Abstract

Previous investigations have used $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy to demonstrate the similarities between lipoproteins and the mobile lipids of an atheromatous plaque. In this study, we tested the hypothesis that $^{13}$C-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 (C$_{19}$,C$_{21}$) of cholesteryl esters (CE), the methine carbon peak of fatty acids (CH$_2$), the choline peak from phospholipids (PL), and the glycerol peak from triglyceride (TG). The UFA/PUFA, UFA/(CH$_2$)$_n$, and PUFA/(CH$_2$)$_n$ ratios are markers of fatty acid saturation. (C$_{19}$,C$_{21}$)/(CH$_2$)$_n$, choline/(CH$_2$)$_n$, and glycerol/(CH$_2$)$_n$ 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/(CH$_2$)$_n$ is 0.26±0.10 in group A versus 0.16±0.04 in group B ($P=.049$). C$_{19}$,C$_{21}$/(CH$_2$)$_n$ 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/(CH$_2$)$_n$ or in the TG or PL ratios. $^{13}$C 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: $^{13}$C-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. 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.

Natural-abundance $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy allows the nondestructive characterization of lipid composition. Because of its broad chemical shift bandwidth, $^{13}$C-NMR provides more information regarding chemical constituents than $^1$H-NMR and has been used for structural and dynamic studies of cholesteryl esters (CE), triglycerides (TG), and phospholipids (PL). Hamilton and Cordes 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, $\omega_6$-9) versus oleate (C18:1, $\omega_9$) content in determining the lipid state (liquid, smectic, or solid) and their spectral characteristics. Hamilton and Cordes and Cushley et al solved the olefinic region of the plaque $^{13}$C-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 $^{13}$C-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
spectroscopic examination they were rewarmed for 1 hour at 37°C in water (50% D$_2$O, 50% H$_2$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 $^{13}$C-spectrum of atheromatous plaque after storage at 4°C. Preliminary data showed that spectra were unchanged when the $^{13}$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 $^{13}$C-NMR spectroscopy.

$^{13}$C-NMR Spectroscopy

A proton-decoupled $^{13}$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. 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$_2$)$_n$, C$_{21}$, 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$_3$)$_4$ peak at 29.9 ppm from tetramethylsilane.$^{11,12}$ 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.$^{18}$

Gas Chromatography

Gas chromatography (GC) was performed according to a previously described technique$^{19}$ 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.

Histology

After spectroscopic examination, the specimens were step sectioned into 2-mm slices perpendicular to the long axis of

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**Fig 1.** $^{13}$C-spectrum of a pathological iliac artery from a 59-year-old patient. Repetition time, 1 second, 14,400 scans. The peak assignments (see References 19 and 22) are: 1, 129.7 ppm: outer methylene carbons of double bonds (CH$_2$-CH=CH-CH$_3$ or CH=CH-CH=CH$_2$); unsaturated carbons in CE, PL, and TG (UFA peak). 2, 128.1 ppm: inner methylene carbons in a series of double bonds (CH=CH-CH=CH$_2$); polyunsaturated fatty acids only (PUFA peak) in CE, PL, and TG. 3, 122.3 ppm: C6 of the cholesterol ring (CE). 4, 73.2 ppm: C3 of the cholesterol ring (CE). 5, 66.1 ppm: choline -(CH$_2$)$_2$N- (PL). 6, 61.9 ppm: $-\text{CH}_2$- in the glycerol backbone (TG). 7, 56.6 ppm: C17 and C14 (CE). 8, 54.2 ppm: choline -(CH$_2$)$_2$N- (PL). 9, 50.2 ppm: C9 (CE). 10, 42.4 ppm: C13 (CE). 11, 39.6 ppm: C24 (CE). 12, 36.6 ppm: C10 and C22 (CE). 13, 33.9 ppm: -CH$_2$-CO- (CE, PL, and TG). 14, 32.1 ppm: -CH$_2$-CH$_2$-CH$_2$- (CE, PL, and TG). 15, 29.9 ppm: (-CH$_2$)$_n$ methine carbons of all long-chain fatty acids (CE, PL, and TG). 16, 28.1 ppm: C2, C12, and C25 (CE). 17, 27.3 ppm: allylic (-CH$_2$-CH=CH$_2$). 18, 25.7 ppm: bisallylic (=CH-CH$_2$-CH$_=\text{CH}_2$). 19, 24.1 ppm: C15 and C23 (CE). 20, 22.8 ppm: last methine (-CH$_2$-CH$_3$). 21, 18.9 ppm: C19 and C21 (CE). 22, 14.1 ppm: methyl (-CH$_3$) (CE, PL, and TG). 23, 11.8 ppm: C18 (CE). The saturation and cholesteryl ester ratios are: UFA/PUFA, 0.84; PUFA/(CH$_2$)$_n$, 0.45; and C19,C21/(CH$_2$)$_n$, 0.70. CE indicates cholesteryl esters; PL, phospholipids; TG, triglycerides; UFA, unsaturated fatty acids; and PUFA, polyunsaturated fatty acids.
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 fuchsine, 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 planimetrized 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 13C-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$_2$)$_n$; (3) peak 2 to peak 15: PUFA/(CH$_2$)$_n$ and (4) peak 21 to peak 15: C19,C21/(CH$_2$)$_n$. The C19,C21/(CH$_2$)$_n$ represents an index of the CE content, and UFA/PUFA, UFA/(CH$_2$)$_n$, and PUFA/(CH$_2$)$_n$ ratios are markers of fatty acid saturation.20-22 In this spectrum, the UFA/PUFA ratio is equal to 0.84, and C19,C21/(CH$_2$)$_n$ 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,C21/(CH$_2$)$_n$ 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$_2$)$_n$ is significantly lower in group B than in group A. The UFA/(CH$_2$)$_n$ ratio does not change significantly with the degree of obstruction.

