestion and steatosis (Figure S1E). We also excluded subjects whose NOKs were unable to provide clinical information.

## Collection of Coronary Arteries and PvAT

Hearts were collected and washed in running water. Agar was injected into the ostia of the coronary arteries to avoid the flattening of the coronary arteries. The right coronary, left main coronary, left anterior descending, and circumflex arteries were dissected with 10 mm of PvAT around the coronary artery trajectories. Afterwards, the PvAT was removed and identified at regular intervals of 15 mm. The coronary arteries were fixed in 4% buffered paraformaldehyde for at least 5 days. The PvAT was fixed for 24 hours.<sup>26</sup> Samples of coronary arteries and PvAT were immersed in paraffin.

## Assessment of Coronary Artery Atherosclerosis

Coronary arteries were cut at 15-mm intervals to match the samples of PvAT. These segments were divided into sections

of 5 mm each to evaluate the atherosclerotic plaques. The sections with the largest arterial obstruction or those suspected of having unstable atherosclerotic plaques were sampled from each coronary artery (periplaque PvAT).<sup>29,30</sup> To define the main lesion, sampling of unstable plaques was preferred over stable ones. Coronary artery sections without macroscopic lesions and at least 15 mm away from any atherosclerotic plaques also represented a control area (distal PvAT).

Afterwards, these samples were stained with hematoxylin-eosin, Verhoeff, and Masson trichrome. The slides were photographed with the aid of a stereomicroscope (SMZ 1000; Nikon, Tokyo, Japan). We measured the areas delimited by the lumen and by the internal elastic lamina using the software ImageJ;<sup>31</sup> and we calculated the percentage of arterial obstruction by dividing the difference of the internal elastic lamina area and lumen area by the internal elastic lamina area, and then multiplying the result by 100.<sup>32</sup> We classified the atherosclerotic lesions according to the American Heart Association criteria.<sup>29</sup> Stable lesions were defined by the presence of fibroatheroma, calcification, lipid core, or whole fibrous cap, whereas the plaque was considered unstable in the presence of ulceration, intraplaque hemorrhage, or total/partial thrombosis (Figures S2 and S3).<sup>29</sup>

## Assessment of PvAT Inflammation

We identified macrophages (Monoclonal Mouse Anti-Human CD68, clone KP1, 1ML Dako-M081401, 1:5000  $\mu$ L), T lymphocytes (Polyclonal Rabbit Anti-Human CD3, 1ML Dako-M045201, 1:1500  $\mu$ L), and B lymphocytes (Monoclonal Mouse Anti-Human CD20cy, clone L26, 1ML Dako-M075501, 1:12000  $\mu$ L) in silanized slides of periplaque and distal PvAT. The slides were scanned with a scanner (Pannoramic Scan 250 Flash III; 3DHISTECH, Hungary) and analyzed with a virtual microscope (Pannoramic Viewer software; 3DHISTECH). The cells were counted into 20 random fields of 600- $\mu$ m diameter.

## Clinical Data

We collected information on sociodemographic variables (age, sex, race, and education). The deceased's medical history was determined using a semistructured interview applied to the NOK; and the medical history includes information on hypertension, diabetes mellitus, CAD, heart failure, dyslipidemia, stroke, physical activity (defined by the performance of domestic, work, or formal physical activities at least 3 times per week), current alcohol use, and current smoking.<sup>33</sup> Body mass index was obtained by direct measurements of weight and height, with the deceased in the supine position without clothes or shoes.

## Statistical Analysis

We estimated a sample size of 78 subjects, considering an effect size of 0.93 for the mean difference in immune cells between CAD and the control group,<sup>14</sup> with power of 90% and  $\alpha$  of 5%.

The dependent variables were the percentage of arterial obstruction (continuous variable) and plaque composition (binary variable). The independent variable was the density of the CD68<sup>+</sup> macrophages, CD3<sup>+</sup> T lymphocytes, and CD20<sup>+</sup> B lymphocytes (continuous variables). The associations of cell density in the periplaque PvAT with the percentage of arterial obstruction were tested using linear regression with robust SEs to account for repeated measures in the same individual (3 arterial segments per person) using the Huber-White sandwich estimators.<sup>34</sup> The associations of cell density in the periplaque PvAT with plaque composition groups were tested using logistic regression with robust SEs through cluster adjustment, where the model tested the odds to unstable over stable plaques. In addition, we compared the cells in the PvAT around stable plaques and in the PvAT around unstable lesions in the same individual using Wilcoxon matched pairs rank test.

We also calculated the differences between the density of immune cells in the periplaque PvAT and the density of these cells in the distal PvAT. We then tested the association of these differences with the percentage of arterial obstruction and plaque composition using linear and logistic regressions with robust SEs, respectively. All the models were adjusted for potential confounders (age,<sup>35–39</sup> sex<sup>40,41</sup>, hypertension,<sup>42–44</sup> diabetes mellitus,<sup>42,45</sup> body mass index,<sup>42,46,47</sup> smoking,<sup>42,48</sup> physical inactivity,<sup>49,50</sup> and alcohol use). The significance level was 5%. The statistical software used was Stata 13.0 (Stata Corp, TX).

## Results

We included 82 participants (mean age=69.0±14.4 years; 50% men; 58% white; and 67% had <4 years of education). Hypertension (74%) and diabetes mellitus (37%) were most frequent risk factors in our sample. Among participants with hypertension, 55% used antihypertensive drugs; and among those with diabetes mellitus, 29% used hypoglycemic drugs and 8% used insulin (Table 1). Most NOK (85.3%) had daily interaction with the deceased. The most advanced lesions in each coronary artery were represented, which resulted in 246 coronary artery samples. In 54 participants with only stable lesions, we found 162 lesions. In 28 individuals with both stable and unstable lesions, we found 41 unstable lesions and 43 stable lesions (Figure S4). The mean percentage of arterial obstruction of proximal lesions was 56.7±26.0%; and of the distal fragments, 17.3±10.3% (Table S1). We tested

**Table 1.** Characteristics of the Sample (n=82)

Variables	Value
Age, y	69.0 (14.4)
Women	41 (50)
White	48 (58)
Education, median (IQR), y	4 (1–6)
Hypertension	61 (74)
Diabetes mellitus	30 (36)
Coronary artery disease	10 (12)
Heart failure	19 (23)
Stroke	10 (12)
Dyslipidemia	14 (17)
Body mass index, kg/m <sup>2</sup>	24.4 (5.8)
Physical inactivity	56 (68)
Smoking	12 (15)
Alcohol use	24 (29)
Antihypertensive drugs	45 (55)
Hypoglycemic drugs	24 (29)
Insulin	7 (8)
Lipid-lowering drugs	6 (7)
Postmortem interval, h	15.6 (3.1)

Data are given as mean (SD) or number (percentage), unless otherwise indicated. IQR indicates interquartile range.

the correlation between the sociodemographic and clinical data with the density of immune cells and found that age and body mass index correlated with the density of CD68<sup>+</sup> macrophages (Table S2). In addition, physical inactivity correlated with CD3<sup>+</sup> T lymphocytes, and no variable correlated with CD20<sup>+</sup> B lymphocytes (Tables S3 and S4).

