Dynamics of intracoronary thrombosis in STEMI and sudden death patients
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A pattern of disperse plaque microcalcifications identifies a subset of plaques with high inflammatory burden in patients with acute myocardial infarction

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Abstract

Aims: Inflammation plays a crucial role in plaque vulnerability. Calcifications can be detected by means of in vivo imaging techniques. The study purpose is to assess a potential association between tissue localization of calcifications and the inflammatory biomarkers, C-reactive protein (CRP), osteopontin and lipoprotein-associated phospholipase A2 (Lp-PLA2), in plaque tissue of patients with acute myocardial infarction (AMI).

Methods and results: Thrombectomy materials obtained from patients with electrocardiographically documented ST-segment elevation type of AMI (STEMI) were histologically screened for presence of thrombus, plaque tissues and calcifications. Size of calcifications was measured morphometrically, and their colocalization with the inflammatory biomarkers macrophages, CRP, osteopontin and Lp-PLA2 was assessed with immunostaining. A total of 171 samples containing plaque tissues were obtained from 562 thrombectomy procedures. Calcifications were observed in 67 (39%) plaque fragments, with diameters ranging from 4 to 170 µm. Plaque tissues with calcifications contained more frequently extracellular CRP and intracellular CRP in macrophages than those without calcifications (85%, 59% versus 64%, 32%, p=0.012 and 0.005 respectively). Similar results were obtained with osteopontin immunostaining (98%, 76% versus 56%, 40%; p<0.001 both). Furthermore, samples with calcifications were immunostained for CRP more intensely than those without calcifications (p=0.001). Finally, 96% of the plaque tissues stained positively for Lp-PLA2, but there was no association with presence of microcalcifications.

Conclusions: A pattern of disperse microcalcifications is positively associated with presence of the inflammatory biomarkers macrophages, CRP and osteopontin in thrombectomy materials of STEMI patients. Based on these findings, we speculate that such microcalcifications could have the potential to serve as a surrogate marker for plaques with high inflammatory burden.
Introduction

By far most cases of acute myocardial infarction (AMI) result from coronary thrombus formation superimposed on disruption of a vulnerable type of atherosclerotic plaque. Many studies have shown that the vulnerability to rupture is usually not a feature of a single (culprit) plaque, but may involve several plaques in the coronary system of a patient (pan coronary vulnerability). Such insights have boosted the search for in vivo markers of plaque vulnerability, by means of high resolution coronary imaging techniques or serum levels of inflammatory proteins, to identify patients with increased cardiovascular risk.

Calcification is an excellent marker for the presence of atherosclerosis, but its role on plaque instability is still unclear. Although coronary calcifications detected by electron beam computed tomography (EBCT) is strongly predictive for adverse cardiovascular outcome, severe calcification is not a feature of coronary luminal narrowing and plaque instability. Moreover, a systematic autopsy study on coronary arteries of sudden coronary death victims revealed that the majority of acute ruptured and thrombosed plaques is only mildly calcified. In addition, a recent in vivo study by using intravascular ultrasound (IVUS) suggested that not the degree, but the pattern of calcifications (multiple, small calcium deposits) could be associated with plaque instability.

In contrast to calcification, inflammation plays a crucial role in plaque vulnerability. Numerous intraplaque inflammatory proteins are capable of degrading the fibrous cap, which may eventually lead to plaque rupture and thrombosis. Furthermore, several inflammatory biomarkers have been elucidated that are associated with adverse cardiovascular outcome, of which particularly C-reactive protein (CRP), osteopontin (OPN) and lipoprotein-associated phospholipase A2 (Lp-PLA2) appear to have a strong predictive value.

With the development of high resolution intravascular imaging techniques, several histological characteristics of vulnerable plaques (thickness of fibrous cap, size of necrotic core) can be identified and quantified in vivo; but detection of the “vulnerability marker” intraplaque inflammation is still a problem that needs to be resolved in clinical practice. In contrast to intraplaque inflammation, calcifications are an excellent marker of atherosclerotic plaques and can be visualized even in micro-dimensions with use of high resolution image techniques. Dystrophic calcification typifies many chronic inflammatory processes in the human body, but a putative relationship between presence of calcifications and pro-inflammatory tissue markers has not yet been studied in vivo in the culprit lesions of AMI patients. This could be of interest, since hypothetically, distinct patterns of calcifications may serve as a surrogate marker of plaque inflammatory activity.

