

13C-NMR Spectroscopy of Human Atherosclerotic Lesions
Relation Between Fatty Acid Saturation, Cholesteryl Ester Content, and Luminal Obstruction

Jean-François Toussaint, James F. Southern, Valentin Fuster, Howard L. Kantor

Abstract Previous investigations have used 13C-nuclear magnetic resonance (NMR) spectroscopy to demonstrate the similarities between lipoproteins and the mobile lipids of atheroma. In this study, we tested the hypothesis that 13C-NMR changes are related to indices of histological severity. We classified 20 human arteries according to their obstruction ratio (OR), defined as the ratio of the plaque area to the area delimited by the external elastic lamina. In group A, OR was <40%, and in group B, OR was >40%. We analyzed at 9.4 T the resonances of unsaturated (UFA) and polyunsaturated (PUFA) carbons, the resonances of the carbons 19 and 21 (C19,C21) of cholesteryl esters (CE), the methine carbon peak of fatty acids (CH2), the choline peak from phospholipids (PL), and the glycerol peak from triglyceride (TG). The UFA/PUFA, UFA/(CH2)n, and PUFA/(CH2)n ratios are markers of fatty acid saturation. (C19,C21)/(CH2)n, choline/(CH2)n, and glycerol/(CH2)n are indices of CE, PL, and TG content, respectively. UFA/PUFA in group A is 1.15±0.34 versus 1.63±0.32 in group B (P=.005). PUFA/(CH2)n is 0.26±0.10 in group A versus 0.16±0.04 in group B (P=.049). C19,C21/(CH2)n in group A is 0.32±0.15 versus 0.63±0.23 for group B (P=.003). No significant difference was found in UFA/(CH2)n or in the TG or PL ratios. 13C spectral examination of human atherosclerosis demonstrates decreased resonances for polyunsaturated fatty acyl chains and cholesteryl esters with increasing obstruction. (Arterioscler Thromb. 1994;14:1951-1957.)

Key Words: 13C-NMR spectroscopy • atherosclerosis • unsaturated fatty acids • oxidation • lipoprotein • humans • mobile lipids

Except for chemical extraction, no technique is effective in characterizing the lipid component of an atheromatous plaque. Intravascular ultrasound or angioscopy in vivo and histopathological examination in vitro generate high-resolution structural information but lack good data with respect to chemical composition.

The need to detect and characterize all stages of atheroma development has become more important inasmuch as unstable coronary syndromes or strokes may result from arterial lesions that have not yet become severely stenotic.1-3 The rupture of atheromatous plaques is a major contributor to the development of myocardial infarction, and it has been demonstrated that plaque vulnerability is dependent on the biochemical characteristics of the lipid pool.4-7

Natural-abundance 13C-nuclear magnetic resonance (NMR) spectroscopy allows the nondestructive characterization of lipid composition. Because of its broad chemical shift bandwidth, 13C-NMR provides more information regarding chemical constituents than 1H-NMR8-10 and has been used for structural and dynamic studies of cholesteryl esters (CE), triglycerides (TG), and phospholipids (PL).11-14 Hamilton and Cordes15 examined plasma lipoproteins and intact atherosclerotic plaques in humans, demonstrating that atheroma had spectral characteristics very similar to those of thermally denatured LDLs, suggesting a comparable chemical composition. They emphasized the importance of CE phase transitions and linoleate (C18:2, ,o6-9) versus oleate (C18:1, ,o9) content in determining the lipid state (liquid, smectic, or solid) and their spectral characteristics. Hamilton and Cordes15 and Cushley et al16 resolved the olefinic region of the plaque 13C-spectrum from an atheromatous aorta, thereby enabling assessment of the relative content of unsaturated fatty acids. Cushley et al examined lipid dispersion from aortic lesions and showed that the narrow resonances present at 37°C arose predominantly from TG and CE in a fluid environment.

We report on a study of human atherosclerotic plaques, testing the hypothesis that lipid alterations as a consequence of progressive atherosclerosis can be detected by variations in 13C-NMR spectral characteristics. We demonstrate that disease severity assessed by histopathology is related to alterations of CEs and fatty acyl chain saturation in the mobile lipid component.

