Characterization of Plasma Lipids in Patients With Malignant Disease by \textsuperscript{13}C Nuclear Magnetic Resonance Spectroscopy and Gas Liquid Chromatography

By Terje Engan, Kristian S. Bjerve, Anne L. Hoe, and Jostein Krane

The purpose of this study was to characterize possible changes in fatty acid composition of plasma lipids associated with malignancy. The very low, low, and high-density lipoproteins were isolated by gradient density ultracentrifugation of plasma from 16 patients with malignant disease and from 15 controls. The triglyceride, esterified cholesterol, and phospholipid constituents of each lipoprotein fraction were isolated, and the fatty acid composition within each lipid component was determined by gas liquid chromatography (GLC). In the 10- to 45-parts-per-million (ppm) region of the carbon-13 (\textsuperscript{13}C) nuclear magnetic resonance (NMR) plasma spectrum, differences were found between patients with malignant disease and controls. The ratio of the 31.6/32.1 ppm resonance intensities was lower in the group of cancer patients. The ratio of the 24.4/24.9 ppm resonance intensities in patients with malignant disease was different from the nonpregnant controls. The NMR changes were interpreted in light of GLC data that indicated derangements in the composition of fatty acids within lipoprotein lipids. In total plasma esterified cholesterol, the relative amount of linoleic acid (18:2, n-6) was lower, whereas oleic acid (18:1, n-9) was higher in the group of patients with malignant disease. In total plasma triglycerides, the amount of oleic acid was higher in the cancer patient group. For total plasma phospholipids, no differences in fatty acid composition between patients and controls were found. Throughout the lipoprotein fractions, the same differences in oleic acid and linoleic acid distribution for triglyceride and esterified cholesterol were found when comparing cancer and control subjects. In conclusion, we found that there are certain differences in the \textsuperscript{13}C NMR spectra and fatty acid profiles between a small and heterogeneous group of cancer patients after they have received their initial treatment and a group of healthy controls. We suggest that carbon NMR spectroscopy could be useful in characterizing malignancy-associated lipid changes.

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LIPID METABOLISM of tumors may be different from that of normal tissue,\textsuperscript{1-7} but the relationship between tumorigenesis and changes in the amounts or composition of circulating plasma lipids is unclear.\textsuperscript{8} Several studies have reported differences in total plasma lipid levels and in relative amounts of plasma lipoproteins between untreated cancer patients and healthy subjects.\textsuperscript{9} Variations in the blood lipid profile after specific cancer treatment have been observed.\textsuperscript{10,11} On the other hand, little is known about the fatty acid composition of lipoprotein lipids in malignant disease. There is some evidence for changes in fatty acid composition in malignancy from studies on blood phospholipids\textsuperscript{12} and triglycerides.\textsuperscript{13}

Nuclear magnetic resonance (NMR) spectroscopy of blood plasma is a rapid method that may simultaneously provide information on multiple molecular compounds, without previous complex sample processing.\textsuperscript{14} In contrast with proton NMR analyses, there are few studies on carbon-13 (\textsuperscript{13}C) NMR analyses of plasma lipids in patients with malignant disease. Resonances of the unsaturated part of the \textsuperscript{13}C NMR spectrum, at 128 to 130 parts per million (ppm), indicate the degree of fatty acid saturation in rat adipose tissue\textsuperscript{15} and in human plasma lipoproteins.\textsuperscript{16,17} By this method, differences in the plasma spectrum between cancer patients and healthy subjects were detected.\textsuperscript{16,17} To further characterize malignancy-associated changes in plasma lipids, we have analyzed plasma and fractionated lipoproteins in patients with malignant disease and in healthy subjects. In addition to obtaining \textsuperscript{13}C NMR spectra, fatty acids were quantified by gas liquid chromatography (GLC).

