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1 **Original article**

2

3 **IMPACT OF FEMORAL OSSIFICATION ON LOCAL AND SYSTEMIC CARDIOVASCULAR**
4 **PATIENTS' CONDITION**

5

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25 **KEYWORDS:** peripheral artery disease – femoral calcifications – osteoid metaplasia – MACE –
26 MALE –inflammation.

27 **Abstract**

28 **Background:** Vascular calcifications are associated with a high cardiovascular morbi-
29 mortality when located in the coronary territory, and with atheromatous plaque stability and
30 stroke when located in carotid arteries. Previous studies have shown that femoral arteries
31 were more prone to calcify and to develop osteoid metaplasia (OM). So far the impact of
32 OM on local and systemic cardiovascular events remains unknown. The aim of this study was
33 to assess the predictive value of OM in histological analysis of femoral plaque for the
34 occurrence of mid- and long-term adverse cardiovascular events. Local inflammation has
35 also been investigated.

36 **Method:** Between 2008 and 2015, 86 femoral atheromatous samples were harvested during
37 femoral endarterectomy (81 patients, 86 limbs) and processed for histomorphological
38 analyses of calcifications and inflammation (monocytes/macrophages and B cells).
39 Histological findings were compared with the long-term patients' follow-up, including major
40 adverse cardiac event (MACE), major adverse limb event (MALE), mortality, Rutherford
41 classification and ankle-brachial index (ABI). Frequencies were presented as percentage,
42 continuous data as mean and standard deviation. A P-value < .05 was considered statistically
43 significant.

44 **Results:** Median follow-up was 42.4 months [26.9 – 58.8]. Clinically, 23 (28%) patients
45 underwent a MACE, and a MALE occurred in 18 (21%) limbs. The survival rate was 87.2% at
46 36 months. Histopathological analysis revealed osteoid metaplasia in 41 (51%) of the
47 samples, without any significant impact on the occurrence of MACE, MALE or mortality.
48 Sheet-like and nodule types seem to influence MACE but it was barely significant (.07 and
49 .05, respectively), and a lack of nodule type was associated with MALE event (P=.01).

50 The inflammatory analysis revealed among patients who presented a MACE, a significantly
51 higher rate on pre-operative white blood cell formulae of monocytes (P=.04) and neutrophils
52 (P=.04), correlated with higher B cells counts in plaque sample. Similarly, MALE were
53 significantly associated with a higher rate of neutrophils in preoperative blood samples
54 (P=.0008) and a higher number of B cells CD20+ in the samples.

55 **Conclusion:** Osteoid metaplasia is part of femoral calcifications in almost 50% of the cases
56 but does not seem to be an independent predictive variable for MACE or MALE. However, a
57 higher rate of B cells infiltration of the plaque and pre-operative neutrophils blood count
58 may be predictive of adverse events during follow up, as well as nodule calcification's type.

59 **1. Introduction**

60 Cardiovascular diseases are the leading cause of mortality worldwide, with a
61 pathophysiology relying mainly on atherosclerosis (1). Coronary artery disease is extensively
62 explored, but less is known about peripheral artery disease (PAD), despite affecting more
63 than 200 million people worldwide in 2010 with severe morbidity and death (2, 3). Previous
64 studies have reported a large heterogeneity of atheroma characteristics depending on its
65 arterial bed location, especially regarding its histo-morphological composition in lipids,
66 intraplaque hemorrhage, inflammatory cells and calcifications, implying that findings from
67 the coronary artery are not necessarily applicable to peripheral territories (4-8).

68 Arterial calcifications in any arterial bed have an impact on plaque stability and
69 complications and are associated with a 3-4 fold higher risk for mortality and cardiovascular
70 events (7). In coronary territory, the calcium score is a well-recognized independent
71 predictive factor for cardiovascular events and death (9-12). In the carotid territory, lipid-rich
72 and less calcified atheroma is associated with plaque instability and cerebrovascular events
73 (6, 13, 14). In the iliofemoral territory, histologic plaque characteristics, including global
74 calcifications, were not associated with secondary cardiovascular event during follow-up
75 (15). However, the predictive value of bone-like arterial calcifications (osteoid metaplasia),
76 preferentially found in the common femoral artery, has not been reported.

77 Direct role of calcifications is still discussed, while their overall presence unarguably
78 associates with increased CV events. Previous studies have shown that small calcifications
79 near the lumen increases the chance of physical rupture of the fibrous cap, due to
80 aggravated tensile strength at the interface between soft tissues (ECM, lipid) and hard
81 material (calcifications) (16, 17). Since the beginning of the statin era, clinical data indicate
82 that plaque erosion occurs in a significant and growing number of cases (18). This type of
83 plaque complications usually intervenes in plaque with abundant fibrosis and SMC, like the
84 so called "stable lesion". Presence and activation of TLR2 signaling and neutrophils seem to
85 actively participate in the development of this types of events (19). In such lesions, extensive
86 calcifications in contact with the lumen could also trigger the formation of occluding thrombi
87 (20). However, macrocalcifications, notably osteoid metaplasia (OM) that develop deeper in
88 the lesion, often near the media/intima interface, could stabilize the lesion, at least in the
89 carotid arteries (13).

90 As advanced plaques almost consistently present both micro and macro, the overall direct
91 effect on plaque complications can be challenging, and most studies often analyze
92 calcifications without characterizing their distinct phenotypes. We chose to investigate this
93 point with the present study.

94 In addition to arterial calcifications, atherosclerosis is commonly associated with persistent
95 inflammation. Roles of innate and humoral immunity in development and evolution or
96 stabilization of atheromatous plaques are well described and are presented as promising
97 therapeutic targets (21, 22). Inflammatory cells are also closely related to plaque
98 calcifications. Monocytes are precursors of osteoclasts, and they can plaque monocyte can
99 exhibit osteoclast-like phenotype with impaired bone resorption activity (23). Macrophage
100 subsets could also indirectly participate in plaque and calcification heterogeneity among
101 vascular beds, as M1 and M2 macrophages are characteristic of carotid and femoral lesions,
102 respectively (24) . Moreover, inflammation can also derive from infiltrating T and B cells, and
103 secretion of inflammatory cytokines can participate in tissue mineralization (25).

104 Inflammatory cells burden differs significantly within arterial beds (e.g. carotid and femoral
105 plaques) but its specific impact on the occurrence of adverse local and global cardiovascular
106 events has not been clearly elucidated (15, 26-28).