The C19,C21/(CH$_2$)$_n$ 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$_3$)$_n$ peak tended to decrease by 10% to 33%.

To examine PLs and TGs, we also measured the ratio of peak 8 to peak 15 ([choline-(CH$_3$)$_3$]/(CH$_3$)$_n$) which characterizes the phosphatidylcholine and sphingomyelin content—polar lipids from the extracellular plaque PL$^{13}$—relative to fatty acyl chain pool, and of peak 6 to peak 15 ([glycerol/(CH$_3$)$_n$]/(CH$_3$)$_n$), 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,C21/(CH$_2$)$_n$ 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 2xC18,2+xC18,3+xC20,3+6xC20,4+6xC22,4. 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$_2$)$_n$ with no significant change in UFA/(CH$_3$)$_n$. 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$_2$)$_n$]. 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$_2$)$_n$</th>
<th>UFA/(CH$_3$)$_n$</th>
<th>C19,C21/(CH$_2$)$_n$</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></td>
<td></td>
<td></td>
<td>0.63±0.23</td>
<td></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>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.32±0.15</td>
<td></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$_2$)$_n$ (peak 1/peak 15), and PUFA/(CH$_2$)$_n$ (peak 2/peak 15); cholesteryl ester ratio: C19,C21/(CH$_2$)$_n$ (peak 21/peak 15). Values are mean±SD.
of 50% to 80% water by weight, and atheromatous core contains 30% to 65% (dry weight) lipid, which corresponds to 6% to 32% of the total weight. Using 1H-NMR spectroscopy, we obtained a lipid/water ratio of 11% for the dissected atheromatous core, suggesting that in some specimens as much as two thirds of the lipid (the immobile, amorphous, or crystalline component) may be undetected in NMR spectroscopic analysis.

This less mobile fraction may still be studied by another NMR imaging sequence using projection reconstruction. This method enables the examination of short T2 lipids. As demonstrated in Table 4, NMR spectroscopic data differ significantly from GC results, suggesting that a significant proportion of the more solid lipids is not observed.15,26

These results suggest that the relation between UFA/PUFA, C19,C21, and OR demonstrated in this study does not apply to the total lipid content (including solid-state lipids such as free cholesterol crystals) but only to the mobile component. The importance of characterizing this component (the "soft" lipids) rests on its contribution to plaque vulnerability and its role in the process leading to plaque rupture through abnormal repartition of circumferential stress.4,7

In his first examination of atheroma and lipoproteins with 13C-NMR, Hamilton and Cordes13 showed that (1) a human vessel containing early fibrous plaques without ulceration or calcification has spectral characteristics comparable to those of denatured LDLs, suggesting an important contribution of lipoproteins to the atheromatous spectrum; (2) a normal artery does not generate any CE peak, as confirmed in our study (spectrum not shown); and (3) the UFA/PUFA ratio of a nonulcerated, noncalcific fibrous plaque, although not explicitly stated in the publication, is approximately 1.1 to 1.2 (taken from Fig 3 of Reference 15). This is consistent with our result of 1.15 for group A, which is composed of similar plaques.

