On-line detection of cholesterol and calcification by catheter based Raman spectroscopy in human atherosclerotic plaque ex vivo

S W E van de Poll, K Kastelijn, T C Bakker Schut, C Strijder, G Pasterkamp, G J Puppels, A van der Laarse

Background: Raman spectroscopy has the unique potential to detect and quantify cholesterol and calcification in an atherosclerotic plaque in vivo.

Objective: To evaluate the sensitivity and specificity of this technique for detecting cholesterol or calcification in human coronary artery and aorta specimens ex vivo, using a compact clinical fibreoptic based Raman system developed for in vivo applications.

Design: From nine coronary arteries and four aorta specimens, 114 sites were evaluated for the presence of cholesterol and calcification by Raman spectroscopy and standard histology. Raman spectra were acquired and evaluated on-line in around five seconds.

Results: The correlation between Raman spectroscopy and histology was $r = 0.68$ for cholesterol and $r = 0.71$ for calcification in the plaque ($p < 0.0001$). Sensitivity and specificity for detecting cholesterol and calcification were excellent; receiver operating characteristic (ROC) analysis for each of the components revealed areas under the curves of $> 0.92$ ($p < 0.0001$). At the optimal cutoff values determined by ROC analysis, positive predictive values of $> 80\%$ and negative predictive values of $> 90\%$ were obtained.

Conclusions: On-line real time catheter based Raman spectroscopy detects accumulation of cholesterol and calcification in atherosclerotic plaque with high sensitivity and specificity.

METHODS

Tissue handling

Nine human coronary artery samples and four human aorta specimens were obtained at necropsy or from explanted hearts from patients undergoing heart transplantation. All samples were snap frozen in liquid nitrogen and stored at $-80^\circ$C until use. For spectroscopic investigation, the arteries were thawed, opened longitudinally, flattened, and positioned on a cardboard surface covered by aluminium foil and attached to a cross table. The samples were immobilised using 0.5 French needles. The tip of the fibreoptic catheter was positioned perpendicular to the arterial surface. Subsequently, the catheter tip was lowered until it was in contact with the tissue. Spectra were obtained in 500 µm steps over the entire diameter of the sample (coronary artery samples) or the full width of the aortic specimen. For correlation with pathology, the initial position of the catheter was marked with a suture through the arterial wall. In addition, a schematic drawing of the arterial inner surface was provided to the pathologist. In all, 228 spectra were obtained (mean (SD), 17 (4) spectra per section). After spectroscopic measurements, the arterial samples were refrozen at $-80^\circ$C and sent for histological evaluation.

Histology

Cross sections of the specimens (7 µm thick) were stained for general morphology (haematoxylin-eosin), collagen (picro-
sirius red), and calcification (von Kossa). The frozen cryosections of the human specimens were stained with oil red \(\text{O}\) for detection of total lipids in the intima of the artery. The lipid content was assessed by the empty spaces in the picro–sirius red stain and the clearly visible lipid droplets in the oil red \(\text{O}\) stains.

Each millimetre of artery was scored for the presence of cholesterol and calcium salts by a histologist who was blinded to the outcome of the study. Quantities of cholesterol and calcification were assessed by eye and expressed as 0%, 5%, 10%, 20%, 30%, 40%, 50%, and > 60% of the arterial surface. In all, 114 sites were evaluated.

Data acquisition and spectral analysis
The compact clinical Raman system and fibreoptic catheters (Enviva Gaser 5, Visonex Inc, Atlanta, Georgia, USA) that were used in the experiments have been described in detail.\(^8\) In brief, the system consists of an 830 nm diode laser (Process Instruments diode laser, Salt Lake City, Utah, USA) which delivers its light through the 400 \(\mu\text{m}\) central core of an optical fibre to the arterial sample. Scattered Raman light is collected in seven surrounding collection fibres (300 \(\mu\text{m}\)). The collection fibres of the catheter were coupled to a modified dispersive imaging spectrometer (System 100, Renishaw, Wotton under Edge, Avon, UK), equipped with a thermoelectrically cooled deep depletion CCD camera.\(^9\)

![Figure 1](https://example.com/figure1.png)