ST-segment elevation myocardial infarction (STEMI) is a subset of AMI and characterized by severe transmural necrosis and high short-term mortality; as results of which emergent revascularization treatment of the occluded coronary artery is recommended for patients with STEMI by means of thrombectomy adjunctive to percutaneous coronary intervention...
(PCI). Materials retrieved from thrombectomy appear suitable for histological study, since they have been shown to contain plaque fragments from culprit lesions of STEMI patients (in addition to thrombus) in up to 40% of cases. In the present study we investigated the relationship between distinct morphologic patterns of plaque calcifications and the intraplaque immunolocalization of macrophages, CRP, osteopontin and Lp-PLA2 with use of the thrombectomy materials.

 Materials and methods

Patient Population
Between January 2005 and December 2006, a total of 562 patients were treated with thrombectomy adjunctive to primary PCI. Patients were eligible if there was evidence of acute myocardial infarction with ST segment elevation of ≥0.2 mV in two or more contiguous leads on the admission electrocardiography and symptoms of <24 hours duration. Thrombectomy procedures were performed as previously described. On admission, all the patients received aspirin 300 mg and unfractionated heparin 5,000 to 10,000 IU. Clopidogrel was administered in a loading dose of 300 or 600 mg directly before or immediately after the procedure and was prescribed for at least 1 month. The use of glycoprotein IIb/IIIa inhibitors and antithrombotic medications was at the discretion of operators. After access into the femoral artery, the flexible thrombectomy catheter was advanced in the infarct-related coronary artery. Before balloon dilatation or stent implantation, several suctions were performed at the site of culprit lesions. The aspirated materials were collected in the collection bottle, which could be used for this study. Information with respect to cardiovascular risk factors was obtained at bedside in the catheterization unit. Serum hsCRP values, measured on admission, were obtained from the electronic hospital record.

Selection of thrombectomy materials
All the aspirated materials were placed in 10% neutral buffered formalin immediately after retrieval, fixed for at least 24 hours and embedded in paraffin. Six-μm-thickness sections were stained with hematoxylin and eosin stains (H&E stain) for histopathological observations including identification of calcifications. Adjacent tissue sections were stained immunohistochemically for evaluation of the intraplaque inflammatory biomarkers. Size of samples was measured macroscopically on glass slices. Only samples of ≥1 mm², and of those only the samples that contained histologically confirmed atherosclerotic plaque tissues, were enrolled in this study (Figure 1).
Morphometric analysis of tissue calcifications and visualization of colocalization between calcifications and macrophages

Morphometric analysis of calcifications was performed on Alizarin red S (ARedS) stained sections. Immuno-enzyme double staining with anti-HAM56 antibody (1:50, Dako, Glostrup, Denmark) and ARedS was used to study the colocalization between calcifications and macrophages. Images of calcifications were acquired at x100 magnification using a Leica DFC500 digital camera mounted on a Leica DM 5000 microscope. Measurement of calcifications was performed automatically by using computer-based image software (Image Pro Plus 5.0, Media Cybernetics). Only the particles ≥4μm were measured for final evaluation, which is around the minimum range of detection of current high resolution imaging techniques.

Evaluation of CRP, OPN and Lp-PLA2 immunostaining

Presence of intraplaque CRP or Lp-PLA2 was evaluated with immunodouble staining combining anti-CRP antibody (rabbit monoclonal Y284, 1:500, Abcam, Cambridge, UK) or anti-Lp-PLA2 antibody (rabbit polyclonal HPA018157, 1:20, Sigma, st. Louis, USA) with anti-CD68 antibody (mouse monoclonal PG-M1, 1:100, Dako). Intraplaque OPN was analyzed with the use of immunotriple staining combining anti-OPN antibody (rabbit polyclonal 1:1000, Abcam), anti-smooth muscle actin (mouse monoclonal 1A4, 1:2000, Dako) and anti-CD68 antibody (PG-M1, 1:500, Dako). Double and triple staining methods were used as previously described. Diaminobenzidine (DAB, Dako), Vector Blue and Vector Red substrate kits (Vector Laboratories) were applied as chromogens. Negative controls were performed by replacing the primary antibodies with non-immune IgG of similar species, subclass and concentration. Steatotic liver tissue was used as positive control for CRP stain, cerebral tissue for Lp-PLA2 stain and post-mortem coronary atherosclerotic plaque served as positive control for OPN stain.