The 13C-spectral similarity between lipoproteins and atheroma was confirmed by Kroon et al13 in an NMR study of VLDL, LDL, and atheromatous plaques from hypercholesterolemic rabbits. Line widths and T1 relaxation times for CE peaks were equivalent in atheroma and lipoproteins, indicating that most of the atheromatous CE was derived from nonmetabolized lipoproteins.

Booth et al characterized atheroma in whole arteries of atherosclerotic rabbits with a two-dimensional 1H,13C spectroscopic study. Their goal was to use the different liquid crystalline phases of CEs to enhance the contrast between normal periadventitial TGs and atheromatous CE. Aortas from rabbits fed a high-cholesterol diet yielded spectra similar to ours, but the authors noticed a considerable variability among animals. In contrast to Kroon's study, the excised aortas were scanned without dissecting the adventitia. Therefore, perivascular TGs, which are highly mobile and in high concentration in the periadventitial adipocytes, may have masked CE signals, which would be of lower intensity.

T1 measurements for 13C-NMR resonances in human atheromatous plaques have not been performed because of the very low SNR in these specimens. Since we did not obtain fully relaxed spectra, T1 variation could have partially contributed to the difference in resonance ratios. Kroon determined the T1 to be 0.52 seconds for the UFA peak of CEs in human lipoproteins at 67.89 MHz. In the same system, Hamilton demonstrated a T1 of 0.32 seconds for PUFA at 15.18 MHz in human lipoproteins. To reduce the saturation effect, we used a TR twice as long as the TR used in these previous studies to analyze atheromatous lesions and obtained comparable spectra in arteries with similar histological characteristics. Despite the longer TR used in the present investigation, we cannot definitively rule out an effect of saturation. We also measured the NOE resulting from the decoupling sequence used in this study. The effect was <2% for most of the peaks (which have a T1 <300 milliseconds) and 6% for the C19,C21 peak with a 2.24-second T1. These enhancements do not alter our conclusions, since the noted changes are much larger than the changes resulting from NOE: 43% for UFA/PUFA and 97% for C19,C21(CH2)4 (Table 1).

Alterations in fatty acid saturation and CEs in atheroma have been described and attributed to lipid and lipoprotein oxidation. This process has been previously studied with 1H and 13C-NMR by using a free radical-mediated copper-catalyzed model of LDL and HDL peroxidation. In these studies, the authors were able to show an increase in the UFA/PUFA ratio as a result of oxidation, similar to our findings in lesions of increasing severity. They demonstrated that UFA/PUFA changes resulted from a decrease in PUFA without changes in UFA. Other changes in the lipid pool were shown by these groups, such as an increase in the lysophosphatidylcholine content and production of

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**Table 2.** Histological and Spectroscopic Data for Groups A and B

<table>
<thead>
<tr>
<th></th>
<th>Glycerol/(CH2)n</th>
<th>C19,21/Glycerol</th>
<th>Choline/(CH2)n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.09±0.05</td>
<td>10.7±4.39</td>
<td>0.16±0.07</td>
</tr>
<tr>
<td>Group B</td>
<td>0.15±0.10</td>
<td>3.99±4.54</td>
<td>0.18±0.12</td>
</tr>
</tbody>
</table>