## CD68<sup>+</sup> Macrophages

The density of CD68<sup>+</sup> macrophages in the periplaque PvAT was positively correlated to the percentage of coronary artery obstruction in the multivariable analysis ( $\beta=2.06$ ; 95% CI=0.71–3.41;  $P=0.003$ ) (Table 2, Figure 1, and Figure 2A). The difference between the density of CD68<sup>+</sup> macrophages in the periplaque PvAT and in the distal PvAT was related to the percentage of coronary artery obstruction ( $\beta=2.85$ ; 95% CI=1.32–4.39;  $P<0.001$ ) (Table 2, Figure 2C). In addition, the density of CD68<sup>+</sup> macrophages in the periplaque PvAT was greater near unstable plaques than near stable plaques (odds ratio=1.15; 95% CI=1.01–1.31;  $P=0.03$ ) (Table 2, Figure 2B). This finding persisted when we compared the stable and unstable lesions in the same individual ( $P=0.008$ ) (Table S5). Macrophages were more frequent in the periplaque PvAT than

**Table 2.** Association Between Immune Cells and Coronary Artery Atherosclerosis (n=246 Artery Segments)

Immune Cell Density	Univariate Model			Adjusted Model*		
<b>CD68<sup>+</sup> macrophages</b>						
% of obstruction	$\beta$	95% CI	<i>P</i> Value	$\beta$	95% CI	<i>P</i> Value
PvATp ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	2.19	0.88 to 3.51	0.001	2.06	0.71 to 3.41	0.003
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	2.30	0.57 to 4.03	0.01	2.85	1.32 to 4.39	<0.001
Unstable plaque composition	OR	95% CI	<i>P</i> Value	OR	95% CI	<i>P</i> Value
PvAT ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	1.12	1.02 to 1.25	0.02	1.15	1.01 to 1.31	0.03
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	1.42	1.14 to 1.78	0.002	1.77	1.28 to 2.47	0.001
<b>CD3<sup>+</sup> T lymphocytes</b>						
% of obstruction	$\beta$	95% CI	<i>P</i> Value	$\beta$	95% CI	<i>P</i> Value
PvATp ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	3.12	−1.17 to 7.41	0.15	3.42	−0.85 to 7.69	0.11
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	1.78	−2.18 to 5.74	0.37	2.15	−1.83 to 6.13	0.28
Unstable plaque composition	OR	95% CI	<i>P</i> Value	OR	95% CI	<i>P</i> Value
PvATp ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	1.31	0.92 to 1.87	0.13	1.37	0.94 to 2.00	0.09
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	1.49	0.85 to 2.59	0.15	1.55	0.77 to 3.11	0.21
<b>CD20<sup>+</sup> B lymphocytes</b>						
% of obstruction	$\beta$	95% CI	<i>P</i> Value	$\beta$	95% CI	<i>P</i> Value
PvAT ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	40.10	7.02 to 73.19	0.01	36.38	1.83 to 70.93	0.03
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>†</sup>	37.66	3.13 to 72.18	0.03	39.49	1.89 to 77.09	0.04
Unstable plaque composition	OR	95% CI	<i>P</i> Value	OR	95% CI	<i>P</i> Value
PvAT ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	26.91	1.38 to 523.65	0.03	23.44	0.67 to 812.92	0.08
Difference: PvATp and PvATd ( $10^{-5}$ cells/ $\mu\text{m}^2$ ) <sup>‡</sup>	13.39	0.54 to 332.03	0.11	8.56	0.16 to 447.22	0.28

OR indicates odds ratio; PvATd, distal perivascular adipose tissue; PvATp, periplaque perivascular adipose tissue.

\*Adjusted for age, hypertension, diabetes mellitus, body mass index, smoking, alcohol use, and physical inactivity.

<sup>†</sup>Linear regression with robust SEs to account for repeated measures in the same individual through cluster adjustment.

<sup>‡</sup>Logistic regression with robust SEs to account for repeated measures in the same individual through cluster adjustment.

in the distal PvAT in the presence of unstable plaques (odds ratio=1.77; 95% CI=1.28–2.47; *P*=0.001) (Table 2, Figure 2D).

### CD3<sup>+</sup> T Lymphocytes

The density of CD3<sup>+</sup> T lymphocytes in the periplaque PvAT was not associated with percentage of obstruction, nor with plaque composition. Moreover, there was no difference in the cell density between the periplaque PvAT and the distal PvAT (Table 2, Figure 1, Table S5, and Figure S5).

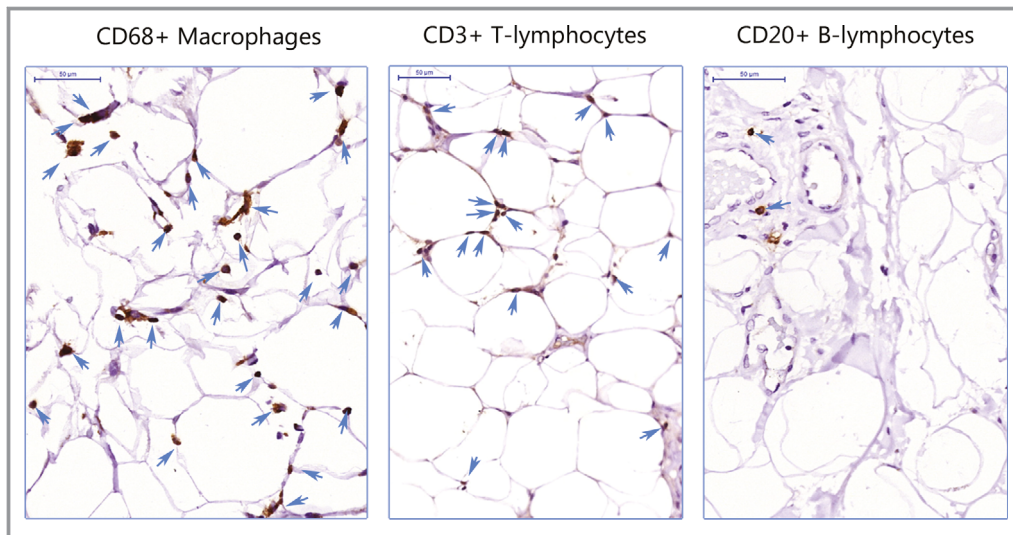
### CD20<sup>+</sup> B Lymphocytes

The percentage of arterial obstruction increased with the density of CD20<sup>+</sup> B lymphocytes in the periplaque PvAT ( $\beta$ =36.38; 95% CI=1.83–70.93; *P*=0.03), and the density of B cells in the periplaque PvAT was greater compared with distal PvAT ( $\beta$ =39.49; 95% CI=1.89–77.09; *P*=0.04) (Table 2, Figures 1 and 3A). We did not find differences in the B-lymphocyte densities in the periplaque PvAT around unstable

plaques compared with the PvAT around stable plaques (odds ratio=23.44; 95% CI=0.67–812.92; *P*=0.08) (Table 2, Figure 3B). The B-lymphocyte densities in the PvAT were similar around stable and unstable lesions in the same individual (*P*=0.58) (Table S5). In addition, the B-lymphocyte densities near unstable lesions were similar in the periplaque and distal PvAT (odds ratio=8.56; 95% CI=0.16–447.22, *P*=0.28) (Table 2, Figure 1, Figure 3A, 3C and 3D).

### Discussion

We found that the densities of CD68<sup>+</sup> macrophages and CD20<sup>+</sup> B lymphocytes in the periplaque PvAT were greater in the presence of larger luminal obstruction by atherosclerotic plaques. In addition, the density of CD68<sup>+</sup> macrophages was greater around unstable lesions than around stable lesions. These findings were more prominent in the PvAT surrounding the most critical atherosclerotic lesions compared with the PvAT near artery regions without macroscopic lesions and distant from the culprit lesions.



**Figure 1.** CD68<sup>+</sup> macrophages, CD3<sup>+</sup> T lymphocytes, and CD20<sup>+</sup> B lymphocytes. Immune cells stained in brown and marked with a blue arrow infiltrated into the perivascular adipose tissue (bar=50 µm).