Methods

Twenty arteries (1 aorta, 2 carotid, 2 femoral, 10 iliac, and 5 coronary arteries) presenting visible signs of atherosclerosis were sampled at autopsy from 16 patients (40 to 86 years old; range, 67.7±15). The causes of death included heart failure (7), pneumonia (3), stroke (3), acute myocardial infarction (2), lymphoma (2), Alzheimer's disease (1), breast cancer (1), and Huntington's disease (1). Each sample included one plaque. The adventitia was microscopically dissected. A segment 1 cm long, including the complete plaque, was then cut from the artery to match the sensitive region of the NMR probe. Samples were immediately frozen and stored at -20°C. Before

Received November 11, 1993; revision accepted August 25, 1994.

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spectroscopic examination they were rewarmed for 1 hour at 37°C in water (50% D\textsubscript{2}O, 50% H\textsubscript{2}O). Since samples were obtained at autopsy, changes that may have occurred between death and autopsy were not studied. Previous studies have shown a full reversibility in the temperature-dependent changes of the \textsuperscript{13}C-spectrum of atheromatous plaque after storage at 4°C.\textsuperscript{11} Preliminary data showed that spectra were unchanged when the \textsuperscript{13}C-spectroscopy analysis was performed after reheating from 4°C or from -20°C, demonstrating that the same fractions of lipid in the liquid phase were analyzed despite the difference in storage temperature. All the plaques were then analyzed by \textsuperscript{13}C-NMR spectroscopy.

\textbf{\textsuperscript{13}C-NMR Spectroscopy}

A proton-decoupled \textsuperscript{13}C-NMR spectrum was acquired at 9.4 T (Bruker, MSL 400) for each artery, using a Waltz decoupling sequence with nuclear Overhauser effect (NOE) and deuterium lock.\textsuperscript{17} The decoupling frequency was centered on the aliphatic region. Probe diameters were 5 mm for the coronary arteries, 10 mm for iliac and carotid arteries, and 20 mm for aortas. Pulse durations were adjusted for optimal signal-to-noise ratio (SNR) of (CH\textsubscript{2})\textsubscript{n}, C\textsubscript{21}, UFA, and PUFA peaks. Optimal pulse durations were 12 microseconds for the 5-mm probe, 16 microseconds for the 10-mm probe, and 30 microseconds for the 20-mm probe. Shimming resulted in a proton line width of 30 to 60 Hz for the water peak. Repetition time (TR) was 1 second. Depending on SNR, the number of scans varied from 12,000 to 40,000. Despite the length of the studies, we did not note any appreciable difference in the spectral characteristics when two studies were performed successively on the same artery. All experiments were done at 37°C. Spectra were corrected for NOE by use of a decoupling sequence applied only during data acquisition: the ratios of interest were not significantly modified by the NOE; data were collected with full NOE to improve SNR. All peaks were referenced to the (CH\textsubscript{3})\textsubscript{3} peak at 29.9 ppm from tetramethylsilane.\textsuperscript{11,12} Each identifiable peak was fit to a Lorentzian shape using the NMR1 program (New Methods Research, Inc). Peak areas were then calculated from the fit data and used to quantify resonance intensity.\textsuperscript{18}

\textbf{Gas Chromatography}

Gas chromatography (GC) was performed according to a previously described technique\textsuperscript{19} on a wall-coated open tubular capillary column, using a temperature program from 150°C to 250°C, increasing 10°C/min, on a Varian 3700 equipped with a flame ionizer. Peaks were quantified with a Hewlett-Packard 3392A integrator.

\textbf{Histology}

After spectroscopic examination, the specimens were step sectioned into 2-mm slices perpendicular to the long axis of...
Fig 2. Histological section of the artery analyzed in Fig 1. Obstruction ratio (OR), 35%. This section shows a fibrous plaque with collagen capping and separating three small lipid regions.

the vessel, fixed in 10% formalin for a minimum of 6 hours, decalcified for 4 hours by alcoholic HCl (10%) and EDTA (0.07%), and rinsed in water. The slice corresponding to the central plane of the scanned plaque at the maximal stenosis site was dehydrated in paraffin. Sections 5 μm thick were prepared from the paraffin-embedded slice and stained with a modified Masson's trichrome: the steps were (1) deparaffinization, (2) rehydration, (3) saturation in aqueous picric acid for 30 minutes, (4) rinsing in water, (5) Verhoeff's elastic staining for 6 minutes (3% hematoxylin, 100% ETOH, 2% ferric chloride hexahydrate, 4% potassium iodide with 2% iodine), (6) rinsing in water, (7) Biebrich scarlet–acid fuchsin staining for 3 minutes (0.5% aqueous Biebrich scarlet, 0.5% aqueous acid fuchsin, glacial acetic acid), (8) rinsing in water, (9) phosphotungstic acid 4% staining for 15 minutes, (10) light green 2% for 3 minutes, (11) differentiation in 1% acetic acid for 30 seconds, (12) dehydration, and (13) mounting with Permount.

