Identification and quantification of macrophage presence in coronary atherosclerotic plaques by optical coherence tomography

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Aims

Vulnerable plaques are characterized by a high macrophage content. We investigated the optical coherence tomography (OCT) capability of identifying coronary plaque macrophage presence using tissue property indexes.

Methods and results

Fifteen epicardial coronary arteries were imaged by OCT and subsequently analysed by histology. Correlating OCT–histological sections were identified and regions of interest (ROIs) were selected on both atherosclerotic plaques and normal appearing vessel tracts. OCT-derived tissue property indexes named normalized standard deviation (NSD), signal attenuation, and granulometry index were applied on ROIs to identify inflamed ROIs defined as a macrophage percentage > 10 by histology. Forty-three paired samples (OCT frame and histology section) were considered suitable as ROIs for analysis. Eleven out of 43 ROIs were considered inflamed and the remaining 32 ROIs were non-inflamed on the basis of histological count of macrophage percentage. All OCT-derived tissue property indexes were positively correlated with macrophage percentage (P = 0.0001 for all). Receiver operating characteristic curve analysis showed that NSD, granulometry index, and signal attenuation had a significant area under the curve (area = 0.906, 0.804, and 0.793, respectively). A two-step algorithm requiring to first apply NSD with a cut-off value of 0.0570 followed by granulometry index was able to identify an inflamed ROI with a sensitivity of 100% and a specificity of 96.8%.

Conclusion

OCT was able to identify and quantify macrophage presence in coronary artery specimens using tissue property indexes. NSD and granulometry index showed the highest accuracy in identifying a significant plaque inflammation, especially if used together in a two-step algorithm.

Keywords

optical coherence tomography • macrophage • vulnerable plaque • normalized standard deviation

Introduction

Coronary plaque rupture is the main cause of myocardial infarction. Plaques with a large lipid pool, a thin fibrous cap, and a high macrophage content are at increased risk of rupture and therefore are considered vulnerable.1

Optical coherence tomography (OCT) is an intravascular imaging technique with a high resolution (20 μm) that enables the assessment of some features of plaque vulnerability: lipid content and fibrous cap thickness.2,3 However, limited data are currently available regarding the OCT capability of identifying macrophages.4 Macrophages are inflammatory cells that exert a central role in plaque destabilization by releasing proteolytic enzymes and other pro-inflammatory mediators that, in turn, can lead to fibrous cap rupture and subsequent plaque thrombosis.5

Previous studies have shown that macrophages can be detected by OCT either using a visual inspection method (punctate regions with a high signal attenuation)6 or applying a dedicated offline software.7 The latter was tested on straight rims of tissue and proved to be able to measure the OCT signal variance named normalized standard

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deviation (NSD) that tended to increase in the presence of a significant macrophage content.

We designed an ex vivo pathologic and OCT study aimed at assessing the ability of multiple OCT-derived parameters, including NSD, granulometry index, and signal attenuation, to identify plaques of coronary specimens with a significant macrophage content using the intravascular frequency-domain-OCT. The final goal was to propose an OCT-based algorithm, capable of identifying plaques with a significant macrophage content with the highest accuracy.

**Methods**

**Specimens**

Fifteen epicardial coronary arteries were harvested from 12 randomly selected formalin-fixed human hearts excised at transplantation from patients with clinical history of coronary artery disease (seven males and five females, mean age 68 ± 3 years). No patient received corticosteroids and/or immunosuppressants before transplantation. Major clinical features are summarized in Table 1. The soft tissue surrounding the arteries was dissected from each coronary artery. Small collaterals were tied off with sutures, and specimens were stored in formalin until OCT assessment. The Institutional Review Board approved the experimental protocol.

**OCT imaging studies**

OCT assessment of coronary specimens was conducted following a validated protocol. The day before OCT examination, specimens were left for 24 h in a warm saline solution at a temperature of 37°C. The distal end of the artery was occluded with a large cork. A 6Fr sheath was sewn into the side arm of the sheath. An intravascular OCT catheter was inserted through the diaphragm of the sheath. OCT images were obtained using an automatic pull-back device applying an acquisition speed of 20 mm/s. The position of the interrogating beam on the tissue was monitored with a visible light-aiming beam that was coincident with the infrared beam. Precise matching of OCT and histology was obtained using a 26-gauge automatic pull-back device applying an acquisition speed of 20 mm/s. The position of the interrogating beam on the tissue was monitored with a visible light-aiming beam that was coincident with the infrared beam. Precise matching of OCT and histology was obtained using a 26-gauge automatic pull-back device applying an acquisition speed of 20 mm/s.