### CD68<sup>+</sup> Macrophages and CAD

The positive correlation between the density of CD68<sup>+</sup> macrophages and the size of the atherosclerotic plaques was previously described.<sup>16</sup> Infiltrations of CD68<sup>+</sup> macrophages in the PvAT were similar between patients with dilated cardiomyopathy and patients with CAD.<sup>17</sup> Previous studies demonstrated an association of chronic CAD with CD68<sup>+</sup> macrophages and polarized macrophages.<sup>12,13,15,16</sup> However, these studies used EAT samples obtained during surgery, where the collection of the sample adjacent to the most important lesion may be difficult.<sup>12,13</sup> This is important because the EAT transcriptomic signature varied according to the anatomical region of the PvAT sampling.<sup>51</sup> In autopsy studies,<sup>15,16</sup> CD68<sup>+</sup> macrophages were associated with stable plaque composition.<sup>15</sup> However, we were the first to show in humans that CD68<sup>+</sup> macrophage density in the PvAT adjacent to unstable coronary lesions was greater than near stable lesions. The association between unstable lesions and macrophages was only investigated in samples of carotid arteries and aorta, where the number of polarized macrophages inside the arterial wall was greater in unstable plaques.<sup>52</sup> This finding suggested that macrophages may be involved with the rupture of the fibrous cap and the progression of disease severity.<sup>52</sup>

In addition, the association between the density of CD68<sup>+</sup> macrophages with plaque size and unstable atherosclerotic plaques was greater in the periplaque PvAT than distal PvAT in the same individual, which emphasizes the potential intrinsic role of CD68<sup>+</sup> macrophages in CAD development. This result is in line with imaging studies, which found more inflammation around the culprit lesion than near nonculprit lesions.<sup>14,19</sup> In the only autopsy study that examined this association, no difference was found in the density of macrophages in

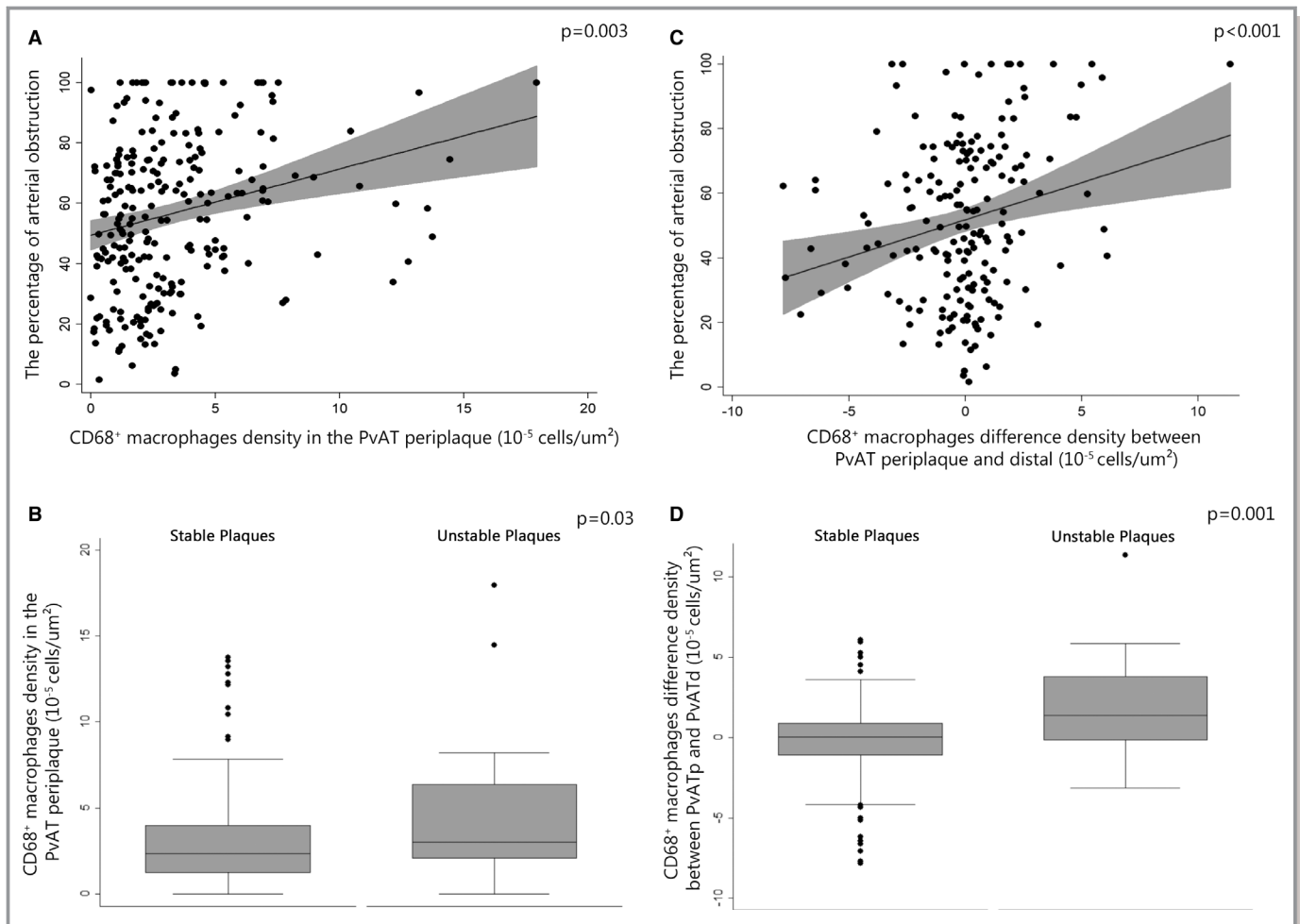
periplaque and distal pvAT,<sup>16</sup> probably because of differences in sample size and distal PvAT sampling. Our result that inflammation is not equally distributed in the EAT is expected because macrophages are cytokine-secreting phagocytic cells that recruit other cells to the site of inflammation.<sup>53</sup> Therefore, macrophages in the PvAT could have an effective role in the atherogenesis severity in humans.

### CD3<sup>+</sup> T Lymphocytes and CAD

We did not find any difference in the density of CD3<sup>+</sup> T lymphocytes, which are a general marker for T lymphocytes,<sup>54</sup> between stable and unstable plaque groups, or any correlation between the density of these immune cells and the percentage of arterial obstruction. However, our results cannot be compared directly with previous studies. Although a greater infiltrate of CD3<sup>+</sup> T lymphocytes, CD68<sup>+</sup> macrophages, and mast cells was found in the EAT, the assessment was qualitative and only in obese individuals.<sup>11</sup> Hirata et al found a greater number of CD8<sup>+</sup> T lymphocytes in PvAT in patients with CAD compared with a control group.<sup>12</sup>

### CD20<sup>+</sup> T Lymphocytes and CAD

The density of CD20<sup>+</sup> B lymphocytes in the PvAT was positively correlated with the percentage of arterial obstruction, but this density was similar between stable and unstable plaques. The relationship of these cells in PvAT adjacent to atherosclerotic plaques has not been yet investigated. Our results suggest that CD20<sup>+</sup> B lymphocytes could play a role in CAD progression.<sup>22</sup> On the other hand, macrophages and T cells seem to play an essential role since the early stages of atherosclerosis development.<sup>22</sup> We used the CD20 marker,