In each thrombectomy sample, CRP, Lp-PLA2 and/or OPN immunostaining was evaluated as either present or absent, and correlated with presence of calcification in the samples. Furthermore, intensity of extracellular CRP and Lp-PLA2 staining was graded into 3 categories: 0 (negative staining), 1 (mild positive staining) and 2 (strong positive staining).

Statistics

Statistical analysis was performed with SPSS 11. Results are expressed as mean ± SD or median with range. In case the data were normally distributed, the 2 groups were compared with unpaired t test. Comparison of non-normal distributed data of two independent groups, Mann-Whitney test was used and the Kruskal-Wallis test was used for ≥3 independent groups. Categorical data were expressed as percentage and evaluated with χ2 test. A p value of ≤0.05 was considered statistically significant.
Results

Patient clinical characteristics
Figure 1 shows the flow chart for selection procedure of study materials. Histologically confirmed materials were obtained from 82% (460/562) of all the thrombectomy procedures. Majority (63%, 289/460) of the materials are composed exclusively of thrombus, whereas 37% (171/460) of the materials consist of both thrombus and atherosclerotic plaque tissue. Only the thrombectomy materials containing plaque tissues (n=171) were included in the current study. Of these, calcifications were present in 39% (67/171) and absent in 61% (104/171) of the samples.

![Flow chart](image)

**Figure 1**: Selection of study materials. Thrombectomy materials obtained during primary PCI were screened and divided into two groups based on presence or absence of calcifications.

Patient characteristics were summarized in Table 1. There were no differences in major risk factors for cardiovascular diseases, in medication or serum hsCRP values between the patients with calcifications in the thrombectomy materials and those without calcifications. The interval from onset of symptom to opening of vessels was in 96% of the patients within 12 hours and no difference was found between the study groups (p=0.95). Pre-infarct unstable angina, defined as unstable angina of <30 minutes in the proceeding 2 weeks, was observed in 23% of all the patients and there was no difference between the two study groups (p=0.22).
### Table 1. Patient clinical characteristics.

<table>
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<th>Calcification (67)</th>
<th>Non-calcification (104)</th>
<th>p value</th>
</tr>
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<tr>
<td>Gender (Male)</td>
<td>68.7%</td>
<td>76.9%</td>
<td>0.29</td>
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<tr>
<td>Age (years; mean, ±SD)</td>
<td>63.5 ± 12.1</td>
<td>63.1 ± 13.2</td>
<td>0.82</td>
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<td>Diabetes mellitus</td>
<td>14.1%</td>
<td>22.1%</td>
<td>0.28</td>
</tr>
<tr>
<td>Hypertension</td>
<td>37.3%</td>
<td>35.6%</td>
<td>0.87</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>50.0%</td>
<td>54.8%</td>
<td>0.80</td>
</tr>
<tr>
<td>Current smoker</td>
<td>43.1%</td>
<td>42.2%</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>38.8%</td>
<td>31.8%</td>
<td>0.41</td>
</tr>
<tr>
<td>Statin</td>
<td>39.4%</td>
<td>33.7%</td>
<td>0.51</td>
</tr>
<tr>
<td>BMI</td>
<td>25.6</td>
<td>26.1</td>
<td>0.16</td>
</tr>
<tr>
<td>(kg/m²; median, range)</td>
<td>17.7-47.8</td>
<td>18.8-41.2</td>
<td></td>
</tr>
<tr>
<td>Serum hsCRP</td>
<td>3.8</td>
<td>2.6</td>
<td>0.24</td>
</tr>
<tr>
<td>(mg/l; median, range)</td>
<td>0.4-7.1</td>
<td>0.9-8.1</td>
<td></td>
</tr>
<tr>
<td>Pre-infarct unstable angina</td>
<td>28.6%</td>
<td>18.8%</td>
<td>0.22</td>
</tr>
<tr>
<td>Event to balloon time</td>
<td>184.5</td>
<td>185.0</td>
<td>0.95</td>
</tr>
<tr>
<td>(min; median, range)</td>
<td>75.0-959.0</td>
<td>64.0-844.0</td>
<td></td>
</tr>
<tr>
<td>Location of lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCA</td>
<td>50.8%</td>
<td>50.5%</td>
<td>0.98</td>
</tr>
<tr>
<td>LCA</td>
<td>44.6%</td>
<td>45.5%</td>
<td></td>
</tr>
<tr>
<td>LCx</td>
<td>4.6%</td>
<td>4.0%</td>
<td></td>
</tr>
<tr>
<td>Size of materials</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(mm; median, range)</td>
<td>3.0</td>
<td>3.5</td>
<td>0.81</td>
</tr>
<tr>
<td>(range)</td>
<td>1.0 – 11.0</td>
<td>1.0 – 18.0</td>
<td></td>
</tr>
</tbody>
</table>