**Histological examination**

After OCT assessment, each coronary arterial segment was pressure fixed in 10% neutral-buffered formalin. Each coronary artery segment was then cut into multiple segments (0.5–1 cm) maintaining reference markers indicated by the OCT examination. After fixation for 48 h, standard paraffin embedding was performed. Each paraffin block was serially cut distal to proximal. From each block, we obtained 180–360 sections that were collected on 60–120 slides (three sections of 5 μm each/slide) until exhaustion of the coronary sample. Serial, adjacent sections were systematically stained with haematoxylin–eosin and movat stain and kept unstained on electrostatic positive slides for immune staining with anti-CD68 (PGM1-Dako).

All slides were collected in a web-assisted database for independent evaluation of the OCT and pathology teams.

**OCT–histology matching and OCT analysis of cross-sections**

OCT–histology matching was carried on with a two-step procedure including cross-section analysis and region of interest (ROI) selection. First, OCT cross-sections of both atherosclerotic plaques and non-diseased vessel segments were matched with the corresponding histological sections using landmarks (side branches, calcifications, ink marks, and needles). Importantly, the same luminal shape was required to obtain OCT and histological pairs (Figure 1). Second, ROIs were drawn on both atherosclerotic and non-diseased OCT cross-sections using 300 × 125 μm (lateral × axial) areas. The same ROI was subsequently selected in the corresponding histological section (Figure 2).

To guarantee a blinded analysis, ROI analysis of macrophage content was performed after having selected all the ROIs by two independent pool of researchers. OCT analyses were performed at Rome Heart Research core laboratory (Rome, Italy) by two expert readers (F.P. and L.D.V.), whereas histological measurements of macrophage content were performed by two pathologists (E.A. and M.A) at Centre for Inherited Cardiovascular Diseases (Pavia, Italy).

Atherosclerotic cross-sections were distinguished into fibrous, lipid, and calcified by OCT as previously reported. Briefly, fibrous plaque exhibited homogeneous highly backscattering (i.e. signal-rich) regions. Calcified plaque was identified by the presence of signal-poor regions with sharply delineated upper and/or lower borders. Lipid-rich plaque showed diffusely bordered, signal-poor regions with overlying signal-rich bands, corresponding to fibrous cap.

A non-diseased vessel cross-section was imaged as a three-layered structure by OCT.

**OCT analysis of ROIs**

ROIs were categorized into two types based on the amount of inflammation on histology, AperioScanner was used to calculate macrophage percentage as the ratio between the area including CD68+ cells and the total area of the ROI. Inflamed ROIs had a percentage of CD68+ cells > 10%, while non-inflamed ROIs had a percentage of CD68+ cells < 10%. Such cut-off value has been already applied in histopathological and ex vivo OCT studies.

OCT analysis of ROI required fibrous cap thickness assessment using an offline proprietary OCT consol (St Jude Medical, Inc., USA) and application of OCT-derived tissue property indexes including signal attenuation, NSD, and granulometry index. These indexes were assessed using a custom developed software written using the Matlab (MathWorks, Inc.).

Signal attenuation refers to the signal loss with depth in the OCT image and was calculated by fitting exponential curves of OCT signal to the signal profile of the pixels within the ROI plus the pixels

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**Table 1** Baseline clinical features of included patients

<table>
<thead>
<tr>
<th>Features</th>
<th>Number of patients</th>
<th>Age, mean (SD)</th>
<th>Male sex, n (%)</th>
<th>Diabetes mellitus, n (%)</th>
<th>Valve heart disease, n (%)</th>
<th>Previous ACS, n (%)</th>
<th>Previous CABG, n (%)</th>
<th>Previous PCI, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>12</td>
<td>68 ± 3 years</td>
<td>7 (58)</td>
<td>4 (33)</td>
<td>3 (25)</td>
<td>12 (100)</td>
<td>2 (16)</td>
<td>12 (100)</td>
</tr>
</tbody>
</table>

ACS, acute coronary syndrome; CABG, coronary artery by-pass; PCI, percutaneous coronary intervention; SD, standard deviation.
immediately beneath the ROI. Each pixel within the ROI was then fitted to an exponential curve, and the attenuation value was computed as the mean over all the radial profiles of the exponents of the best fitting curves. The NSD parameter measures the scattered signal by a tissue that leads to an intense light variation. NSD was calculated as previously described by Tearney et al. as the ratio between OCT signal variation and the difference between the maximal and minimal OCT signals of the cross-section.