The histological slide was photographed, and the enlarged image was planimetered with a GRAF-Pen digitizing tablet, a Tektronics 3010 graphics monitor, and a VAX 3000 computer. We defined the obstruction ratio (OR) as the ratio of the plaque area to the area delimited by the external elastic lamina. This ratio is a good index of atherosclerotic disease severity because it accounts not only for luminal area alterations, as the degree of stenosis does in angiographic studies, but also for disease involvement inside the arterial wall. Comparing NMR imaging of human atheromatous arteries sampled under physiological pressure with histological data obtained in the manner we used here showed no significant changes resulting from the fixation and staining.12 We defined two groups of plaques on the basis of the OR: group A (11 arteries) with an OR <40% (20±14%) and group B (9 arteries) with an OR >40% (75±19%).

Results

The $^{13}$C-spectrum of the iliac artery of a 59-year-old patient is shown in Fig 1. The peaks are assigned by use of known chemical shifts11,12 and are described in the legend. Peaks 3 and 4 arise from the C6 and C3 carbons,
respectively, of CEs. Free cholesterol would generate similar peaks but shifted upfield by approximately 2 ppm. The absence of these shifted peaks indicates that free cholesterol does not contribute appreciably to this spectrum.\(^{15}\)

We calculated the following intensity ratios: (1) peak 1 to peak 2: unsaturated/polyunsaturated fatty acid ratio (UFA/PUFA); (2) peak 1 to peak 15: UFA/(CH\(_n^2\)); (3) peak 2 to peak 15: PUFA/(CH\(_n^2\)) and (4) peak 21 to peak 15: C19,21/(CH\(_n^2\)). The C19,21/(CH\(_n^2\)) represents an index of the CE content, and UFA/PUFA, UFA/(CH\(_n^2\)), and PUFA/(CH\(_n^2\)) ratios are markers of fatty acid saturation.\(^{20-22}\) In this spectrum, the UFA/PUFA ratio is equal to 0.84, and C19,21/(CH\(_n^2\)) is 0.70. The histological section of this artery, with an OR of 35%, is shown in Fig 2.

The spectrum of a stenosed iliac artery from a 61-year-old patient is shown in Fig 3. In contrast to the arterial spectrum presented in Fig 1, there is a reversal of the relative olefinic peak amplitudes, with a calculated UFA/PUFA ratio of 1.98, and a decrease in the CE peaks of the aliphatic region (peaks 10,11,12,16,19,21, and 23). C19,21/(CH\(_n^2\)), is 0.22. The histological section is shown in Fig 4: the OR is 61%.

As shown in Table 1, the UFA/PUFA ratio of group B is significantly higher than that of group A. The PUFA/(CH\(_n^2\)) is significantly lower in group B than in group A. The UFA/(CH\(_n^2\)) ratio does not change significantly with the degree of obstruction.

The C19,21/(CH\(_n^2\)) ratio is significantly lower in group B than group A. Though they did not reach statistical significance, the ratios of other CE peaks (peaks 3, 4, 7, 10, 11, 16, 19, and 23) with respect to the (CH\(_n^2\)) peak tended to decrease by 10% to 33%.

To examine PLs and TGs, we also measured the ratio of peak 8 to peak 15 ([cholesterol+(CH\(_n^2\))]/([CH\(_n^2\)]), which characterizes the phosphatidylcholine and sphingomyelin content—polar lipids from the extracellular plaque PL—relative to fatty acyl chain pool, and of peak 6 to peak 15 ([glycerol+(CH\(_n^2\))]/([CH\(_n^2\)]), which reflects the quantity of TG with respect to the fatty acyl chain pool. Results are shown in Table 2. No significant difference was found between group A and group B. Also, glycerol/UFA and glycerol/PUFA did not reach statistical significance (data not shown). Finally, no relation was found between UFA/PUFA or C19,21/(CH\(_n^2\)) and patient age or lesion site.