Continuous variables are expressed as mean ± SD or median with range. Categorical variables are expressed as percentages. RCA: right coronary artery, LCA: left coronary artery, LCx: left circumflex; BMI: body mass index; hsCRP: high sensitive C-reactive protein.

**Calcification score in plaque tissue**

All together, a total of 1699 calcium particles were measured in the sections that contained calcifications. Calcifications in the samples were disperse microcalcifications with diameters ranging from 4 µm to 170 µm and a median of 9 µm (Figure 2A and 2B).
Figure 2: Morphometric analysis of calcifications.
2A – Immuno-enzyme double staining with anti-HAM56 antibody and Alizarin Red S (x 200 magnification). Calcifications stained in red as shown with arrows and macrophages stained in blue. 2B – Pooled data of diameters of all the measured calcifications; Range: 4-170 µm; Median: 9 µm.

In situ colocalization of calcifications with plaque cells, C-reactive protein, osteopontin and lipoprotein-associated phospholipase A2
Immunohistochemistry was used to compare the in situ presence of inflammatory biomarkers in plaque tissues containing microcalcifications with those without microcalcifications. Because of the limited size of aspirated samples, only 119/171 (70%) of the selected thrombectomy materials were available for CRP staining, 115/171 (67%) for OPN staining and 45/171 (26%) thrombectomy samples were available for Lp-PLA2 staining. CD68+ foam cells were nearly always located inside lipid rich plaque tissue in both groups (93% of tissues with microcalcifications and 86% of tissues without microcalcifications, p=0.38).

If present, extracellular CRP staining was found throughout lipid plaque tissue (Figure 3A, 3B and 3C), whereas extracellular OPN staining appeared bound to microcalcifications (Figure 3E). Immunotriple staining with anti-CD68, anti-smooth muscle actin and anti-OPN antibodies showed that cytoplasmic OPN was present in macrophages only, and not in smooth muscle cells. As shown in Table 2, both intra- and extracellular immunostaining of CRP and OPN was found more frequently in plaque tissues with microcalcifications than those without microcalcifications (p<0.05 for all). Furthermore, intensity of CRP immunostaining was greater in samples with microcalcifications than those without microcalcifications (p=0.001, Figure 3D).