107 It would be clinically beneficial if patients at higher risk for secondary cardiovascular adverse
108 event could be identified after a common femoral endarterectomy by a histological analysis
109 of calcification type and inflammation burden of their atheromatous sample. The aim of this
110 study was to assess whether femoral osteoid metaplasia was predictive for the severity of
111 both cardiovascular systemic disease and local peripheral arterial disease during long term
112 follow up after common femoral endarterectomy. Secondary objectives included the
113 assessment of other types of vascular calcifications and of the immunohistochemical
114 (inflammation) composition of the femoral plaque.

115 **2. Methods**

116

117 This study consisted of monocentric matching cohorts, with retrospective longitudinal
118 analysis of prospective data and has been approved by the local ethics committee (research
119 protocol #PFS09–014, authorized on Dec 23, 2009 by the Agence de Biomédecine; French
120 Research Ministry n° DC-2008–402; CNIL n° 1520735 v 0). It was designed for patients from
121 ECLA and ECLAGEN cohorts (see below) who underwent surgical common femoral
122 endarterectomy prior to a bypass or patch closure, to gather long term clinical data. Prior to
123 the beginning of this study, every patient was contacted by phone and mail, in order to get
124 an informed consent. When the patients were not reachable by either phone, mail or when
125 their personal doctor had lost contact, they were considered lost and not possible to follow
126 up. The last ultrasound date would then be considered as the date of latest news.

127

128 **2.1. Patients and biological samples**

129 *2.1.1. ECLA and ECLAGEN cohorts*

130 Recruitment and analysis conducted on the ECLA cohort (between 2008 and 2010) and the
131 ECLAGEN cohort (between 2011 and 2015) has been described previously (6). Consecutive
132 patients who underwent femoral endarterectomy were included. Atheromatous plaques and
133 blood samples were collected for histopathological analysis and biological analysis,
134 respectively. Healthy samples for femoral artery were collected from deceased non-
135 atheromatous organ donors.

136 Inclusion criteria for ECLA and ECLAGEN cohorts were adults (age over 18 and under 90 years
137 old), and open surgical treatment needed for *de novo* atheromatous lesions. Criteria of
138 exclusion were pregnant women, restenosis, non-atheromatous lesions (dysplasia, post-
139 traumatic, inflammatory) and thrombosis. Blood collection was performed following fasting,
140 the day of the surgery. Plasma were shortly obtained after centrifugation and stored at -
141 80°C.

142 *2.1.2. Clinical data collection*

143 Patients' perioperative characteristics were collected from the original database. Follow-up
144 assessment was performed retrospectively using patients' files and information from the
145 general practitioner. Items collected were: global mortality, cardiovascular mortality,
146 cerebrovascular event, myocardial infarction, unstable angina, major above-ankle

147 amputation and major re-intervention, ankle-brachial index (ABI), and pre-operative white
148 blood cell formulae.

149

150 **2.2. Histological and immunological analysis**

151 *2.2.1. Original human biological samples collection and processing*

152 The process for histological analysis of the endarterectomy samples has been previously
153 described (6, 29). Briefly, in ECLA and ECLAGEN studies, tunica adventitia was removed and
154 samples were immediately fixed in formalin for paraffin embedding and histology. After
155 removal of the tunica adventitia, plaques or arterial sections were cut longitudinally in four
156 samples and conditioned as follows: (i) fixation in formalin for paraffin embedding and
157 histology, (ii) fixation in paraformaldehyde for OCT[®] embedding and histology, (iii) storage in
158 RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and mRNA extraction, (iv)
159 snap freezing and storage at -80°C.

160 *2.2.2. Histological analysis*

161 Ten serial sections (3µm) from the middle of the atherosclerotic lesion were processed.
162 These sections were stained with hematoxylin, eosin and Masson's trichrome for plaque
163 phenotype analysis and calcification characterization (sheet-like calcifications, nodules, clear
164 center/micro-calcifications and/or osteoid metaplasia). Calcifications subtypes were scored
165 binary (yes/no). Microcalcifications were defined as small speckled calcified particles
166 (<40µm) usually found within the lipid-rich core of the lesion (30).

167 *2.2.3. Immunological analysis*

168 Macrophages and B lymphocytes were stained with CD68 antibody (M0876, Dako[®], Glostrup,
169 Denmark) and CD20 antibody (ab9475, Abcam[®], Cambridge, UK), respectively, and images
170 acquisition was obtained with the NanoZoomer digital slide scanner (Hamamatsu Photonics[®],
171 Hamamatsu, Japan). Fluorescent staining allowed to manually quantify the number of CD20+
172 cells, and Image-Pro Plus[®] 6.0 (Media Cybernetics, Rockville, USA) was used to calculate the
173 positive area for CD68 staining. Results were expressed as a number of cells (CD20+) or as a
174 percentage of the stained area (CD68+) over the area of the plaque (intima) for each slide.

175

176 **2.3. Primary and secondary endpoints**

177 Major adverse limb events (MALE) criteria for patients presenting plaque composition with
178 osteoid metaplasia were the primary composite endpoint of the study. MALE is defined by

179 major above-ankle amputation and / or major re-intervention on the index limb, including
180 new bypass graft, interposition graft revision, or thrombectomy/thrombolysis (31).
181 Secondary endpoints of the study were the assessments of the following event(s) among
182 patients presenting osteoid metaplasia in the histological composition of their plaque. Major
183 adverse cardiac events (MACE) comprised any cardiovascular mortality, cerebrovascular
184 disease, myocardial infarction and / or unstable angina events. Other secondary endpoints
185 understood global mortality, last follow-up Rutherford classification and the assessment of
186 the inflammatory burden (CD68+ and CD20+ infiltration) among patients presenting a MACE
187 and/or MALE. Rutherford classification and ABI were collected in order to evaluate clinical
188 status improvement at different times during follow-up (32, 33).

189

190 **2.4. Statistical analysis**

191 Continuous data were presented as mean \pm SD or median [quartiles] based on the
192 distribution. Demographic and comorbidity data were recorded per patient. The χ^2 or Fisher
193 exact test (based on expected frequency) was used to compare categorical variables
194 between groups. Categorical variables were presented as counts (proportions). The t-test
195 was performed to test for statistical differences in continuous parameters between two
196 groups.