Figure 1  OCT–histology matching and pair identification. An OCT cross-section was matched with the correlating histology section using landmarks. A lipid plaque with a thin fibrous cap is imaged by OCT (A) and histology (B). The luminal shape appears as a dome with a straight base (arrows) and a large arch (asterisks) confirming the correct matching between OCT and histology and leading to an OCT–histology pair.

Figure 2  ROI selection. A 300 × 125 μm (lateral × axial) area was selected in both OCT cross-section and histology section. Fibrous cap thickness and OCT-derived tissue property indexes (NSD, signal attenuation, and granulometry index) were assessed on OCT ROI (A). Correlating histology ROI was used to assess macrophage percentage by counting CD68+ cells (B).
The granulometry index represents a size filter of the structures imaged by OCT allowing to measure elements of a specific range of dimensions. It was obtained projecting the ROI in polar co-ordinates and applying a sieving process to the pixels intensity sized between 30 and 150 μm. Granulometry index was subsequently assessed by measuring the ratio between pixels in the specific range and speckle noise.

Statistical analysis
Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Categorical variables are presented as frequencies and analysed with the χ² test or Fisher’s exact test. Most continuous variables were not normally distributed as assessed by the Kolmogorov–Smirnov test for normality and thus were presented as median and inter-quartile range (IQR), and logarithmic transformation was applied to the data to allow Student’s t-test to be used. The relationship between macrophage content and fibrous cap thickness, NSD, signal attenuation, and granulometry index was assessed by Pearson correlation coefficient.

Receiver operating characteristic (ROC) curves were constructed by comparing the true-positive rate (sensitivity) with the false-positive rate of inflamed ROI for identifying the best cut-off value for NSD, attenuation, and granulometry index. An algorithm based on a combination of tissue property indexes was tested to achieve the highest accuracy in identifying inflamed from non-inflamed ROIs.

Results

OCT analysis of cross-sections and ROIs
A total of 43 paired samples (OCT cross-section and histology section) from 18 atherosclerotic plaques and 25 non-diseased vessel tracts were perfectly coincident and considered suitable as ROIs for analysis.

OCT analysis of selected cross-sections showed that a lipid plaque was present in 15 out of 18 (83%), a calcific plaque in 2 out of 18 (11%), and a fibrotic plaque in 1 out of 18 (6%) (Table 2).

The immunostain-based count of macrophage provided the basis for the definition of inflamed ROIs (n = 11) and non-inflamed ROIs (n = 32). In inflamed ROIs, the median value of macrophage percentage was significantly higher than in non-inflamed ROIs (15.6% IQR, 12.0–27.5 vs. 0.24% IQR, 0.10–0.58, P = 0.0001).

ROI analysis showed that NSD, granulometry index, and signal attenuation were significantly higher in inflamed ROIs. Conversely, fibrous cap thickness was significantly lower in inflamed ROIs (Table 3).

Correlations and cut-off values of OCT indexes for identifying inflamed ROIs
NSD, signal attenuation, and granulometry index showed a significant linear correlation with the percentage of macrophage on histology, while a significant inverse correlation was found for fibrous cap thickness (Table 4). A ROC curve analysis was applied to assess the most accurate OCT value able to distinguish between inflamed and non-inflamed ROIs. NSD, granulometry index, and signal attenuation had a significant area under the curve (0.906, 0.804, and 0.793, respectively) (Figure 3 and Table 5). An NSD value of 0.0570 was able to identify inflamed ROIs with a sensitivity of 90.9% and a specificity of 87.5%, leading to one false-negative case and four false-positive cases. A granulometry index cut-off value of 1.044 showed a sensitivity of 90.9% and a specificity of 87.5%, leading to one false-negative case and four false-positive cases (Table 5).