Immunostainable Lp-PLA2 was present in 96% of plaque tissues containing thrombectomy samples, strictly localized in the areas with extracellular lipid and macrophages (vulnerable plaque tissue) (Figure 3F). However, there were no differences observed in presence (Table 2) or staining intensity of Lp-PLA2 between calcified and non-calcified plaque tissues (p=0.15).
Figure 3: Immunostaining for intraplaque inflammatory biomarkers: macrophages, C-reactive proteins (CRP), osteopontin (OPN) and lipoprotein-associated phospholipase A2 (Lp-PLA2).
Panel A – negative CRP staining: no CRP immuno-activity; only macrophages stained in red (low power view of x 40 magnification). “T” indicates thrombus compartment; “Pl” indicates plaque compartment.
Panel B – mild positive CRP staining: CRP stained in light blue and macrophage stained in red. Panel C – strong positive CRP staining: CRP stained diffusely in dark blue; arrow shows macrophage with intracellular CRP stained in purple. Panel D – Extracellular CRP in atherosclerotic plaques was significantly more intensely stained in samples with microcalcifications compared to those without microcalcifications (p=0.001). Chi-square test is used for statistical analysis. Panel E – Extracellular osteopontin stained in red and macrophages in brown, (x 200 magnification). Arrow shows macrophage with intracellular OPN. Panel F – Immunodouble staining with anti-Lp-PLA2 antibody and anti-CD68 antibody, (x 200 magnification). Extracellular Lp-PLA2 stained in blue and macrophage in red, arrow shows macrophage with intracellular Lp-PLA2.
Table 2. Overview of results.

<table>
<thead>
<tr>
<th></th>
<th>Calcification group</th>
<th>Non-calcification group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcification diameters (µm; median, range)</td>
<td>9, 4-170</td>
<td>86%, (57/66)</td>
<td>0.38</td>
</tr>
<tr>
<td>Macrophages</td>
<td>93% (49/53)</td>
<td>86% (57/66)</td>
<td>0.38</td>
</tr>
<tr>
<td>Extracellular CRP</td>
<td>85% (45/53)</td>
<td>64% (42/66)</td>
<td>0.012</td>
</tr>
<tr>
<td>Intracellular CRP in macrophages</td>
<td>59% (31/53)</td>
<td>32% (21/66)</td>
<td>0.005</td>
</tr>
<tr>
<td>Extracellular OPN</td>
<td>98% (49/50)</td>
<td>56% (36/65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intracellular OPN in macrophages</td>
<td>76% (38/50)</td>
<td>40% (26/65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>32% (16/50)</td>
<td>43% (29/65)</td>
<td>0.18</td>
</tr>
<tr>
<td>Extracellular Lp-PLA2</td>
<td>96% (22/23)</td>
<td>96% (21/22)</td>
<td>1</td>
</tr>
<tr>
<td>Intracellular Lp-PLA2 in macrophages</td>
<td>61% (14/23)</td>
<td>46% (10/22)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as median with range. Categorical variables are expressed as percentages. Chi-square test is used for statistical analysis. CRP indicates C-reactive protein; OPN indicates osteopontin; Lp-PLA2 indicates lipoprotein-associated phospholipase A2.

Discussion

Thrombectomy adjunctive to primary PCI, which is currently the treatment of choice for patients with STEMI in our institution, provides a unique opportunity to retrieve large numbers of tissue samples from culprit atherosclerotic lesions related to onset myocardial infarction. In the present study we demonstrated that thrombectomy materials can be used as ex vivo materials for histopathological investigation of potential biomarkers for plaque vulnerability in STEMI patients. The primary results of this study show that disperse calcium deposits with dimensions of microcalcifications (median=9 µm, range 4-170 µm), can be found in 39% of the thrombectomy samples containing plaque tissues, and are strongly associated with immunostaining of the biomarkers for cardiovascular risk CRP and OPN in plaque tissues, but not Lp-PLA2.

Microcalcifications in culprit lesions of STEMI patients
Although calcification is a well known feature of coronary atherosclerotic plaques, its potential role in onset of plaque instability remains controversial. An autopsy study by Sangiorgi et al showed that coronary calcification was proportional to overall plaque burden, but was not associated with the severity of anatomic stenosis. However, with the introduction of electron beam computed tomography (EBCT), coronary calcification can be analyzed quantitatively in vivo, and several clinical studies have shown that the EBCT-derived coronary calcium score can be considered as a strong predictor of adverse cardiovascular prognosis, even in asymptomatic patients. Also size of calcifications seems to be important. Virmani et al revealed that calcifications in 65% of acute ruptured plaques of sudden coronary death victims were radiographically so-called “speckled calcifications”. Similar results were obtained by Ehara et al who, with use of IVUS, compared calcifications in culprit lesions...
of acute coronary syndromes with those of chronic stable angina. They observed multiple, small calcium deposits (so-called “spotty calcifications”) in the tissues of patients with acute coronary syndromes, in contrast to patients with stable angina who had only few but large calcifications. Our findings of disperse microcalcifications in 39% of the thrombectomy specimens containing plaque tissues which are all obtained from culprit plaques underlying STEMI, confirm and extend these previous observations by measuring precisely the range of sizes of the microcalcifications in plaque tissues.