197 Survival rate curves for outcomes (MALE, MACE, global mortality) were plotted and
198 calculated using the Kaplan-Meier method. Cox proportional-hazards regression analysis
199 with stratification on cohort [with Hazard Ratios (HR) and confidence intervals (CI)] was were
200 used to evaluate the independent risk of clinical factors and of blood cell count for MALE,
201 MACE or death. From univariate analysis, we selected variables with P-value <0.25
202 (statistical criterion) and looked at multicollinearity between variables. For the multivariate
203 models, we kept significant variables. Variables were eliminated from highest to lowest p-
204 values but remained in the final model if the P-value was less than .05 or seem to be
205 confounders (more than 10% change in estimate). Final multivariable Cox model was
206 stratified by cohort and by sex. No interaction between the stratification variables and the
207 predictor of interest (other variables in the model) were found so we presented no subgroup analysis
208 for each stratum.

209 For patients who died before the final follow-up examination or for patients lost during the
210 follow-up, the status of the last follow-up examination was recorded. For histological

211 analyses, two investigators (TQ and OE) performed the morphologic observations
212 independently and blindly, after an inter-observer concordance test. Immunohistological
213 analyses were conducted by one observer (AD) in a blinded fashion. A P-value $<.05$ was
214 considered statistically significant. Data were analyzed with the SAS packages (SAS Institute
215 Inc version 9.4, Cary, NC).

216

217 **3. Results**

218

219 **3.1. Population**

220 From ECLA and ECLAGEN cohorts, we selected 44 and 50 patients respectively (47 and 54
221 samples because of 3 and 4 bilateral surgeries, respectively) who underwent surgical femoral
222 endarterectomy. Among these patients, 13 were excluded: one refused to give her consent
223 for participating in this study, three presented aberrant surgical indications, five because of
224 plaque damage (inconclusive histology analysis), and 4 did not show any calcification on
225 histological analysis. Finally, 86 femoral plaques and 81 patients were included. Main
226 characteristics of the population were summarized in Table I. The median follow-up was 42.4
227 months [26.9 – 58.8]. Most of them were Rutherford stages 2 and 3 (65 limbs, 76.5%) with a
228 mean ankle brachial index (ABI) prior to revascularization of 0.59 ± 0.25 . The surgery
229 consisted in aorto-bifemoral bypass (9%), femoro-femoral or femoro-popliteal bypass with
230 local femoral endarterectomy (56%) or isolated endarterectomy and patch (35%).

231

232 **3.2. Clinical results**

233 Twenty limbs (23.3%) were treated for critical limb ischemia. The median follow-up was 42.4
234 months [26.9 – 58.8]. In-hospital major complications rate was 6.2% (5 patients), including: 1
235 death (day 65); 2 major cardiac events; 2 major amputations on the index limb. At 30 days,
236 15 patients (9%) encountered a +3 clinical improvement, 39% a +2 and 30% a +1, while 11%
237 did not report improvement and one reported major deterioration. At the last follow-up, 19
238 additional patients died, including 7 deaths related to a cardiovascular adverse event
239 (Supplemental Figure 1). The survival rate was 95.0% at 1-year and 85.1% at 3-years. At 36
240 months, 21%, 43% and 21% of patients reported a +3, +2 and +1 improvements; 10%
241 reported no change; 2 patients reported a -1 deterioration.

242

243 **3.3. MACE and MALE occurrence during follow-up**

244 Twenty-three patients (28.4%) suffered from MACE, including: 7 strokes, 9 myocardial
245 infarctions, 5 unstable anginas and 2 cardiovascular deaths (Figures 1 and 2). A medical
246 history of stroke or renal impairment was significantly associated with the occurrence of
247 MACE (30%, $P=.001$ and 22%, $P=.03$, respectively) during follow-up (Table I).

248 Moreover, 17 patients (21.0%) suffered from MALE (including 7 patients who presented
249 both MACE and MALE, one patient suffered from MALE on each limb): 5 major amputations
250 (median 66 days [19.5 – 913 days]); 5 additional bypasses; 2 graft infections (treated with
251 allograft replacement); 1 bypass embolectomy; 2 surgical closure of false-aneurysms, 6 redo
252 surgeries due to local restenosis (Figure 1 and 3). None of the patients' characteristics except
253 for the Rutherford classification, were significantly correlated with MALE.

254

255 **3.4. Impact of calcification types on MACE and MALE occurrence**

256 Eighty-six atheromatous plaques from 81 patients underwent histological analyses. Osteoid
257 metaplasia was found in 41 (51%) of them. Sheet-like calcifications and nodules were
258 identified in a large majority of the samples (78 and 81% respectively). History of smoking
259 were associated with no osteoid metaplasia ($P=.01$). Patients with OM were older ($69.8\pm$
260 9.5 vs 64.6 ± 8.5 respectively, $P=.01$). Calcifications types were similar between men and
261 women.

262 Statistical analysis with multivariate Cox models adjusted by sex and cohort did not show any
263 significant influence of osteoid metaplasia and microcalcifications on MALE or MACE (Table
264 II). Sheet-like types seem to influence MACE ($P=.04$) but not nodule type ($P=.32$). A lack of
265 nodule type was associated with MALE event ($P=.003$).

266

267 **3.5. Biological and histological inflammatory burden**

268 Pre-operative blood sample analysis of the ECLA cohort ($n=38$ patients) showed a
269 significantly higher rate of neutrophils within patients suffering from MALE ($P=.0003$, Table
270 II) in multivariate analysis. These results correlated with histological findings about
271 inflammatory infiltration of the plaques. CD20+ (lymphocyte B) staining was significantly
272 higher among patients who experienced a MACE or a MALE. No correlations were found
273 with CD68+ staining (Figure 4).

274 4. Discussion

275

276 Arterial calcifications are associated with a higher morbi-mortality in coronary disease, and
277 with atheromatous plaque stability in the carotid territory. Clinical data are still lacking for
278 lower limb atherosclerosis. In our study, we report the lack of significant impact of intimal
279 calcification types on local severity or general cardiovascular condition of the patient.
280 Especially, the presence of osteoid metaplasia in femoral atheromatous plaque does not
281 correlate with a higher local or general impact of the vascular disease (MACE and MALE
282 criteria) and cannot be used as predictor score for cardiovascular morbi-mortality. Even if
283 more than 5000 patients would have been studied (simulation analysis), the presence of
284 osteoid metaplasia in femoral atheromatous plaque stay a non-significant predictor score for
285 cardiovascular morbi-mortality (n=5265: HR=0.92[0.83;1.03], P=.1425). Sheet-like
286 calcifications showed a trend on influencing the occurrence of a MACE and nodule
287 calcification types on influencing MALE.