MATERIALS AND METHODS

Subjects

The study was approved by the ethics committee of the medical faculty of the University of Trondheim (Trondheim, Norway), and informed consent was obtained from each subject. Blood samples were collected from 15 (14 females, 1 male) healthy, unmedicated members of the hospital staff including 6 women in the third trimester of pregnancy. In addition, 16 nonpregnant patients (11 females, 5 males) with malignant disease were included. The cancer was biopsy proven in all patients at the time of their initial diagnosis. Except in two cases, new biopsy samples were not taken at the time of this study. Nine patients suffered from metastatic breast cancer, and the others had metastatic cancer of the thyroid, testis, prostate, lung, or large bowel. One patient had breast cancer diagnosed >25 years before metastases. For the other 15 patients, the mean time from primary diagnosis to metastatic disease was, on average, 16 (range, 0 to 63) months, and the interval from diagnosis of metastatic disease to blood sampling was, on average, 11 (range, 0 to 45) months. Eight patients had metastatic disease at the time of primary diagnosis. Four patients were treated by progestogens (medroxyprogesterone acetate 500 mg twice or megestrol acetate 160 mg once daily) or low-dose corticosteroids (prednisone 10 mg/d); another 5 were treated by localized external radiation therapy using linear accelerators; and 7 patients had no antitumor treatment at the time of blood sampling. No patients had major surgery within 6 months before sampling, but 2 underwent a biopsy 7 and 3 weeks before
Table 1. Selected Characteristics of Patient and Control Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Controls (n = 18)</th>
<th>Cancer Patients (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>42 ± 12</td>
<td>60 ± 16*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67 ± 13</td>
<td>64 ± 15</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168 ± 7</td>
<td>169 ± 87</td>
</tr>
<tr>
<td>BMI</td>
<td>24.2 ± 3.4</td>
<td>23.0 ± 4.0</td>
</tr>
</tbody>
</table>

The age, weight, height, and calculated BMI were compared between the group of patients with malignant disease and the group of healthy subjects. The results are given as mean ± SD.

Abbreviation: BMI, body-mass index.

* Significantly different from controls (P = .002).
† BMI was calculated as weight in kilograms divided by the square of the height in meters.

Biomarker sampling, respectively. Six patients had received cytotoxic chemotherapy of various regimens (5-fluorouracil and mitomycin C every 3 weeks; low-dose doxorubicin weekly; cisplatin, VP-16, and bleomycin every 3 weeks). Four of the 6 patients were also treated with hormonal acting agents or radiation therapy as described above. No patients had been given cytotoxic chemotherapy within the last 3 weeks before blood sampling. The mean time from last chemotherapy to blood sampling was 6 (range, 3 to 13) weeks. To confirm that the results of NMR or fatty acid analyses were not simply caused by effects of hormonal or radiation therapy, analyses were done excluding patients under treatment and patients who had previously received cytotoxic agents. The results were not materially different between the use of either method (data not shown). Data on age, weight, height, and body-mass index are shown in Table 1. The characteristics of the participants and the medical records of the patients were reviewed without knowledge of the NMR test results.

Blood Sampling

Fasting blood samples were collected prospectively and selected for NMR analysis by persons who were not involved in the analysis or the evaluation of the results. The fasting blood samples were drawn into EDTA tubes and the plasma was immediately separated.

The plasma samples were coded with random numbers and the aliquots were submitted in random order for NMR spectroscopy and lipid analysis, and performed blind to clinical data. Lipoprotein isolation and NMR measurements were usually done immediately after the plasma samples were drawn. The aliquots for chemical lipid analysis were stored at −80°C.

Lipoprotein Isolation and Characterization

The reagents used in this part of the study were potassium bromide (KBr), sodium chloride (NaCl), and sucrose, all from E. Merck, Darmstadt, Germany. With small modifications, we used a KBr density gradient ultracentrifugation method described elsewhere.8 Aliquots of 6.6-mL EDTA-plasma were layered by 1.006 g/cm3 NaCl solution in 14-mL polyallomer tubes (Kontron Instruments, Zurich, Switzerland) and centrifuged in a Kontron swinging bucket TST 41/14 rotor (Kontron Instruments) at 39,000 rpm (gmax, 250,000) for 15.5 hours, at 4°C. Very low density lipoprotein (VLDL) was isolated by aspirating the upper 2.5 mL of the tube. The infranatant was resuspended, 3 mL were transferred to new ultracentrifugation tubes, and KBr and sucrose were added to obtain a density of 1.22 g/cm3. Thereafter, the samples were layered successively with 3 mL of 1.21 g/cm3 NaCl/KBr solution followed by distilled water and centrifuged at 41,000 rpm (300,000 gmax) for 24 hours using the same rotor and temperature. The low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions, clearly visible as yellow well-separated bands, were removed by side puncture using 25 gauge needles (Terumo Corporation, Leuven, Belgium). The purity of the lipoprotein fractions obtained by this method was controlled by agarose gel electrophoresis (Beckman Paragon Lip0 Gel, Beckman Instruments Inc, Brea, CA). The total amounts of cholesterol and triglyceride in the fractions were analyzed using enzymatic colorimetric methods with commercial kits (CHOD/PAP for cholesterol and GPO-PAP for triglycerides; Boehringer-Mannheim, Mannheim, Germany). Aliquots of the lipoprotein fractions were taken for fatty acid analysis. Blood sampling and isolation of lipoprotein lipids were performed in six different rounds, and the distribution of cancer patients and healthy subjects within each round differed because of the random procedure. In the first three rounds, only total plasma phospholipids were isolated. In rounds 4 through 6, both total plasma