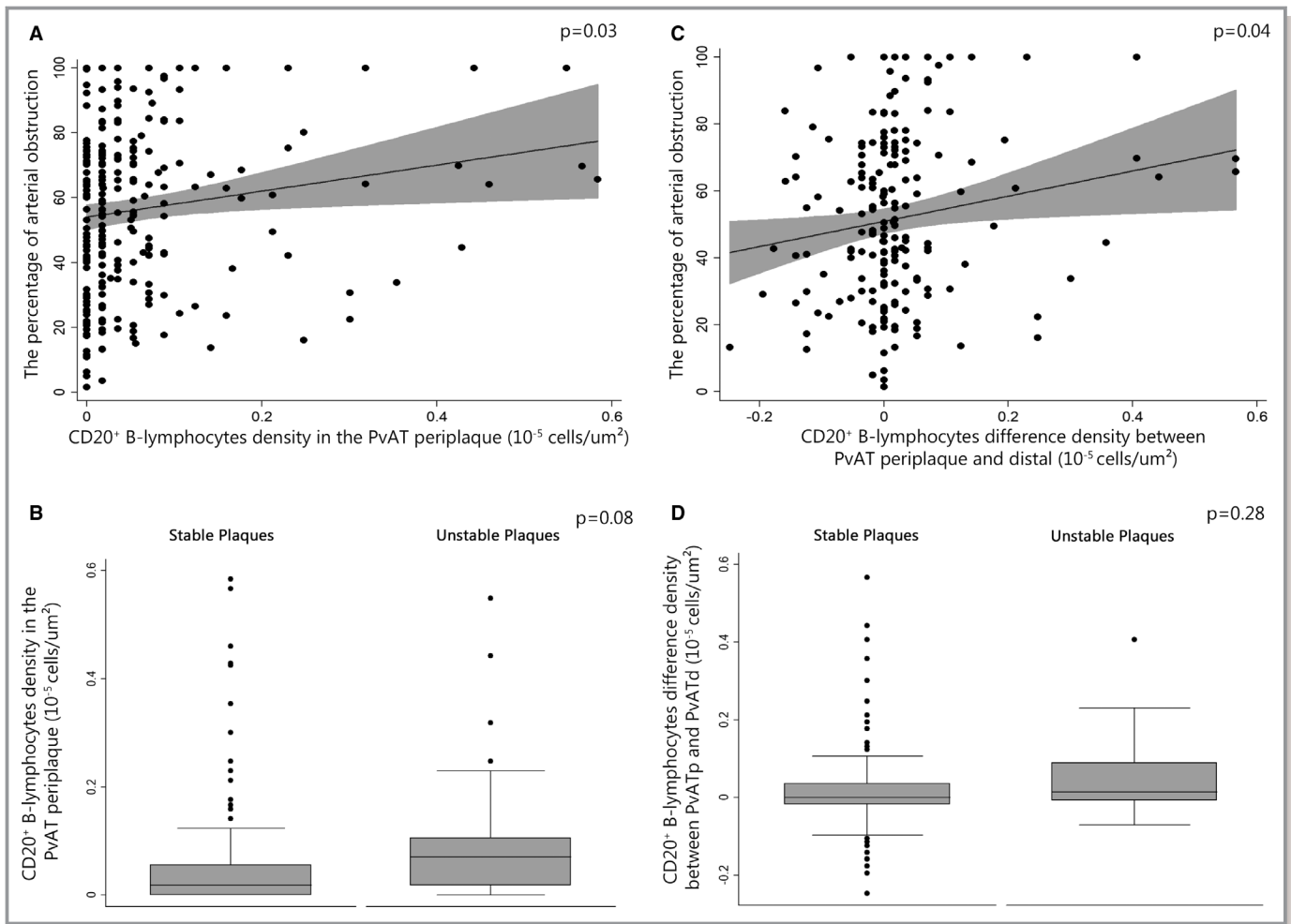
**Figure 2.** Association between CD68<sup>+</sup> macrophages and atherosclerosis. **A**, Scatterplot of the density of macrophages in periplaque perivascular adipose tissue (PvAT) with the percentage of arterial obstruction. **B**, Box plot of the density of macrophages in periplaque PvAT in stable and unstable plaque groups. **C**, Scatterplot of the difference of the macrophage density in periplaque PvAT and distal PvAT with the percentage of arterial obstruction. **D**, Box plot of the difference of macrophage density between periplaque PvAT and distal PvAT in stable and unstable plaque groups.

which was related to atherosclerosis progression in animal models.<sup>55,56</sup> Treatments with anti-CD20 attenuated the atherosclerosis expression.<sup>55,56</sup> The transfer of CD20<sup>+</sup> cells to lymphocyte-deficient mice increased the plaque size and macrophage accumulation in these lesions.<sup>56</sup> In addition, we did not find any difference in the density of B lymphocytes between stable and unstable atherosclerotic plaques. This finding may be caused by the short time interval between the acute coronary event and the individual's time of death. Activated B cells may not have had enough time to complete the process of recruitment, differentiation, and migration to unstable plaque site. In mice models, an increase in B-lymphocyte number in the heart muscle was only present after 5 days of myocardial infarct induction.<sup>57</sup> More studies should be conducted in experimental models to determine the response time of B-lymphocyte infiltration in the PvAT after acute coronary artery lesions.

### Can Inflammation in the Periplaque PvAT Promote Coronary Atherosclerosis?

Periplaque PvAT may promote atherosclerosis<sup>3</sup> through 2 possible mechanisms. First, the PvAT can communicate with the atherosclerotic plaque through the infiltration of adipocytes in the adventitial layer,<sup>58</sup> as we observed in our study. Second, the vasa vasorum that infiltrates the PvAT could bring the inflammatory cells from the PvAT to the coronary artery.

The question of whether the inflammation in PvAT is cause or consequence of atherosclerosis remains unknown. A recent experimental study using apolipoprotein E-deficient mice suggested that inflammation in the PvAT precedes atherosclerotic plaque formation.<sup>59</sup> Moreover, the infiltration of leukocyte in the PvAT was greater than in the vessel wall,<sup>59</sup> and the PvAT expresses genes associated with the production of complex lipids, which are also present in



**Figure 3.** Association between CD20<sup>+</sup> B lymphocytes and atherosclerosis. **A**, Scatterplot of the density of B lymphocytes in periplaque perivascular adipose tissue (PvAT) with the percentage of arterial obstruction. **B**, Box plot of the density of B lymphocytes in periplaque PvAT in stable and unstable plaque groups. **C**, Scatterplot of the difference of the B-lymphocyte density in periplaque PvAT and distal PvAT with the percentage of arterial obstruction. **D**, Box plot of the difference in density of B lymphocytes between periplaque PvAT and distal PvAT in stable and unstable plaque groups.

atherosclerosis.<sup>51,60</sup> Macrophages containing lipoproteins were found only in the periplaque PvAT, suggesting that these macrophages carried the lipoproteins from the PvAT to the artery wall.<sup>60</sup> Although these experimental lines of evidence point to the causality direction from the PvAT to coronary artery wall, a complex bidirectional communication of inflammatory cells between the arterial wall and the PvAT may be present.<sup>61</sup>