288

289 For years, appearance of vascular calcifications (VCs) in atheromatous plaques has been
290 considered as a passive phenomenon, but is now known to be a highly regulated process, for
291 which research still aims to find the precise molecular mechanisms (34-41). Vascular
292 calcifications and their impact on clinical status and on evolution of the cardiovascular risk
293 have first been described on coronary arteries. Several studies have led to recognize
294 coronary-artery calcium (CAC) as an independent risk factor for cardiovascular morbidity and
295 mortality, in both coronary and peripheral territories (7, 10, 11). Calcium score – or Agatston
296 score (9) – can either be considered as a factor of stability or instability of the atherosclerotic
297 plaque depending on imaging characteristics of the calcifications themselves, as well as a
298 predictive factor for restenosis (42). However, such scores do not take into account
299 calcification heterogeneity in atherosclerotic plaques. Previous studies reported that in the
300 carotid territory, microcalcifications are related to plaque instability and a higher risk of
301 cerebrovascular disease. In contrast, large and extensive calcification in deeper layers of the
302 carotid plaques could stabilize the lesions (12, 14, 43).

303 We first reported that femoral lesions have a high prevalence of osteoid metaplasia as
304 compared with other arterial beds (6, 29, 30). Osteoid metaplasia has all the characteristics
305 of fully mature bone tissue, with osteoblasts and osteocytes embedded in a dense

306 extracellular osteoid matrix made of collagen, and even fatty and vascular structures similar
307 to bone marrow. In carotid arteries, it has been demonstrated that osteoid metaplasia was
308 rare but positively associated with asymptomatic, stable plaques. The authors concluded
309 that femoral osteoid metaplasia may have a critical impact that needs to be assessed,
310 justifying our study.

311 Derksen et al. reported an inverse relation between the calcification-content and the
312 restenosis rate after femoral endarterectomy on ultrasound duplex at 12 months (44). In this
313 study, 60% of femoral plaques presented with more than 40% of calcifications, and these
314 samples were associated with a reduced risk of restenosis, but the implication of VCs on
315 postoperative event was not reported. In another recent study, Van Haelst et al. assessed
316 the impact of 7 plaque histological features on secondary cardiovascular events in 497
317 patients undergoing iliofemoral endarterectomy (15). Histological analysis included
318 calcifications but not calcification types. Calcified plaques were univariably associated with
319 secondary cardiovascular event, but not in multivariable analysis. To complete such
320 analyses, we decided to focus on calcification type and test whether different subtypes of
321 calcifications — especially osteoid metaplasia — were associated with more postoperative
322 clinical events (both locally and systemically).

323 Pathophysiology of atherosclerosis is widely reckoned as a progressive inflammatory-lipid
324 disorder with recruitment of macrophages (innate immunity) and T cells (adaptive immunity)
325 in the early stages (21, 22, 45). Hypotheses have emerged to explain and relate calcification
326 burden with inflammation in the artery wall, main cell candidates being macrophages and
327 vascular smooth muscle cells (VSMC). Indeed, macrophages can release matrix vesicles that
328 could mediate calcium and phosphate nucleation, and can show some osteoclastic-like
329 properties (46-48), while VSMCs can transdifferentiate into osteochondrogenic cells (49, 50).
330 On the other hand, presence of calcification can also trigger and sustain local inflammation,
331 with production of proinflammatory cytokines (IL-1, IL-6) (51). In the early stages of
332 atherosclerosis, monocytes infiltrate the intima layer of artery walls, where they turn into
333 macrophages that ingest oxidized low-density lipoproteins (ox-LDL) and become foam cells.
334 Later on, macrophages can also produce cytokines that perpetuate the inflammation process
335 locally, and MMPs that degrade collagen matrix, hence favoring microcalcification, plaque
336 destabilization and ultimately rupture (21, 34, 45). In humans, macrophages (expressing
337 CD68 cell marker) can be separated into at least two subtypes called M1 and M2 (52, 53). In

338 the carotid arteries, CD68+ staining is higher in unstable, symptomatic plaques (54) and
339 displays mostly an M1 phenotype, while M2 macrophages are associated with stable
340 asymptomatic carotid plaques (55). When compared with the femoral territory, CD68+
341 staining is higher in carotid plaques (they are more cellular) (6), associated with a high
342 presence of M1 macrophages while femoral plaques show more M2 macrophages (24).

343 Several small populations of innate immune cells have important roles at different stages of
344 atherosclerosis development, but macrophages are the main innate immune effector cell
345 type in the plaque. T cells that respond to autoantigenic components of LDL particles
346 orchestrate plaque development; T helper type 1 (TH1) cells promote atherosclerosis,
347 regulatory T cells are protective and TH17 cells promote plaque stability. Many connections
348 between the immune system and metabolism exist; acute inflammation induces
349 hypertriglyceridemia, whereas chronic inflammation has more complex effects. Novel
350 approaches such as anti-inflammatory therapies, T-cell-based treatments or vaccination
351 against LDL, could potentially reduce cardiovascular inflammation and protect against the
352 development of atherosclerosis.

353 Following these hypotheses, we decided to look more specifically for inflammation
354 characteristics in patients' pre-operative blood and histological samples. Interestingly, we
355 found a significant correlation between the monocytes blood count and secondary
356 cardiovascular adverse events. Concordantly, Dopheide et al found an increase in circulating
357 pro-inflammatory monocytes count in patients suffering from critical limb ischemia (CLI),
358 speculating that systemic inflammation in atherosclerosis might increase the severity of the
359 disease (56). Zimmermann et al demonstrated an increase in CD68+ cells within femoral
360 plaques from CLI patients, but this finding was not confirmed by our histological analysis of
361 CD68+ (57). Additional analyses with specific staining of macrophage subtypes are required
362 to better understand the monocyte/macrophage infiltration of femoral plaques and its role
363 on secondary cardiovascular events. In contrast, our analysis of CD20+ was significantly
364 associated with MACE and MALE. Humoral immunity B cells are known from mice
365 experimentations, to be involved with atherosclerosis, and B-cell depletion to protect
366 against atherosclerosis. Moreover, patients with splenectomy are at higher risk of death by
367 myocardial infarction (58), and we know from the CANTOS trial, that in human, modulation
368 of the IL-6 signalling pathway, at least with canakinumab, associates with reduced
369 cardiovascular event rates, independent of lipid lowering (59). We confirm these results in

370 our CD20+ histological analysis, with a significantly higher risk of MALE and MACE among
371 patients with high B-cells infiltration. These results are encouraging to develop novel clinical
372 approaches such as anti-inflammatory therapies, or B or T-cells based treatment or
373 vaccination, that could potentially reduce cardiovascular inflammation and protect against
374 the development of atherosclerosis.