**Figure 1** Example of the Raman modelling procedure for quantification of proteins, calcification, and lipids, as described by Brennan and colleagues.\(^4\) (A) Raman spectrum after correction for background signal. This spectrum was obtained in 60 seconds from an atherosclerotic specimen using the fibreoptic catheter system. (B) Raman model spectra. From top to bottom: \(\beta\) carotene, calcification, adventitial fat, esterified cholesterol, cholesterol, delipidised atherosclerotic artery (protein 2), delipidised normal artery (protein 1). (C) Raman spectrum (black) and model fit (grey). The fit parameters are shown in the inset.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Correlation between the fit parameters calculated from Raman spectra acquired in 60 seconds (x axis), and the fit parameters calculated from Raman spectra that were acquired in respectively 5 seconds, 3 seconds, and 1 second (y axis). Panels A, B, and C: cholesterol; panels D, E, and F: calcification.
The software for real time data analysis has been described in detail. All spectra were analysed by means of a previously developed and validated least squares fitting routine, which uses Raman spectra of the main components of the arterial wall—that is, proteins, triglycerides, cholesterol, cholesteryl esters, calcium salts, and β carotene. To determine the molecular composition of the arterial wall, the relative weight percentages of the various components calculated by this fitting routine were then rescaled to add up to 100%.

An example of the fitting procedure is shown in fig 1.

Correlation with histology
Contributions of two adjacent Raman spectra (0.5 mm) were combined to obtain an average value of total cholesterol (TC) or calcium salts (CS) per mm of coronary artery. These data (n = 114) were compared with the data provided by histology. Receiver operating characteristic (ROC) analysis was used to determine sensitivity and specificity of Raman spectroscopy for detecting cholesterol and calcification.

Mainly because of cutting artefacts in the calcified sections, it was difficult to define the exact place of the catheter; hence correlation could be improved by histological analysis of larger segments. To this purpose, contributions of four adjacent Raman spectra were averaged. This averaged contribution was subsequently compared with the histopathological diagnosis of 2 mm artery segments (n = 57).

Data acquisition measurements
From a total of 54 spots with varying histopathological characteristics, a set of Raman spectra was obtained with collection times varying between 1 and 60 seconds. At each collection time (1 s, 2 s, 3 s, . . . 60 s), the Raman spectrum was fitted using the model developed by Brennan and colleagues. Fit contributions of protein, cholesterol, triglycerides, and calcification are presented as weight percentages. Fit contributions of β carotene are presented as arbitrary units.

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Short collection times (1–5 seconds) and at 60 seconds, did not achieve for data obtained with a minimum of 5 seconds of acquisition. Bland–Altman analysis showed that the differences between the values of cholesterol or calcification at acquisition. Excellent correlation coefficients were acquired in 60 seconds. For quantification of calcification (panels D to F) in, respectively, 5 seconds, 3 seconds, and 1 second, and the fit contributions calculated from spectra that were obtained in 60 seconds. For quantification of calcification, the correlation coefficients for each of the acquisition times were excellent ($R^2 > 0.99$), even for Raman data acquired with collection times as short as 1 second. For quantification of cholesterol, excellent correlation coefficients were achieved for data obtained with a minimum of 5 seconds of acquisition. Bland–Altman analysis showed that the differences between the values of cholesterol or calcification at short collection times (1–3 seconds) and at 60 seconds, did not vary in any systematic way.

Areas containing large quantities of cholesterol (or calcification) on histological evaluation showed large contributions in the Raman spectrum. There was a highly significant correlation between the quantitative data from histological analysis and the data obtained by Raman spectroscopy ($r = 0.68$ and $0.71$ for cholesterol and calcification, respectively; $p < 0.0001$). Qualitative detection of cholesterol and calcification was excellent; sensitivity and specificity for detecting even small pockets of cholesterol and calcification were high, as analysed by ROC analysis (fig 3, panels A and B). For cholesterol and calcification, areas under the ROC curve were $> 0.92$. ROC analysis was repeated on histological evaluations of 2 mm sections and resulted in areas under the curve of 0.95 (fig 3, panels C and D). For both evaluations, optimal cut off values for obtaining equal sensitivity and specificity were 4% for detecting cholesterol and 6% for detecting calcification. At these cut off values, sensitivity, specificity, and positive and negative predictive values are indicated in table 1.

Figure 4 shows the results of ROC analysis when using β carotene (a vitamin with high lipid solubility) to predict the presence of cholesterol in the atherosclerotic lesion. Areas under the curve were 0.92 (1 mm intervals) and 0.97 (2 mm intervals). Sensitivity and specificity were comparable to those for cholesterol, and are indicated in the grey areas in table 1.

The fit contributions at shorter collection times were then compared with the data obtained from Raman spectra that were acquired in 60 seconds. For each component, correlation coefficients were determined. In addition, Bland–Altman analysis was used to determine whether systematic errors were encountered and whether the correlation between the shorter collection times and the reference data obtained in 60 seconds was appropriate.