**Table 3.** Fatty Acid Composition of Five Atheromatous Plaques, Analyzed by Gas Chromatography

<table>
<thead>
<tr>
<th></th>
<th>C14,0</th>
<th>C16,0</th>
<th>C16,1</th>
<th>C18,0</th>
<th>C18,1</th>
<th>C18,2</th>
<th>C18,3</th>
<th>C20,3</th>
<th>C20,4</th>
<th>C22,4</th>
<th>C24,0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>27.0</td>
<td>1.0</td>
<td>8.9</td>
<td>29.6</td>
<td>26.3</td>
<td>0.0</td>
<td>0.0</td>
<td>6.5</td>
<td>0.0</td>
<td>0.0</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>
<td>26.0</td>
<td>0.2</td>
<td>0.7</td>
<td>3.9</td>
<td>0.6</td>
<td>0.0</td>
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<tr>
<td>3</td>
<td>0.0</td>
<td>36.0</td>
<td>0.0</td>
<td>5.5</td>
<td>34.3</td>
<td>18.0</td>
<td>0.0</td>
<td>0.0</td>
<td>6.2</td>
<td>0.0</td>
<td>0.0</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>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
<td>8.1</td>
<td>0.0</td>
<td>0.0</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>
<td>18.0</td>
<td>0.3</td>
<td>2.6</td>
<td>0.0</td>
<td>0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are percent fatty acyl content.
These crystals are the hallmark of monohydrate crystals, when in excess of a 1:1 molar ratio of CE/(CH\textsubscript{n}). This inhibition leads to the toxic effect of oxidized lipoproteins as described by Brown and Goldstein.

Further support for the reduction in fatty acid saturation resulting from lipoprotein oxidation comes from the studies by Zhang et al\textsuperscript{34} and Jialal and Chait,\textsuperscript{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 malonaldehyde\textsuperscript{36} or 4-hydroxy-nonenal\textsuperscript{37} and an increase in the content of thiobarbituric acids.\textsuperscript{38} Esterbauer et al\textsuperscript{39-42} showed a loss of PUFA that was noted only after a significant drop of all antioxidants (\alphatocopherol [vitamin E], \beta-carotene, or retinyl stearate). The loss of PUFAs has been ascribed to their low resistance to oxidation,\textsuperscript{39,40} and it has been shown to have clinical implications with respect to atherosclerosis prevention.\textsuperscript{39-41,43} The low content of PUFAs also influences the cytotoxic effects of oxidized LDL\textsuperscript{44} and alters the lipid phase transition and fluidity.\textsuperscript{13,45-48} The decreased fluidity, studied by the broadening of the \textsuperscript{13}C phosphocholine methyl resonance and by the line width changes of the carbons C3 and C6 of the cholesterol ring, showed a loss of PUFA that was noted only after a significant drop of all antioxidants (\alphatocopherol [vitamin E], \beta-carotene, or retinyl stearate). The decrease 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.\textsuperscript{13,47}

Our investigation also demonstrates a decrease in the resonance of the carbons 19 and 21 of the CEs 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 NMR invisible. No significant free cholesterol resonances were found in our spectra as assessed by the chemical shift of peaks 3 and 4.\textsuperscript{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.\textsuperscript{35,49-52}

Several authors have published data on the TG content of atherosclerotic plaque. Lundberg,\textsuperscript{53} using thin-layer chromatography, showed a 10.6% TG content, and Katz and Small,\textsuperscript{54} 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\textsubscript{n})\textup{6} (peak 6/peak 15, shown in Table 2), as well as glycerol/PUFA 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\textsubscript{n})\textup{6} ratio (peak 21/peak 15) is primarily due to an alteration in isotropic CE content.

**Conclusions**

Using \textsuperscript{13}C-NMR spectroscopy to examine human atheromatous plaques, we have demonstrated that the resonances from polysaturated 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**

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**References**


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**Table 4. Obstruction Ratio and UFA/PUFA Ratios Calculated From NMR and GC, for the Five Arteries in Table 3**

<table>
<thead>
<tr>
<th>OR</th>
<th>NMR</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>1.157</td>
</tr>
<tr>
<td>2</td>
<td>0.11</td>
<td>1.448</td>
</tr>
<tr>
<td>3</td>
<td>0.55</td>
<td>1.406</td>
</tr>
<tr>
<td>4</td>
<td>0.79</td>
<td>1.993</td>
</tr>
<tr>
<td>5</td>
<td>1.300</td>
<td>1.613</td>
</tr>
</tbody>
</table>

OR indicates obstruction ratio; UFA, unsaturated fatty acids; PUFA, polyunsaturated fatty acids; NMR, nuclear magnetic resonance; and GC, gas chromatography.

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**Conclusions**

Using \textsuperscript{13}C-NMR spectroscopy to examine human atheromatous plaques, we have demonstrated that the resonances from polysaturated 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.

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