375 This study has several limitations. First is the retrospective design of the study regarding long
376 term clinical follow-up. Despite a big work of recovery, some patients remained lost for
377 follow-up. Analyses were made on 81 patients maximum. We lack power to show effect of
378 calcifications on cardiovascular adverse events. Moreover, most of the patients are
379 claudicants, less likely than CLI patients to suffer from reinterventions and amputations.
380 Looking specifically into this population may lead to different results. Also, we did not
381 examine different slides in a single patient. The assumption that the examination of the
382 culprit lesion is consistent in the total plaque might be a study limitation. Finally,
383 extrapolation of our results to female patients is severely limited, due to the very low
384 number of female (9) in our cohorts.

385 To conclude, osteoid metaplasia, an osteochondral calcification found preferentially in the
386 common femoral artery, does not appear to impact the occurrence of secondary adverse
387 cardiovascular events. In our findings, systemic patients' blood monocyte count may have a
388 predictive impact, with a significant correlation with CD20+ (lymphocyte B) plaque infiltrates
389 and adverse cardiovascular events. More studies are required to assess the predictive value
390 of atheromatous plaque features on mid- and long-term secondary cardiovascular adverse
391 events in the lower limb territory.

392

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405

406 **6. Competing interests**

407 Blandine Maurel has been paid a consulting / honorarium by Cook. Yann Gouëffic has been
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409 consulting / honorarium by Abbott, Bard, Biotronik, Boston Scientific, Cook, Medtronic,
410 Vygon, Spectranetics, Terumo and WL Gore.

411

412

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584

585

586 **Figure Legends**

587

588 **Figure 1.** Kaplan-Meier curves for MACE and MALE for male patients.

589

590 **Figure 2.** Occurrence of MACE.

591 MACE stands for major adverse cardiac event and consists of a composite endpoint
592 constituted with each of the described events. 24 patients (26.7%) suffered from a MACE
593 during follow-up.

594

595 **Figure 3.** Occurrence of MALE.

596 MALE stands for *major adverse limb event* and is a composite endpoint constituted of each
597 category of sub-described clinical event. Major amputations are defined by all above-the-
598 ankle amputations of the index limb. All other criteria include major reintervention related
599 to these causes. 21 limbs (21.7%) encountered a MALE during follow-up (7 patients
600 underwent bilateral surgery).

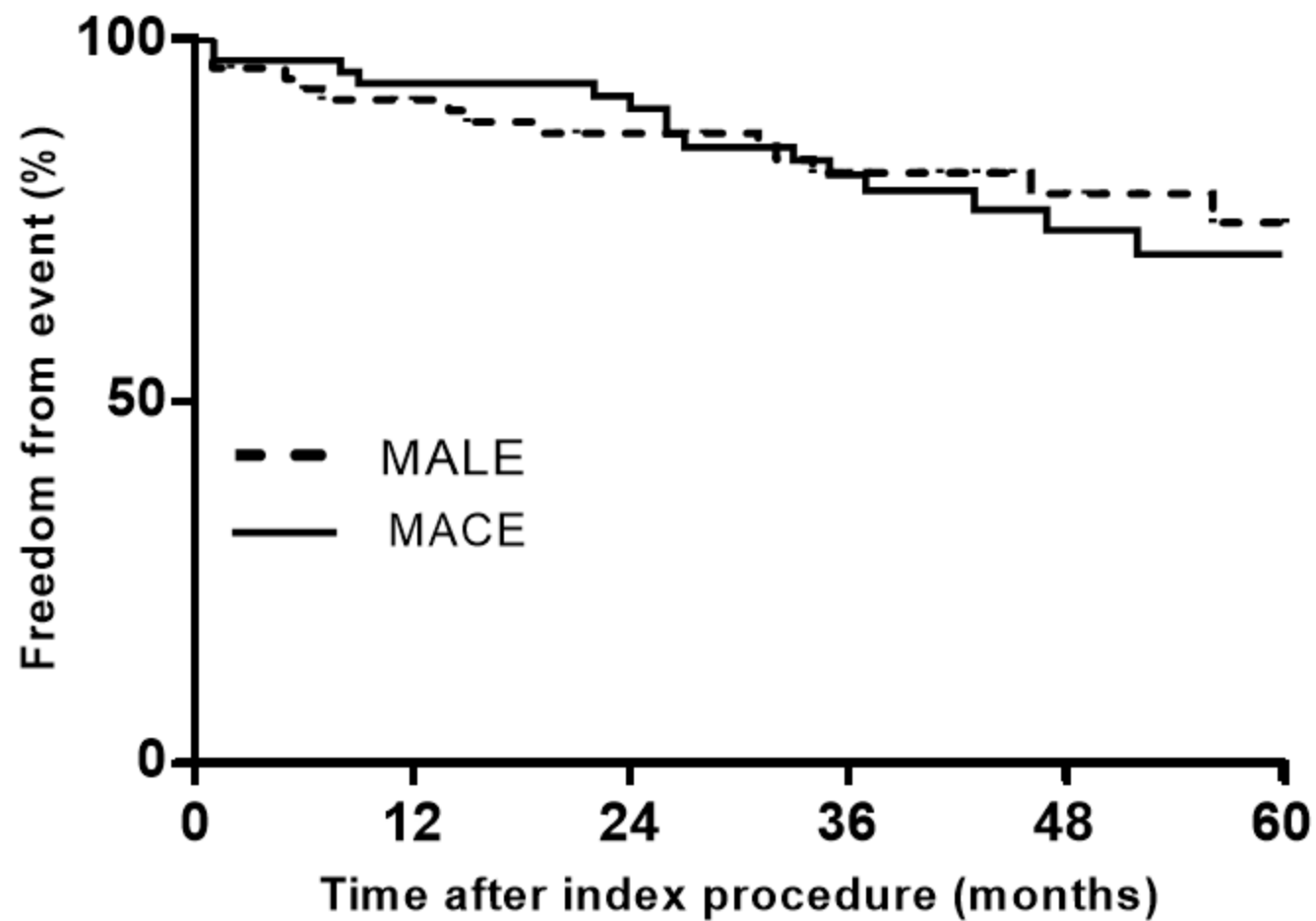
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602 **Figure 4.** Inflammation burden in femoral plaques.