We analyzed five plaques by GC and NMR. Results of fatty acid analysis by GC are given in Table 3. To compare NMR and GC analyses of fatty acid composition, a correction factor has to be determined for each fatty acid to take into account its contribution to the UFA and PUFA peaks in the NMR spectrum. For example, in the arachidonic acid molecule (C20,4), 8 carbon atoms are involved in the series of 4 double bonds: 2 carbons will contribute to the UFA peak (2 outer carbons of the series of 4 double bonds), and 6 carbons to the PUFA peak (6 inner carbons of the series). From GC results, the corrected UFA/PUFA ratio is 2×(C16,1)+2×(C18,1)/2×(C18,2)+4×(C18,3)+4×(C20,3)+6×(C20,4)+6×(C22,4). UFA/PUFA calculated from NMR and GC results, as well as ORs of the 5 arteries, are given in Table 4. No significant correlation between NMR and GC could be assessed.

**Discussion**

In this study we demonstrate that the mean UFA/PUFA ratio of group A lesions (composed primarily of uncomplicated, nonulcerated fibrous plaques) is 1.15, while in the more stenotic lesions of group B this ratio increases by 42%. This increase in UFA/PUFA results from a 38% decrease in PUFA/(CH\(_n^2\)), with no significant change in UFA/(CH\(_n^2\)). These two latter ratios represent the relative amount of polyunsaturated fatty acids and unsaturated fatty acids, respectively, compared with the fatty acid pool [all fatty acid chains contribute to the signal intensity of the methine carbon peak: (CH\(_n^2\)]. We also relate a decrease of a CE resonance, corresponding to the carbons 19 and 21 of the cholesterol ring, to the increase of obstruction. No significant variation of visible TG or PL is noted compared with the fatty acid pool.

The \(^{13}\)C-spectra we analyze in this investigation of human atherosclerosis are derived predominantly from the mobile atheromatous lipids, which generate relatively narrow resonances. The arterial wall is composed

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**Table 1.** Histological and Spectroscopic Data for Groups A and B

<table>
<thead>
<tr>
<th>OR</th>
<th>UFA/PUFA</th>
<th>PUFA/(CH(_n^2))</th>
<th>UFA/(CH(_n^2))</th>
<th>C19,21/(CH(_n^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>20±14%</td>
<td>1.15±0.34</td>
<td>0.26±0.10</td>
<td>0.28±0.08</td>
</tr>
<tr>
<td>Group B</td>
<td>75±19%</td>
<td>1.63±0.32</td>
<td>0.16±0.04</td>
<td>0.24±0.05</td>
</tr>
</tbody>
</table>

P=.005 P=.049 P=.213 P=.003

OR indicates obstruction ratio; UFA, unsaturated fatty acids; and PUFA, polyunsaturated fatty acids. Saturation ratios: UFA/PUFA (peak 1/peak 2), UFA/(CH\(_n^2\)) (peak 1/peak 15), and PUFA/(CH\(_n^2\)) (peak 2/peak 15); cholesterol ester ratio: C19,21/(CH\(_n^2\)) (peak 21/peak 15). Values are mean±SD.
TABLE 3. Fatty Acid Composition of Five Atheromatous Plaques, Analyzed by Gas Chromatography

<table>
<thead>
<tr>
<th>1,2,3</th>
<th>4,5</th>
<th>6,7</th>
<th>8,9</th>
<th>10,11</th>
<th>12,13</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14,0</td>
<td>C16,0</td>
<td>C16,1</td>
<td>C18,0</td>
<td>C18,1</td>
<td>C18,2</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>27.0</td>
<td>1.0</td>
<td>8.9</td>
<td>29.6</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>27.1</td>
<td>2.0</td>
<td>4.6</td>
<td>34.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>36.0</td>
<td>0.0</td>
<td>5.5</td>
<td>34.3</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>27.3</td>
<td>0.0</td>
<td>6.2</td>
<td>34.4</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>32.0</td>
<td>9.0</td>
<td>2.0</td>
<td>32.3</td>
</tr>
</tbody>
</table>

Values are percent fatty acyl content.
conjugated dienes and oxadienes. These changes are not detectable with the methodology implemented in this study.