### Limitations and Strengths

Our study has several strengths. We investigated the association of B lymphocytes and T lymphocytes in human PvAT with coronary atherosclerosis using autopsy material. Basic research using human samples is scarce because of the decreased number of autopsies performed worldwide. In addition, we compared the density of these cells in unstable

plaques, and no previous study has performed analyses with adjustment for confounders. However, some limitations should be considered. First, it is possible that a systemic inflammatory process could be triggered by the autopsy procedure. However, the stability of the cell counts is unknown in autopsy studies. Nonetheless, we excluded subjects with sepsis diagnosis, as well as other cardiac or systemic inflammatory conditions. In addition, the post-mortem interval of all samples was <24 hours (mean postmortem interval=15.6 hours), which probably limited the inflammatory process after death.<sup>62</sup> Moreover, previous studies used samples obtained during surgery,<sup>11–13</sup> which probably triggers more intense inflammatory responses than the autopsy procedure, mainly when extracorporeal circulation is used.<sup>25,63</sup> Second, we did not measure serum levels of inflammation and could not adjust our analyses for the presence of this important variable. Third, we cannot establish



relations of causality because this is a cross-sectional study. Finally, we did not follow participants during life, and clinical variables were reported by the NOK. To minimize bias, we only included reliable NOKs. Using this approach, our interview showed high accuracy in previous studies.<sup>32,33,64,65</sup> Future research in human samples will be necessary to show if specific types of macrophages, B lymphocytes, and T lymphocytes with proatherogenic or atheroprotective roles in the PvAT may have different associations with atherosclerosis pathogenesis.<sup>20</sup>

In conclusion, a greater density of macrophages and B lymphocytes in the periplateau PvAT was associated with an increase in atherosclerotic obstruction. Moreover, the density of CD68<sup>+</sup> macrophages was greater in the periplateau PvAT near unstable plaques. Finally, the number of macrophages and B lymphocytes was higher in the PvAT surrounding critical plaques than in segments of PvAT without atherosclerosis.

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# **SUPPLEMENTAL MATERIAL**

**Table S1. Descriptive analysis of coronary artery atherosclerosis and immune cells in the perivascular adipose tissue.**

<b>Variables</b>	<b>Mean (SD) or n (%)</b>
<b>Proximal fragment</b>	
. Percentage of arterial obstruction	56.7 (26.0)
. Unstable Atherosclerotic plaques	41 (16.7%)
. Density of macrophages ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	2.44 (1.31; 4.33)
. Density of T-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0.619 (0.301; 1.07)
. Density of B-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0.02 (0; 0.07)
<b>Distal fragment</b>	
. Percentage of arterial obstruction	17.3 (10.3)
. Unstable Atherosclerotic plaques	0 (0%)
. Density of macrophages ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	2.34 (0.99; 4.39)
. Density of T-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0.601 (0.380.4; 1.12)
. Density of B-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0.02 (0; 0.05)
<b>Difference proximal and distal fragment</b>	
. Density of macrophages ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0.07 (-1.08; 1.10)
. Density of T-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	-0.03 (-0.35; 0.40)
. Density of B-lymphocytes ( $10^{-5}$ cells/ $\mu\text{m}^2$ ), median (IQR)	0 (-0.02; 0.03)

**Table S2. Association of density of CD68<sup>+</sup> macrophages in the PvAT with sociodemographic and clinical data (n=246).**

<b>Density of CD68<sup>+</sup> macrophages (10<sup>-5</sup> cells/μm<sup>2</sup>)</b>	<b>β</b>	<b>95% CI</b>	<b>p<sup>†</sup></b>
<b>Age (years), mean (SD)</b>	0.052	0.017; 0.088	0.004
<b>Female, n (%)</b>	0.492	-0.581; 1.564	0.36
<b>White, n (%)</b>	0.240	-0.868; 1.349	0.66
<b>Education (years), median (IQR)</b>	-0.072	-0.194; 0.049	0.23
<b>Hypertension, n (%)</b>	-0.578	-1.954; 0.796	0.40
<b>Diabetes, n (%)</b>	-0.389	-1.428; 0.649	0.45
<b>CAD symptoms, n (%)</b>	-0.461	-1.891; 0.968	0.52
<b>Cardiac failure, n (%)</b>	-0.298	-1.341; 0.746	0.57
<b>Stroke, n (%)</b>	0.271	-1.029; 1.571	0.67
<b>Dyslipidemia, n (%)</b>	-0.286	-1.622; 1.051	0.67
<b>BMI, mean (SD)</b>	-0.140	-0.254; -0.027	0.01
<b>Physical inactivity, n (%)</b>	0.524	-0.627; 1.675	0.36
<b>Current smoking, n (%)</b>	0.951	-1.017; 2.920	0.33
<b>Current alcohol use, n (%)</b>	-0.414	-1.542; 0.714	0.46

<sup>†</sup>Linear regression with robust standard errors to account for repeated measures in the same individual

**Table S3. Association of density of CD3<sup>+</sup> T-lymphocytes in the PvAT with sociodemographic and clinical data (n=246).**

<b>Density of CD3<sup>+</sup> T-lymphocytes (10<sup>-5</sup> cells/<math>\mu</math>m<sup>2</sup>)</b>	<b><math>\beta</math></b>	<b>95% CI</b>	<b>p<sup>†</sup></b>
<b>Age (years), mean (SD)</b>	0.004	-0.004; 0.013	0.30
<b>Female, n (%)</b>	0.004	-0.281; 0.289	0.97
<b>White, n (%)</b>	-0.156	-0.459; 0.147	0.30
<b>Education (years), median (IQR)</b>	-0.025	-0.059; 0.007	0.12
<b>Hypertension, n (%)</b>	-0.162	-0.569; 0.245	0.43
<b>Diabetes, n (%)</b>	-0.034	-0.324; 0.255	0.81
<b>CAD symptoms, n (%)</b>	0.029	-0.300; 0.359	0.86
<b>Cardiac failure, n (%)</b>	0.078	-0.285; 0.441	0.67
<b>Stroke, n (%)</b>	0.488	-0.155; 1.131	0.13
<b>Dyslipidemia, n (%)</b>	-0.200	-0.455; 0.054	0.12
<b>BMI, mean (SD)</b>	-0.014	-0.034; .008	0.22
<b>Physical inactivity, n (%)</b>	0.246	0.0007; 0.492	0.049
<b>Current smoking, n (%)</b>	0.212	-0.208; 0.632	0.31
<b>Current alcohol use, n (%)</b>	-0.054	-0.312; 0.203	0.67

<sup>†</sup>Linear regression with robust standard errors to account for repeated measures in the same individual

**Table S4. Association of density of CD20<sup>+</sup> B-lymphocytes in the PvAT with sociodemographic and clinical data (n=246).**

<b>Density of CD20<sup>+</sup> B-lymphocytes (10<sup>-5</sup> cells/<math>\mu</math>m<sup>2</sup>)</b>	<b><math>\beta</math></b>	<b>95% CI</b>	<b>p<sup>†</sup></b>
<b>Age (years), mean (SD)</b>	0.0004	-0.0005; 0.001	0.35
<b>Female, n (%)</b>	0.005	-0.021; 0.031	0.69
<b>White, n (%)</b>	-0.020	-0.047; 0.007	0.20
<b>Education (years), median (IQR)</b>	0.002	-0.001; 0.005	0.20
<b>Hypertension, n (%)</b>	0.013	-0.018; 0.045	0.40
<b>Diabetes, n (%)</b>	-0.019	-0.044; 0.005	0.11
<b>CAD symptoms, n (%)</b>	-0.017	-0.059; 0.025	0.43
<b>Cardiac failure, n (%)</b>	-0.013	-0.035; 0.009	0.23
<b>Stroke, n (%)</b>	-0.010	-0.033; 0.013	0.40
<b>Dyslipidemia, n (%)</b>	0.018	-0.017; 0.052	0.31
<b>BMI, mean (SD)</b>	-0.002	-0.004; 0.0002	0.07
<b>Physical inactivity, n (%)</b>	0.012	-0.017; 0.040	0.41
<b>Current smoking, n (%)</b>	0.036	-0.012; 0.084	0.13
<b>Current alcohol use, n (%)</b>	0.014	-0.015; 0.045	0.33