603 Box-and-whisker plots represent percentage of CD68+ stained area (A) and number of
604 CD20+ cells (B) in femoral plaques (intima layer) of our patients, comparing the
605 inflammation infiltration rates of monocytes/macrophages (CD68+) and B lymphocytes
606 (CD20+) in patients who suffered (versus not) from MALE, MACE, MALE and/or MACE, or
607 global mortality. None of the differences were statistically significant.

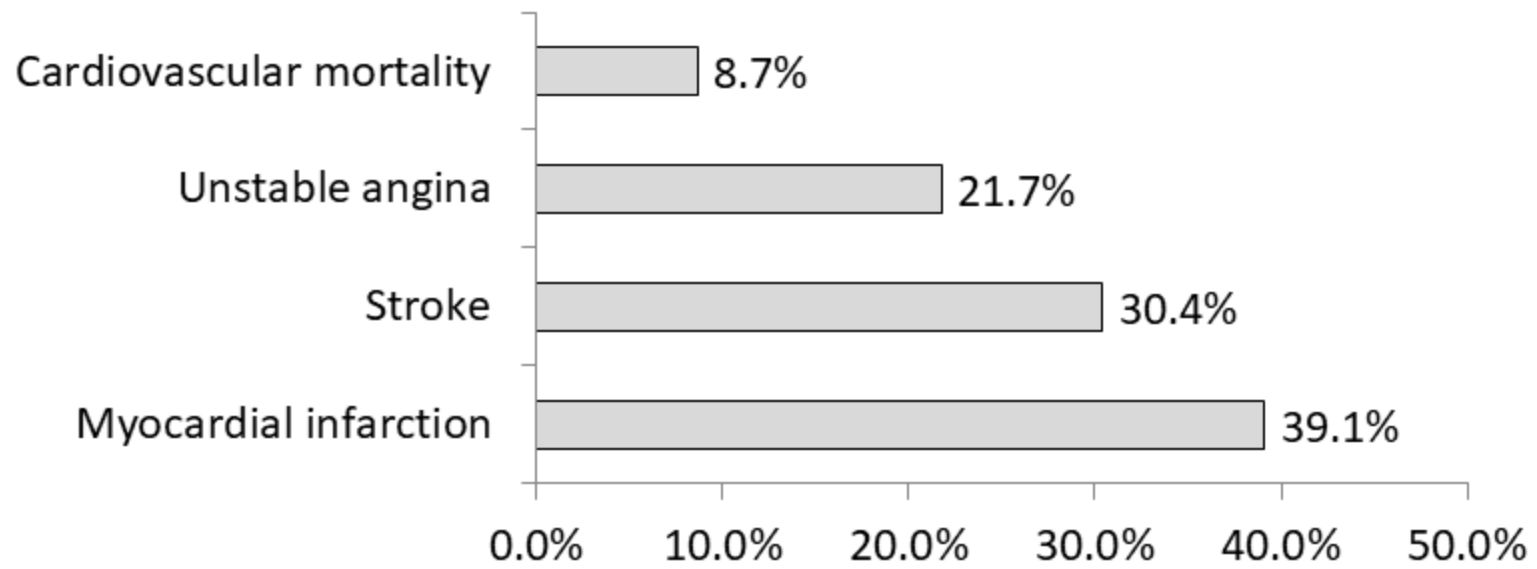
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Males