Further support for the reduction in fatty acid saturation resulting from lipoprotein oxidation comes from the studies by Zhang et al.34 and Jialal and Chait,35 who described the effects of oxidation on fatty acyl chain double bonds, demonstrating a 55% loss of PUFA chains, with the production of reactive aldehydes such as malonaldehyde36 or 4-hydroxy-2-nonenal37 and an increase in the content of thiobarbituric acids.38 Esterbauer et al.38-39 showed a loss of PUFA that was noted only after a significant drop of all antioxidants (α-tocopherol [vitamin E], β-carotene, or retinyl stearate). The loss of PUFAs has been ascribed to their low resistance to oxidation,39,40 and it has been shown to have clinical implications with respect to atherosclerosis prevention.39,40-45 The low content of PUFAs also influences the cytotoxic effects of oxidized LDL.46 It alters the lipid phase transition and fluidity.13,45-48 The decreased fluidity, studied by the broadening of the \(^1^C\) phosphocholine methyl resonance and by the line width changes of the carbons C3 and C6 of the cholesterol ring at order-disorder transition, has been proposed as a mechanism for atherosclerosis progression through a limited accessibility of HDLs or esterifying enzymes to CE in the smectic phase.13,47

Our investigation also demonstrates a decrease in the resonance of the carbons 19 and 21 of the CE with respect to the pool of all fatty acids in more stenosed plaques. C19 and C21 are located on the edge of the cholesterol ring and therefore have less restricted motion than inner ring carbons, making them well suited for NMR analysis. The decrease of the C19,C21 peak of CE is consistent with previous studies by Jialal and Chait35 and Shaikh et al.49 showing that the oxidation of the cholesterol moiety of lipoproteins caused a 55% decrease of CE. The degree of unsaturation of the surrounding fatty acids and PLs influences cholesterol oxidation through a propagation reaction as demonstrated by Sevanian and McLeod.50

Another factor accounting for the decreased resonances from CEs in the most stenotic lesions could be the inhibition of the reesterification cycle by the cytotoxic effect of oxidized lipoproteins as described by Brown and Goldstein.51 This inhibition leads to the accumulation of free cholesterol, which precipitates as monohydrate crystals, when in excess of a 1:1 molar ratio with PLs.52,53 These crystals are the hallmark of advanced lesions53 and have very short T2 relaxation times resulting from the solid phase,15,25 making them NMR-invisible. No significant free cholesterol resonances were found in our spectra as assessed by the chemical shift of peaks 3 and 4.15 However, since GC was not used to measure the cholesterol or CE content, we do not demonstrate that the CE decrease shown by NMR is related to an increase of free cholesterol. Nor do we demonstrate that UFA/PUFA changes reflect a change in saturation of the CE fatty acyl chains. However, these hypotheses are consistent with the data in the literature.35,49,52

Several authors have published data on the TG content of atherosclerotic plaque. Lundberg,52 using thin-layer chromatography, showed a 10.6% TG content, and Katz and Small,44 using discontinuous sucrose density gradient centrifugation, measured a 7.8% TG content. They showed that almost all of the TG is in the isotropic liquid phase, indicating that TG should fully contribute to the high-resolution NMR signal. In our experiments, the ratios glycerol/(CH\(_2\)_n) (peak 6/peak 15, shown in Table 2), as well as glycerol/UFAs and glycerol/PUFA, did not reach statistical significance when the two groups of atherosclerotic plaques were compared. These results suggest that the observed change in CE/(CH\(_2\)_n) ratio (peak 21/peak 15) is primarily due to an alteration in isotropic CE content.

Conclusions

Using \(^1^C\)-NMR spectroscopy to examine human atheromatous plaques, we have demonstrated that the resonances from polyunsaturated fatty acyl chains and CEs decrease as vessel obstruction increases. These results suggest a loss of double bonds and a decrease of CEs in the mobile lipid pool when plaque progresses. NMR spectroscopy, as a nondestructive biochemical analysis technique, provides a powerful tool to study the variation of lipid content and state associated with the progression of atherosclerosis.

Acknowledgments

We gratefully acknowledge the support of the Harold M. English Fund (Dr Toussaint), National Institutes of Health grant RO1-HL-39371, the Eugene H. and Patricia C. Remmer Fund, and the Ruth and Frank Stanton Fund. We thank Drs Erik Falk, Tannenbaum MA, Alexopoulos D, Hjemdahl-Monsen CE, Leavy J, Weiss M, Borrico S, Gordin R, Fuster V, and Jerry Ackerman for their helpful advice, Janice White for assistance in manuscript preparation, and Star Sevilla for histological staining. We would also like to recognize Drs M. Laposta and M. Pins, and Joanne Brown for their efforts in performing the gas chromatography analysis. We would especially like to thank the staff and members of the MGH-NMR Center for the use of the NMR systems and for helpful discussions.

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13C-NMR spectroscopy of human atherosclerotic lesions. Relation between fatty acid saturation, cholesteryl ester content, and luminal obstruction.

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doi: 10.1161/01.ATV.14.12.1951

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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