Proposed OCT-derived algorithm to identify inflamed ROIs
A two-step algorithm was applied onto OCT ROIs to distinguish between inflamed and non-inflamed ROIs with the goal to achieve the maximal accuracy. The first step required the application of an NSD cut-off value of 0.0570. In the presence of NSD values comprised in a grey zone (between 0.040 and 0.072) (Figure 4), a second step based on the granulometry index was then applied, using a cut-off value of 1.197. This two-step algorithm was able to identify an inflamed ROI, with a sensitivity of 100% and a specificity of 96.8% (Table 6).

Discussion

In the present ex vivo study, we applied OCT on coronary artery segments to compare OCT frames and immunostained sections of coronary plaques with the major aim of assessing whether the resolution of OCT allows the identification of macrophage clusters in vivo. OCT is proved to be able to characterize the typical major traits of plaque vulnerability such as lipid pool and fibrous cap thickness. As the OCT resolution is close to the dimensions of human macrophages/fibrous cells present in atherosclerotic plaques, OCT is potentially capable of detecting macrophages.

Macrophages have many and relatively large organelles, sustaining their high metabolic activity, that tend to scatter light very efficiently. This leads to either a high attenuation coefficient or a high backscatter coefficient of OCT images. Thus, when imaged

<table>
<thead>
<tr>
<th>OCT-defined plaque type</th>
<th>Case</th>
<th>Fibrous plaque</th>
<th>Lipid plaque</th>
<th>Calcific plaque</th>
<th>Normal vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-inflamed ROIs</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Inflamed ROIs</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>25</td>
</tr>
</tbody>
</table>
by OCT, macrophages appear as ‘bright spots’ with a high signal attenuation behind.7

Tearney et al.7 proved this concept applying the NSD value. An excellent accuracy was obtained with 100% sensitivity and specificity to identify a significant macrophage density within the fibrous cap. However, this study only included sections of carotid plaques. In addition, when the same group applied such an approach in an intravascular in vivo study, there was a minimal difference in NSD value between the culprit plaque and remote regions.13

We designed our study to identify a significant macrophage content in coronary atherosclerotic plaques by OCT-derived indexes and fibrous cap thickness using immunohistology as the gold standard. As a novelty, we also applied texture analysis named granulometry, which is based on a sieving process, for identifying macrophages.

In our study, 11 out of 43 ROIs showed by histology a significant macrophage content, defined as a macrophage content >10%. Fibrous cap thickness showed a negative correlation with the percentage of macrophage (Table 4) while signal attenuation showed a positive correlation suggesting that plaques with features of vulnerability such as large lipid pool and thin fibrous cap may have a higher degree of inflammatory cells.

Consistently, both NSD and granulometry index showed a positive correlation with macrophage content (Table 4).

To identify OCT cut-off values able to distinguish inflamed vs. non-inflamed ROI, OCT tissue property indexes were tested using ROC curves. NSD, granulometry index, and signal attenuation showed significant areas under the curve (Table 5). However, signal attenuation led to a greater number of false-positive cases, showing an impaired capability of identifying macrophages in the presence of lipid pools. Previous studies showed that signal attenuation was able to distinguish among plaque types. In fact, lipid pools lead to a higher signal attenuation than fibrous or calcium, causing a ‘signal-poor’ region.10,11 However, macrophages affect the OCT signal mimicking lipid material with a typical high attenuation and high signal scattering.10,11 To increase the ability to distinguish between lipid material and macrophages, we tested a software program that applies OCT acoustic parameters (NSD and granulometry index) and is potentially able to identify inflammatory cells.

Indeed, both NSD and granulometry index were not able to correctly classify all the ROIs, when applied alone. As a result, we tested a two-step algorithm first based on the use of NSD and as a second step, in case of borderline values, on the adoption of the granulometry index. This two-step algorithm was able to distinguish inflamed from non-inflamed ROIs with a sensitivity of 100% and a specificity of 96.8%.