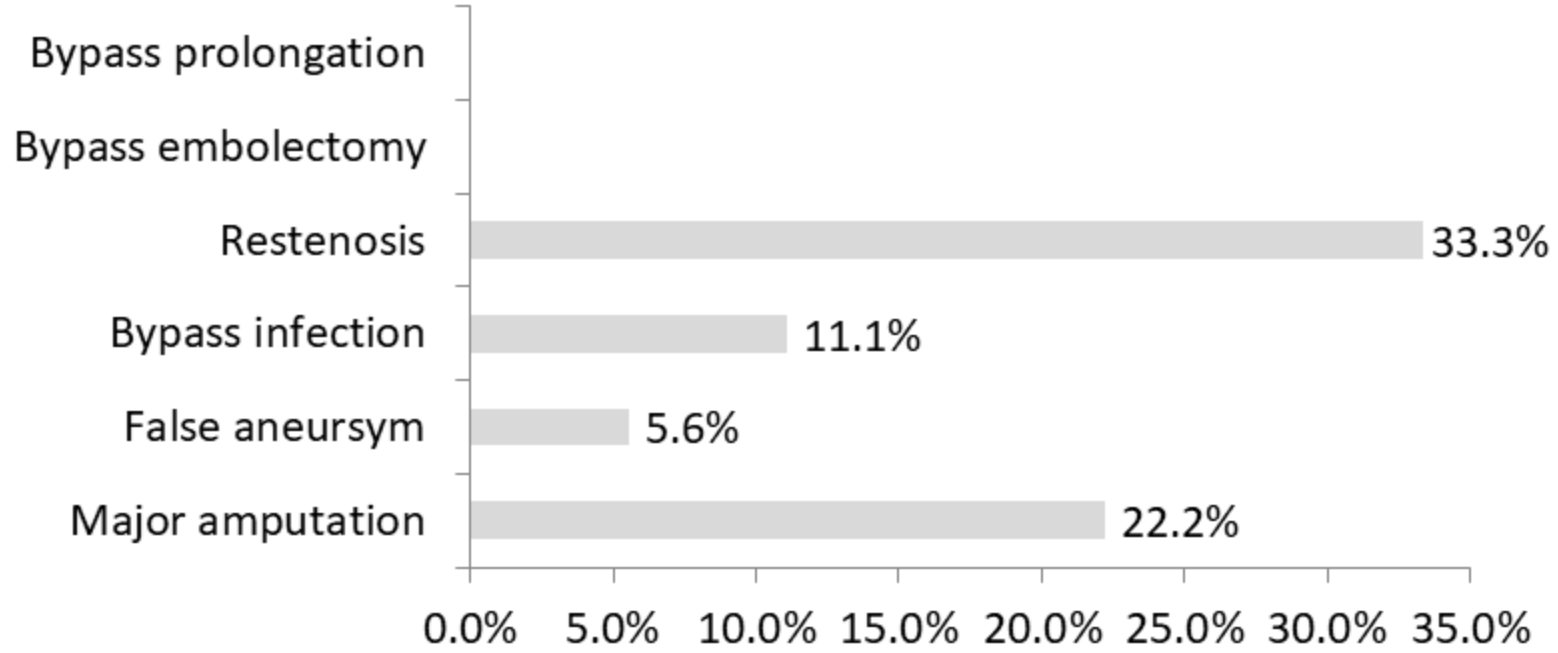


		0	12	24	36	48	60
MACE	Nb at risk	72	58	53	39	26	12
	% cumulative survival	100%	90,0%	86,7%	77,9%	70,6%	67,4%
MALE	Nb at risk	77	61	52	40	26	14
	% cumulative survival	100%	88,1%	83,6%	78,3%	75,5%	71,6%

MACE
n=23



MALE
n=18



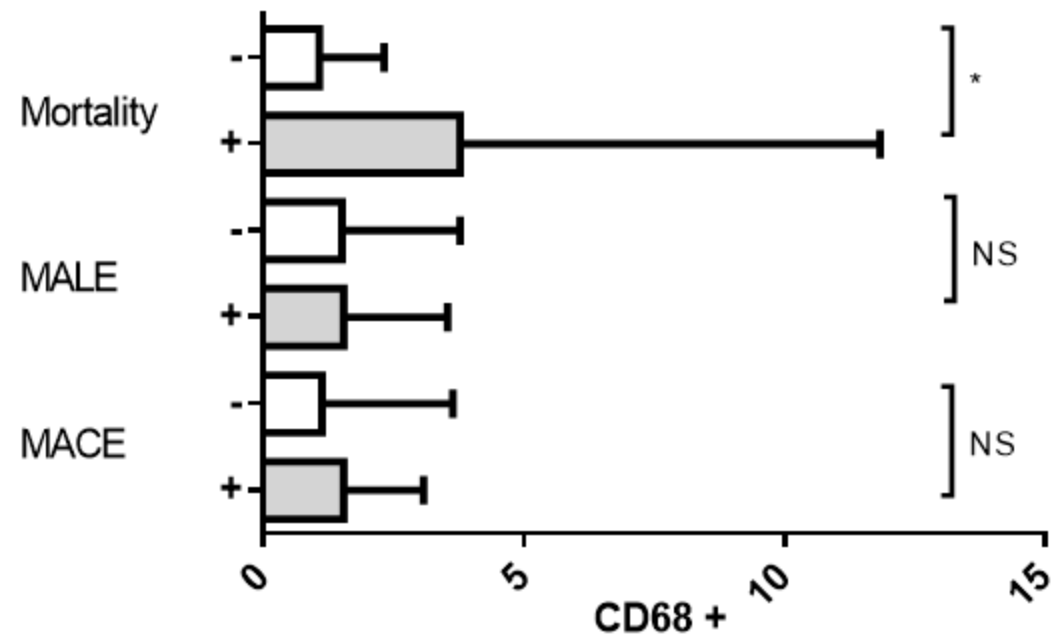
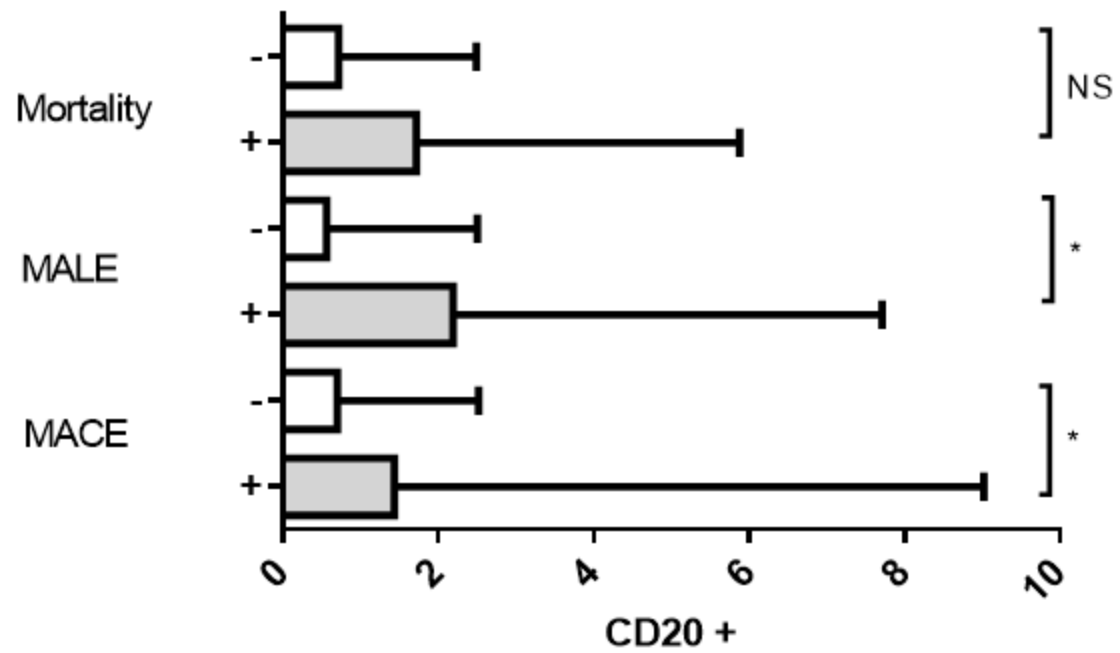


Table I. Demographic and histological characteristics of patients.