<sup>†</sup>Linear regression with robust standard errors to account for repeated measures in the same individual

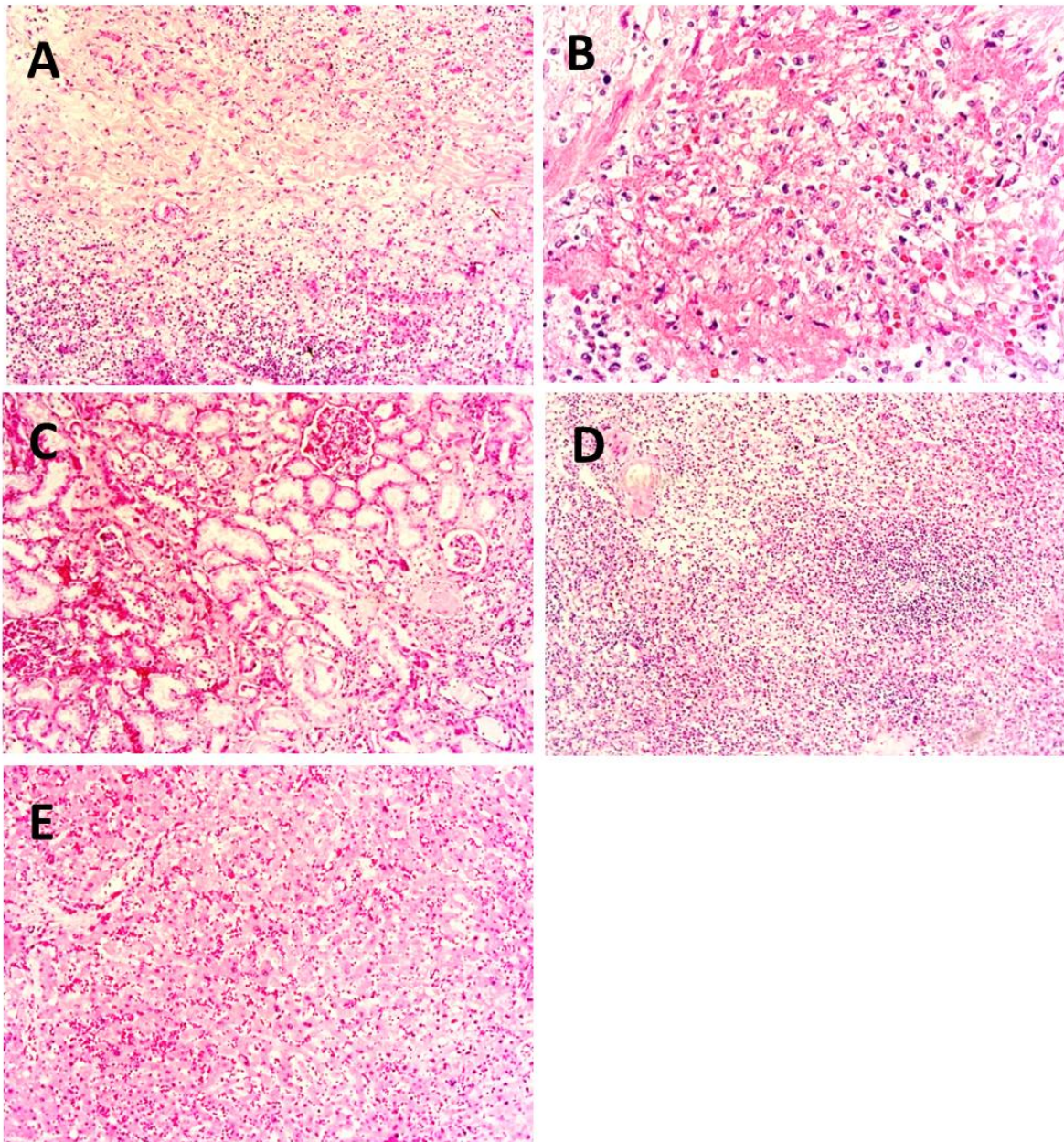
**Table S5 Comparison of immune cells between perivascular adipose tissue of unstable atherosclerosis and perivascular adipose tissue of stable atherosclerosis in the same individual (n=26 matched pairs).**

<b>The density of immune cells (<math>10^{-5}</math> cells/<math>\mu\text{m}^2</math>)</b>	<b>Perivascular adipose tissue of unstable atherosclerosis</b>	<b>Perivascular adipose tissue of stable atherosclerosis</b>	<b>p<sup>¤</sup></b>
. <i>CD68<sup>+</sup> macrophages</i>	2.94 (1.93; 6.85)	3.09 (0.97; 4.33)	0.008
. <i>CD3<sup>+</sup> T-lymphocytes</i>	0.70 (0.28; 1.29)	0.54 (0.42; 1.11)	0.19
. <i>CD20<sup>+</sup> T-lymphocytes</i>	0.07 (0.02; 0.01)	0.035 (0.02; 0.07)	0.58