**Conclusions**

Intravascular OCT was able to identify and quantify macrophage presence in coronary artery specimens using tissue property

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**Table 3** OCT findings in the inflamed and non-inflamed ROIs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Inflamed ROIs</th>
<th>Non-inflamed ROIs</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>11</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Fibrous cap thickness [μm (IQR)]</td>
<td>90 (60–100)</td>
<td>130 (110–160)</td>
<td>0.009</td>
</tr>
<tr>
<td>Signal attenuation</td>
<td>0.043 (0.048–0.034)</td>
<td>0.029 (0.036–0.025)</td>
<td>0.001</td>
</tr>
<tr>
<td>NSD</td>
<td>0.075 (0.060–0.084)</td>
<td>0.39 (0.031–0.051)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Granulometry index</td>
<td>1.16 (1.12–1.37)</td>
<td>0.58 (0.37–1.10)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

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**Table 4** Correlations between OCT findings and macrophage contents by histology

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSD</td>
<td>0.67</td>
<td>0.00001</td>
</tr>
<tr>
<td>Fibrous cap thickness</td>
<td>−0.60</td>
<td>0.00001</td>
</tr>
<tr>
<td>Signal attenuation</td>
<td>0.52</td>
<td>0.00001</td>
</tr>
<tr>
<td>Granulometry index</td>
<td>0.53</td>
<td>0.00001</td>
</tr>
</tbody>
</table>

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**Figure 3** ROC curve analysis. ROC curves for inflamed ROI using NSD (blue line), signal attenuation (marrow line), and granulometry index (green line).
Table 5  Cut-off values of OCT-derived parameters for identifying macrophage presence

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cut-off value</th>
<th>Area under the curve</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>False negative</th>
<th>False positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSD</td>
<td>0.057</td>
<td>0.906</td>
<td>90.9</td>
<td>12.5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Granulometry index</td>
<td>1.044</td>
<td>0.804</td>
<td>90.9</td>
<td>28.1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Signal attenuation</td>
<td>0.335</td>
<td>0.793</td>
<td>81.8</td>
<td>37.5</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

Figure 4  NSD assessment of inflamed ROI. Scatter plot displaying NSD values on the horizontal axis and macrophage percentage on the vertical axis. NSD cut-off value of 0.057 is marked by a vertical dashed line. Four false-positive (FP) and one false-negative cases were detected using NSD compared with histology. A grey zone area was designed to include the false-negative case and three out of four false-positive cases.

Table 6  OCT-based algorithm to identify macrophage content

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>Cut-off value</th>
<th>False negative</th>
<th>False positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>First step</td>
<td>NSD</td>
<td>0.057</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Second step</td>
<td>Granulometry index</td>
<td>1.197</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Limitations

OCT assessment of coronary artery specimens was obtained in formalin-fixed samples. Although the vessels were cleared with warm saline solution before OCT interrogation, we cannot exclude that formalin may have affected OCT signals. However, this event is unlikely if we take into account the fact that formalin fixation does not influence the presence, absence, and distribution of CD68+ cells.

Conflict of Interest: F.P. is a consultant for St. Jude Medical. The remaining authors have no conflicts of interest to declare.

References

A 74-year-old female patient with a history of deep venous thrombosis of the left axillary vein, presented with an unexpected relapse of ischaemic stroke. The transoesophageal echocardiography demonstrated normal interatrial septum and pulmonary veins. During contrast infusion using a left arm venous access, we noted a simultaneous opacification of the left and right cavities (Panel B, Supplementary data online, movie 1). Contrast injection into the right arm, obtained opacification only of the right cavities (Panel A, Supplementary data online, movie 2). Another injection into the left arm allowed visualization of a massive contrast flow (Panel C arrow, Supplementary data online, movie 3) through the left upper pulmonary vein (LUPV). These findings suggested an abnormal shunting of the left arm venous flow towards the LUPV. A computed tomography scan showed an ancient occlusion of the brachiocefalic vein (Panel D arrow) and a normal superior vena cava (SVC). The venous angiography of the left arm demonstrated both the occlusion of the left innominate vein (Panel E yellow arrow), with abundant collateralization towards the right side at the neck level and through internal mammary veins, and the opacification of the LUPV through collaterals (Panel E red arrow) coming from the left subclavian vein (LSV). Given her age and the multiple neurological sequelae, no interventional treatment was proposed. Abnormal venous return of the left upper lobe into the left innominate vein has been described, usually associated with partial anomalous venous connection (PAVC). It is a rare entity, only 3% of PAVC cases. It has not yet been noted as a retrograde pressure escape valve in consequence of an acquired systemic venous obstruction.

Supplementary data are available at European Heart Journal – Cardiovascular Imaging online.