		Total	MACE	<i>P-value</i>	MALE	<i>P-value</i>	Global mortality	<i>P-value</i>
Population, n		81 pts & 86 limbs	23 / 81 pts		18 / 86 limbs		19 / 81 pts	
Male, n (%)		72 (89.0)	19 (83)	-	17 (94)	-	15 (79.0)	-
Age (y), mean ± SD		67.3 ± 9.3	69.7 ± 9.4	.21	68.8 ± 8.9	.23	70.9 ± 10.7	.09
BMI (kg/m ²), mean ± SD		25.8 ± 4.8	26.5 ± 3	.74	25.4 ± 3.6	.15	25 ± 3.2	.13
Follow-up (m), median [quartiles]		42.4 [26.9 - 58.8]						
History								
History of smoking, n (%)		32 (40)	11 (48)	.17	8 (44)	.86	6 (32)	.86
HTN, n (%)		64 (79)	20 (87)	.86	17 (94)	.13	16 (84)	.62
Dyslipidemia, n (%)		59 (73)	17 (74)	.55	14 (78)	.65	15 (79)	.93
Diabetes, n (%)	Type I	5 (6)	1 (4)	.12	2 (11)	.27	2 (11)	.53
	Type II	21 (26)	9 (39)		6 (33)		7 (37)	
Coronary events, n (%)		45 (56)	13 (57)	.76	10 (56)	.73	12 (63)	.55
Cerebrovascular disease, n (%)		9 (11)	7 (30)	.009*	4 (22)	.1	4 (22)	.37
Renal impairment, n (%)		12 (15)	5 (22)	.06*	3 (17)	.36	7 (37)	.0007*
Creatininemia (μmol/L, mean ± SD)		92.2 ± 58.8	92.3 ± 41.1	.18	89.2 ± 47.5	.87	129.9 ± 104.2	.0002*
Treatments								
Statins, n (%)		66 (81)	18 (78)	.43	15 (83)	.82	17 (89)	.95
Anti-platelet agents, n (%)		72 (89)	21 (91)	.63	15 (83)	.31	14 (74)	.10
PAD								
Rutherford, n (%)	1 - 3	62 (77)	15 (65)		11 (61)		9 (47)	
	4	7 (9)	3 (13)	.14	2 (11)	.06	2 (11)	.38
	5 - 6	12 (15)	5 (22)		5 (28)		8 (42)	.001*
ABI, mean ± SD		0.6 ± 0.3	0.5 ± 0.3	.57	0.6 ± 0.3	.28	0.6 ± 0.3	.95
Surgical procedure								
Endarterectomy and patch, n (%)		28 (35)	6 (26)		5 (28)		5 (26)	
Ipsilateral femoral bypass, n (%)		46 (57)	14 (61)		11 (61)		13 (68)	
Aorto-bifemoral bypass, n (%)		7 (9)	3 (13)		2 (11)		1 (5)	
Hospital stay (d), median [quartiles]		8 [7 - 10]	9 [7 - 15]		13.3 [8 - 14]		10 [7 - 17]	

First column presents results of the whole cohort, then separated in subgroups regarding MACE, MALE and global mortality.

All values are presented as mean ± SD or median [25th – 75th percentiles) for continuous data, or as number of patients (percentage) for frequency data. P-values are considered statistically significant when <.05 (*). P-values were results from univariate Cox regressions with stratification on cohort and gender.

Abbreviations: MACE = major adverse cardiac events (including cardiovascular mortality and/or unstable angina and/or myocardial infarction and / or stroke), MALE = major adverse limb events (including major above-the-ankle amputation and / or major reintervention on the index limb), y = years, m = months, d = days, BMI = body mass index, kg/m² = kilograms per meter square, HTN = hypertension, PAD = peripheral artery disease, ABI = ankle-brachial index, pts = patients, n/a = not applicable.

Table II. Histological and biological features for the population, MACE and MALE groups.

	Total (81)	MACE (23)	<i>P-value</i>	HR	95% CI	<i>P-value adjusted</i>	MALE (18)	<i>P-value</i>	HR	95% CI	<i>P-value adjusted</i>
Histological features											
Microcalcifications. n (%)	16 (20)	3 (13)	.53	0.50	0.12 ; 2.00	.33	3 (17)	.76	1.20	0.28 ; 5.08	.80
Sheet-like calcifications. n (%)	63 (78)	21 (91)	.13	9.28	1.14 ; 75.2	.04	15 (83)	.69	1.367	0.34 ; 5.49	.67
Nodules. n (%)	66 (81)	18 (78)	.19	0.55	0.17 ; 1.80	.32	12 (67)	.04	0.13	0.04 ; 0.50	.003*
Osteoid metaplasia. n (%)	41 (51)	12 (52)	.86	1.03	0.31 ; 3.36	.96	9 (50)	.74	0.763	0.25 ; 2.26	.62
White blood cells formulae											
White blood cells (G/L). mean ± SD	7.2 ± 2.4	7.9 ± 3.3	.10	1.17	0.97 ; 1.41	-	8.2 ± 4	.01*	1.293	1.05 ; 1.59	-
Neutrophils (G/L). mean ± SD	4.4 ± 2.1	5.1 ± 3	.08*	1.19	0.98 ; 1.44	-	5.5 ± 3.7	.0001*	1.60	1.247 ; 2.06	.0003
Lymphocytes (G/L). mean ± SD	1.9 ± 0.8	2 ± 0.9	.13	1.478	0.89 ; 2.42	-	2 ± 1	.62	1.15	0.67 ; 1.96	-
Monocytes (G/L). mean ± SD	0.7 ± 0.2	0.7 ± 0.3	.06*	8.57	0.90 ; 81.6	-	0.7 ± 0.3	.97	1.05	0.08 ; 13.4	-
Histological inflammation											
CD20 +. median[quartiles]†	0.7 [0.14 - 2.51]	1.44 [0.19 - 5.74]	.04*	1.08	1.00 ; 1.17	-	1.95 [0.19 - 18.88]	.03*	1.06	1.01 ; 1.13	-
CD68 +. median[quartiles]†	1.13 [0.36 - 3.49]	1.54 [0.75 - 2.83]	.99	1.00	0.86 ; 1.16	-	1.37 [0.48 - 2.34]	.66	0.59	0.54 ; 1.47	-

First column presents results of the whole cohort then separated in subgroups regarding MACE and MALE.

All values are presented as mean ± SD for continuous data, or as number of patients (percentage) for frequency data. For histological features correlation was calculated and is presented as hazard ratio (HR) with its 95% confidence interval (IC95). P-values are considered statistically significant when <.05 (*). P-values were results from univariate Cox analyses with stratification on cohort and gender. P-values adjusted were results from multivariate Cox analyses with stratification on cohort. † n = 38 plaque analysis from the ECLA cohort.

Abbreviations: MACE = major adverse cardiac events (including cardiovascular mortality and/or unstable angina and/or myocardial infarction and / or stroke). MALE = major adverse limb events (including major above-the-ankle amputation and / or major reintervention on the index limb). HR = hazard ratio. IC95 = 95% confidence interval.