<sup>¤</sup> Wilcoxon matched pairs signed rank test

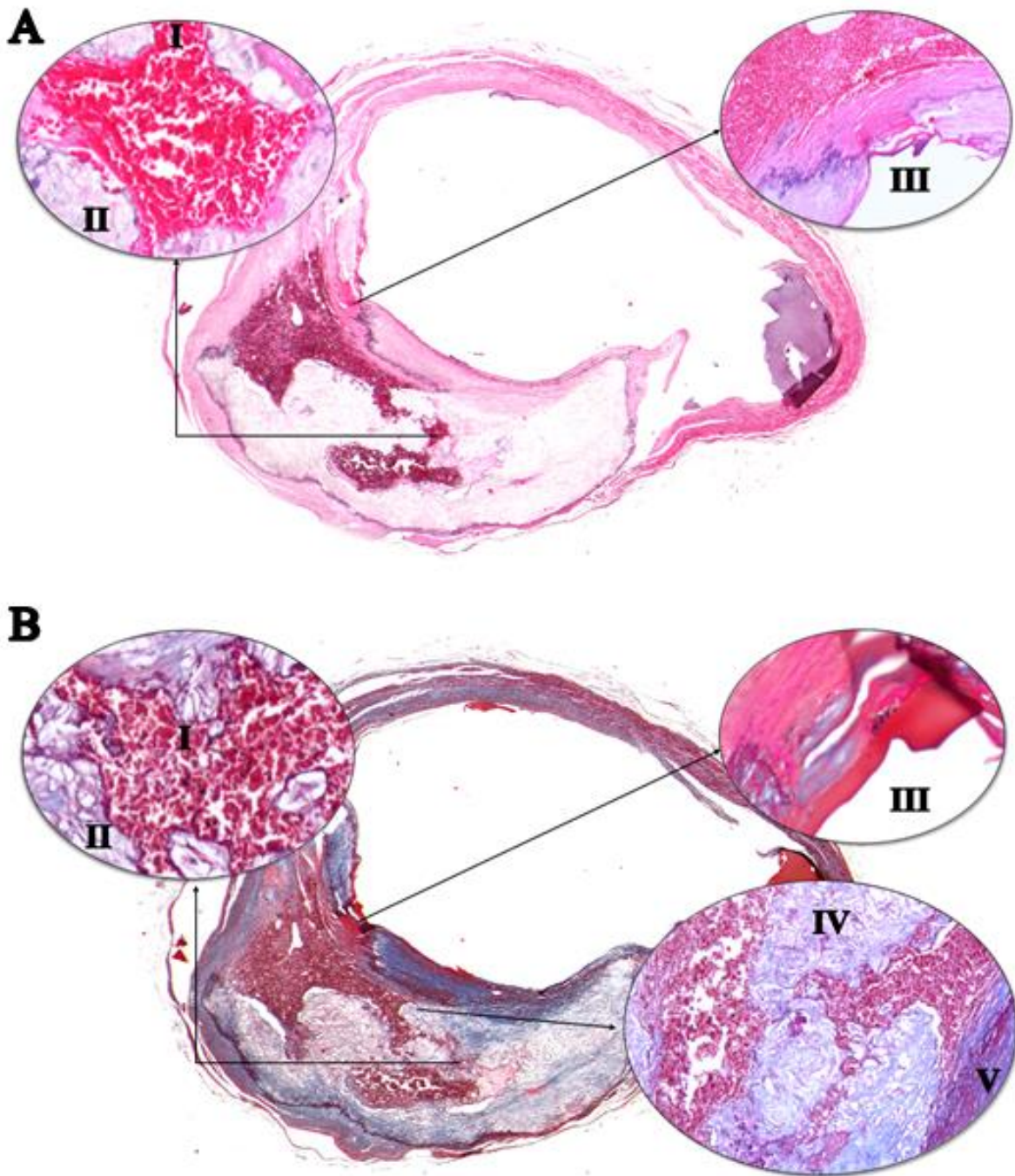


**Figure S1. Signs of sepsis detected during the anatomopathological post-mortem exam.**



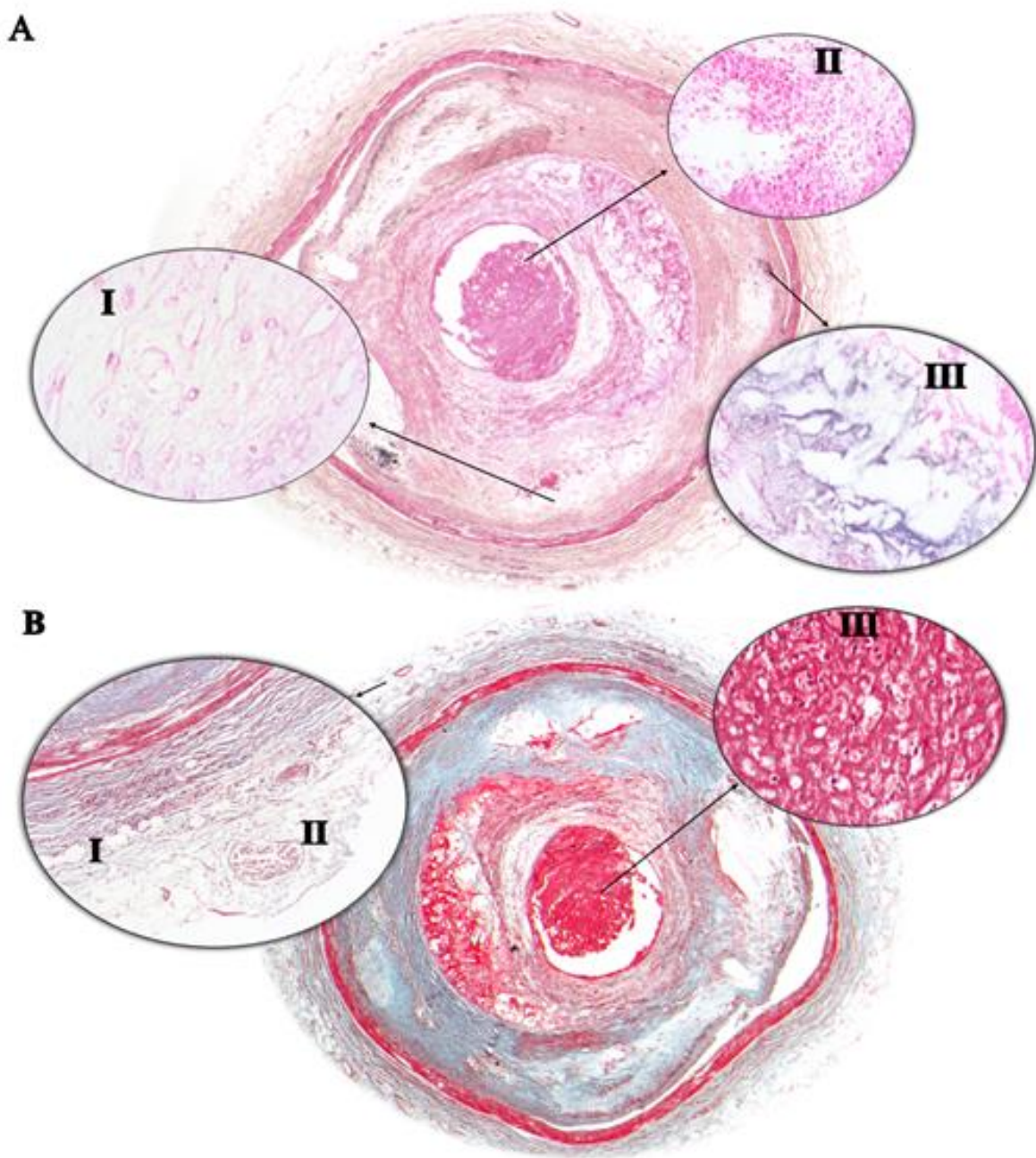
A: microscopic photograph (10x magnification) of lung with neutrophil infiltration due to bronchopneumonia. B: microscopic photograph (40x magnification) of neutrophil infiltrate in the lung. C: microscopic photograph (40x magnification) acute tubular necrosis responsive to shock in the kidney; D: microscopic photograph (10x magnification) of acute splenitis; E: microscopic photograph (10x magnification) of hepatic congestion and steatosis secondary to bronchopneumonia.