Moreover, such microcalcifications could play an active role in the process of plaque destabilization prior to rupture. A recent in vitro study suggested that microcalcifications with a diameter of 1 µm or less could lead to apoptosis of human vascular smooth muscle cells, and these cells are considered to play a protective role in plaque stability.24 In addition, several studies demonstrated that minute cellular-level calcium particulates could increase local stress in fibrous cap and lead to plaque rupture.25, 26

Association between calcification and inflammatory biomarkers
The concept that inflammation plays a crucial role in plaque vulnerability is widely accepted9, and the application of inflammatory biomarkers as tools in cardiovascular risk stratification has become a hotspot. Among all the biomarkers, the acute phase protein CRP is studied most extensively and considered to be a robust predictor of poor cardiovascular outcome.9-11 Several clinical studies have investigated the putative relationship between serum CRP levels and coronary calcification. In the Framingham Heart Study a positive association was reported between level of CRP in the blood and subclinical coronary calcification detected door EBCT.27 In contrast, the SIRCA study showed that serum hsCRP was not at all associated with coronary calcifications in men, and only weakly in women.28 The present study also did not reveal any difference in serum hsCRP levels between patients with- and without plaque microcalcifications in their thrombectomy materials. But, when we applied CRP-immunostaining on the same specimens of all the patients, it was shown that the plaque tissues with microcalcifications contain more frequently immunostainable CRP, including CD68+ macrophages with intracytoplasmic CRP. Moreover, the extracellular CRP was immunostained more intensely in the plaque tissues with microcalcifications than those without microcalcifications. These findings showed that CRP inside the plaques associated with microcalcifications appears to be a far more common feature as can be appreciated from measurement of serum hsCRP levels. Still such low levels of intraplaque CRP synthesis can be of relevance, since CRP exerts several pro-inflammatory effects inside the plaque29, which may further increase plaque vulnerability.

Osteopontin (OPN) is another important biomarker and strongly associated with poor cardiovascular prognosis.12 In addition, OPN plays an important role in migration of macrophages and inhibits ectopic calcification in human body.30, 31 Similar to our results on CRP immunostaining, we found a positive association between extracellular immunostainable

Atherosclerotic plaque (micro)calcifications and inflammatory biomarkers | 45
OPN, OPN containing macrophages and the presence of microcalcifications in plaques. Such observations endorse the outcome of recent in vitro studies on macrophages co-cultured with calcium particles, which revealed that microcalcifications induce a proinflammatory effect in macrophages, as became evident by their secretion of proinflammatory cytokines.32 Lp-PLA2, as a calcium-independent phospholipase A2, is another novel inflammatory biomarker. Serum levels and in situ expression in carotid atherosclerosis of Lp-PLA2 are positively associated with adverse cardiovascular outcomes, independently of traditional risk factors.13, 14 We found positive Lp-PLA2 immunostaining in nearly all the plaque fragments containing extracellular lipid and macrophages (the vulnerable plaque components), which illustrates the importance of the protein as a biomarker. However, in contrast to our findings on CRP and OPN, we found no positive association between presence of microcalcifications and plaque Lp-PLA2 immunopositivity, so apparently a pattern of disperse microcalcifications can not serve as a surrogate marker for in situ presence of this protein.

Conclusion
In this study we have shown that when microcalcifications are present in plaque fragments of STEMI patients, they frequently colocalize with the inflammatory biomarkers CRP and OPN; as such, a subgroup of plaques with high inflammatory burden can be identified. As a future perspective, we may speculate that these microcalcifications in plaques, which can be detected by means of various in vivo imaging techniques, could serve as a potential surrogate marker for intraplaque inflammatory activity. However, the application of the microcalcifications as a marker for plaque instability needs to be confirmed, since the sensitivity, specificity and predictive value of this marker is still undefined without comparative data on non-culprit lesions.
References

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