**RESULTS**

Figure 2 shows the correlation between the fit contributions of Raman spectroscopy for cholesterol (panels A to C) and calcification (panels D to F) in, respectively, 5 seconds, 3 seconds, and 1 second, and the fit contributions calculated from spectra that were obtained in 60 seconds. For quantification of calcification, the correlation coefficients for each of the acquisition times were excellent ($R^2 > 0.99$), even for Raman data acquired with collection times as short as 1 second. For quantification of cholesterol, excellent correlation coefficients were achieved for data obtained with a minimum of 5 seconds of acquisition. Bland–Altman analysis showed that the differences between the values of cholesterol or calcification at short collection times (1–3 seconds) and at 60 seconds, did not vary in any systematic way.

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**DISCUSSION**

Our paper shows that Raman spectroscopy is capable of accurate detection of deposits of cholesterol and calcium salts in coronary arteries and aortic tissue, using a fibreoptic catheter and spectroscopic system that can be employed in a clinical setting. Acquisition times for reliable detection of cholesterol accumulation can be reduced to approximately 3–5 seconds, while accurate detection of calcification can be achieved in 1 second. There were highly significant correlations between the amount of cholesterol and calcification in the Raman spectrum and the quantity detected by histology. The sensitivity and specificity of Raman spectroscopy for detecting cholesterol and calcification within an atherosclerotic lesion were high and comparable with other spectroscopic techniques. 10–12

At a cut off value of, respectively, 0.04 (wt/wt) and 0.06 (wt/wt), sensitivity and specificity for detecting cholesterol and calcification were approximately equal. This is comparable with results of Römer and colleagues, 7 who found similar cut off values using large sensitive laboratory equipment and longer collection times. In this respect, it is remarkable that with a compact and relatively straightforward Raman system, equipped with catheters that are far from optimal, similar values were obtained. Apparently, the model developed by Brennan and colleagues 6 is very robust, which shows promise for in vivo applications of the technique.

Statistical analysis

Statistical analysis was done with SPSS software (SPPS Inc, Chicago, Illinois, USA).

Positive and negative predictive values for detection of cholesterol obtained in the present study are comparable with corresponding values obtained with fibreoptic techniques such as near-infrared reflectance spectroscopy or time resolved fluorescence spectroscopy. However, the latter data were acquired with large laboratory equipment and have not yet been applied to an in vivo situation. 11,12

Quantitative data provided by histology and Raman spectroscopy showed a highly significant but only moderate correlation. Previous Raman spectroscopic studies have achieved better correlations between Raman data and values obtained with chemical assays ($r > 0.95$) or oil red O staining ($r = 0.87$). 6,14 Thus in our study the quantitative data of the Raman results do not always match the quantitative data obtained by histological evaluation. There may be several explanations for this discrepancy. First, when atherosclerotic tissue is stained, areas containing cholesterol stain entirely red with oil red O, and hence the area is quantified accordingly. However, only a small fraction of the atheroma contains cholesterol; other molecules, such as proteins and necrotic cell material, contribute to the Raman signal but are disregarded by the histology. Second, an important drawback of the use of
the current prototype catheters is the fact that the excitation and collection volumes of the probe are poorly defined. Experiments in our laboratory have shown that the excitation/collection depth of the prototype catheters should be approximately 0.8 mm. A rough estimate of the sample volume of the catheter is approximately 0.8 x π x 0.5² = 0.63 mm³. However, owing to the organisation of the quartz fibres and the presence of complex filters in the tip of the catheters, the light reflected from the first 200 µm of tissue is not collected. Improvements to the catheters is a major facet of Raman research in our laboratory, and new catheters are under construction.

Our study shows that β carotene can be an accurate marker for the detection of plaque lipid. In previous studies, a close correlation has been found between the amount of lipid in a plaque and the peak intensity of (highly lipophylic) β carotene in spectrum. The Raman scattering cross section of β carotene is much larger than that of cholesterol. Thus it will be easier to detect β carotene in a complex multicomponent Raman spectrum such as the spectrum of atherosclerotic artery tissue. Sensitivity and specificity for detecting lipid using β carotene were approximately 92% and 95%, respectively (2 mm artery segments). It would be of interest to investigate whether oral administration of carotenoids could facilitate the detection and quantification of cholesterol in an atherosclerotic plaque by Raman spectroscopy in vivo.

Conclusions
We have found that the sensitivity and specificity of catheter based Raman spectroscopy for on-line detection of cholesterol and calcification within a human atherosclerotic plaque are high, and that reliable Raman spectra can be obtained in approximately 3-5 seconds. Previous studies using Raman spectroscopy have shown that this catheter technique can be applied in vivo in the presence of blood flow. As soon as improved flexible (sideways viewing) Raman catheters are developed, Raman spectroscopy may be applied to assess plaque vulnerability and to evaluate the effects of drugs on plaque composition in vivo.

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