**Figure S2. Coronary artery with intraplaque hemorrhage.**



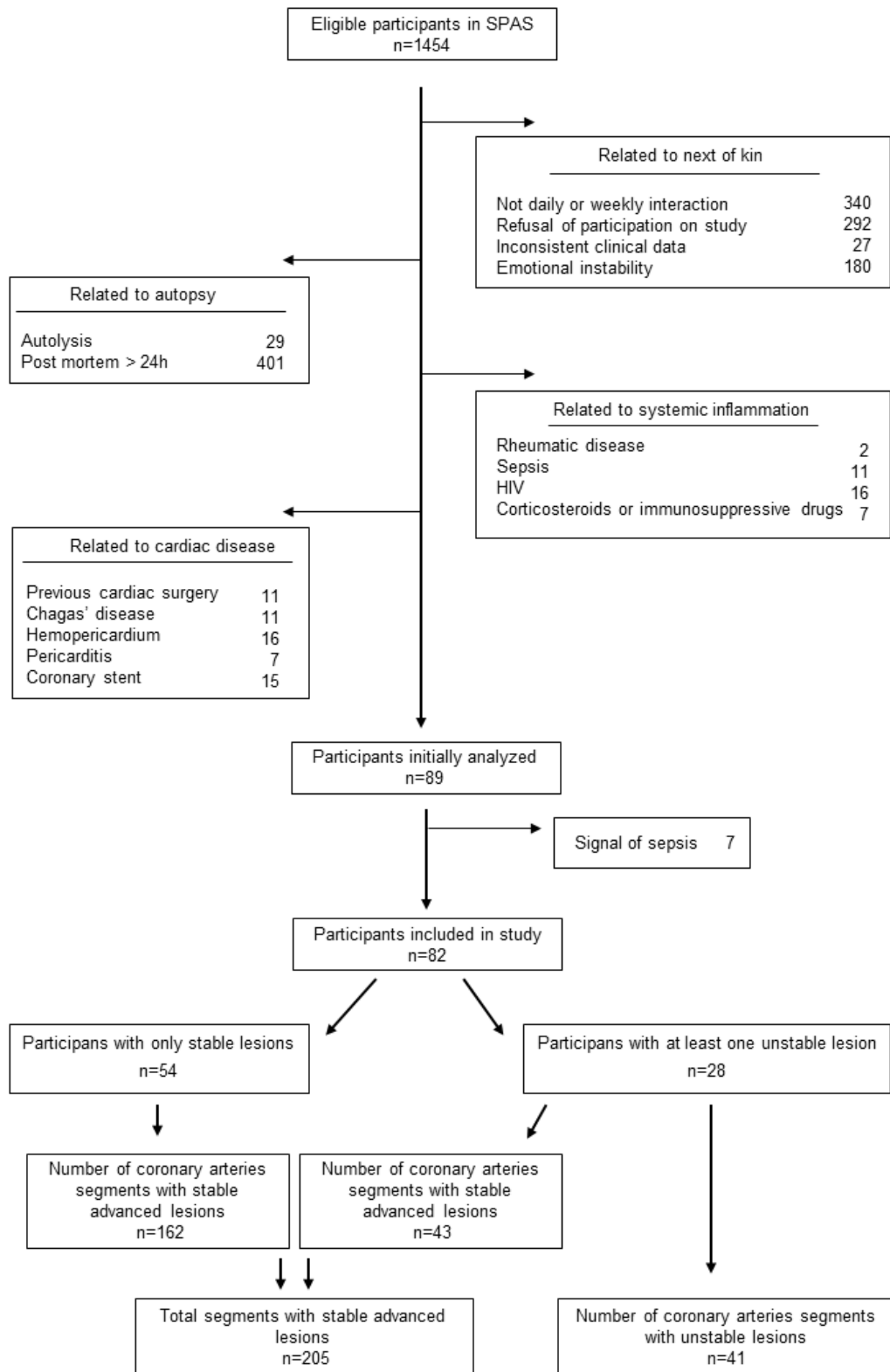
A. Coronary artery fragment stained with haematoxylin-eosin (2x of magnification). I: erythrocytes (40x of magnification); II: lipid core (40x of magnification); III: narrowing of the fibrous cap adjacent to the intraplaque hemorrhage (40x of magnification). B. Coronary artery fragment stained with Masson's trichrome (2x of magnification). I: erythrocytes (40x of magnification); II: lipid core (40x of magnification); III: narrowing of fibrous cap adjacent to intraplaque hemorrhage (40x of magnification); IV: lipid core and intraplaque hemorrhage (10x of magnification) and V: fibroses (10x of magnification).

**Figure S3. Coronary artery with thrombus.**



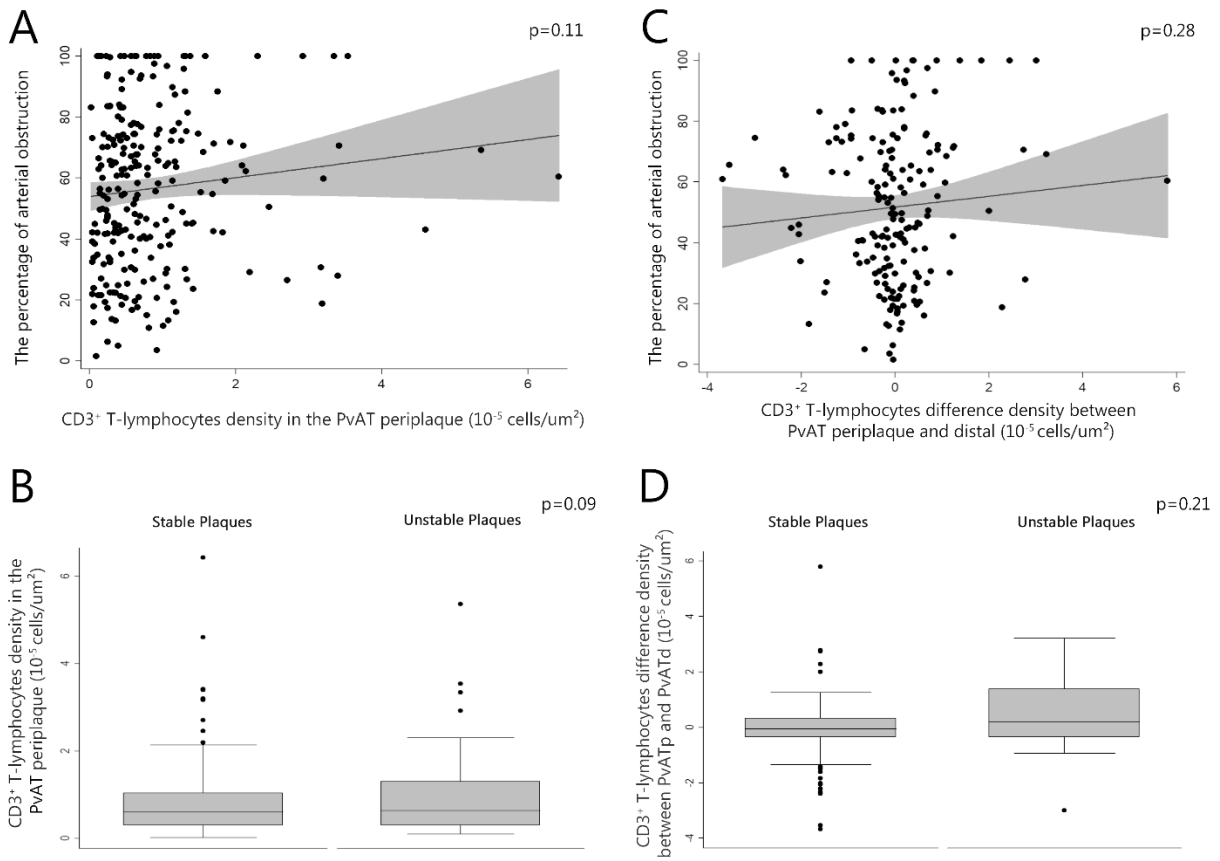
A. Coronary artery fragment stained with haematoxylin-eosin (2x of magnification). I: lipid core (40x of magnification); II: erythrocytes inside the thrombus (40x of magnification); III: calcification (40x of magnification). B. Coronary artery fragment stained with Masson's trichrome (2x of magnification). I: Periplaque perivascular adipose tissue near the adventitia layer (10x of magnification); II: *vasa vasorum* (10x of magnification); III: thrombus (40x of magnification).

**Figure S4. Flowchart of study participants.**



SPAS: São Paulo Autopsy Service. HIV: Human Immunodeficiency Virus.

**Figure S5. Association between CD3<sup>+</sup> T-lymphocytes and atherosclerosis.**



PvATp: periplaque perivascular adipose tissue; PvATd: distal perivascular adipose tissue. A: Scatter plot of the density of CD3<sup>+</sup> T-lymphocytes in periplaque PvAT with the percentage of coronary artery obstruction. B: Box plot graph of the density of CD3<sup>+</sup> T-lymphocytes in periplaque PvAT by stable and unstable plaque groups. C: Scatter plot of the difference of the CD3<sup>+</sup> T-lymphocytes density in periplaque PvAT and distal PvAT with the percentage of coronary artery obstruction. D: Box plot graph of the difference density of CD3<sup>+</sup> T-lymphocytes between periplaque PvAT and distal PvAT by stable and unstable plaque groups. PvATp: periplaque perivascular adipose tissue; PvATd: distal perivascular adipose tissue.