Table 2. Fatty Acid Composition of Total Plasma Lipoproteins, Grouped According to the Presence or the Absence of Cancer

<table>
<thead>
<tr>
<th>Fatty Acid*</th>
<th>Control (n = 4)</th>
<th>Cancer (n = 5)</th>
<th>Control (n = 9)</th>
<th>Cancer (n = 10)</th>
<th>Control (n = 4)</th>
<th>Cancer (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids</td>
<td>31.5 ± 4.2</td>
<td>31.7 ± 4.8</td>
<td>43.8 ± 0.7</td>
<td>44.6 ± 1.0</td>
<td>11.1 ± 2.1</td>
<td>13.0 ± 0.6</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>41.8 ± 4.3</td>
<td>47.6 ± 3.7</td>
<td>12.3 ± 1.0</td>
<td>13.5 ± 2.0</td>
<td>20.8 ± 2.6</td>
<td>20.4 ± 6.4</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>22.5 ± 1.1</td>
<td>17.5 ± 4.9</td>
<td>34.5 ± 2.6</td>
<td>31.2 ± 3.5</td>
<td>65.3 ± 3.4</td>
<td>56.5 ± 6.5†</td>
</tr>
<tr>
<td>(n-3) acids</td>
<td>4.0 ± 1.3</td>
<td>3.1 ± 0.9</td>
<td>9.3 ± 2.7</td>
<td>10.7 ± 4.7</td>
<td>2.9 ± 0.8</td>
<td>3.0 ± 1.0</td>
</tr>
</tbody>
</table>

The relative proportion of fatty acid is expressed as a weight percentage of the total amount of fatty acids, and the results are given as mean ± SD.

* No. of carbon atoms: no. of unsaturated carbon-carbon bonds and position of the first double bond counting from the methyl carbon end.
† Oleic acid.
‡ Significantly different from controls (P = .001).
§ Linoleic acid.
‖ Significantly different from controls (P ≤ .01).
¶ Arachidonic acid.

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and lipoprotein fractions (VLDL, LDL, and HDL) were analyzed in the following way: in round 4, phospholipids were measured only; in rounds 5 and 6, both triglycerides and cholesterol ester were measured. In this way, the samples were chosen randomly, not specifically. This also explains the different number of sample measurements shown in Tables 2 through 5. All plasma samples, solutions, isolated fractions, pipettes, tubes, and the rotor were kept at 4°C, and all manipulations were performed on ice. All fractions were isolated by the same person. The addition of dye described in the original method was omitted because it interfered with the determination of cholesterol and triglyceride.

**Fatty Acid Analysis of Total Plasma Lipoproteins**

To determine phospholipid fatty acid composition, fatty acid concentrations in total serum phospholipids and in phospholipids from isolated lipoprotein fractions were measured essentially as described previously. Total lipids were extracted with n-butanol, and phospholipids were isolated on BondElut aminopropyl columns (Analytichem Int, Harbor City, CA) after adding diheptadecanoylglycerophosphocholine and butylated hydroxytoluene (Sigma Chemical Co, Dorset, England) as internal standard and antioxidant, respectively. The phospholipid fraction was transmethylated with 14% BF3 in methanol and fatty acid methyl esters extracted into isoctane before GLC analysis. A human control serum stored at -70°C was included to monitor analytical performance. The day-to-day precision expressed as the coefficient of variation (n = 55) for octadecenoate, oleate, linoleate, eicosatetraenoate, eicosapentaenoate, docosahexaenoate, and total phospholipid fatty acids was 3.3, 4.9, 3.1, 4.0, 3.8, 4.3, 6.6, and 3.1% at mean concentrations of 169.5, 117.7, 214.5, 18.1, 120.0, 68.7, 121.8, and 1,246 mg/L, respectively.

To determine triglyceride and cholesterol ester fatty acid composition, aliquots of serum or isolated lipoprotein fractions were extracted with chloroform-methanol according to Bligh and Dyer. Trinonadecanoin and heptadecanoyl-cholesterol were added as internal standards, and butylated hydroxytoluene as antioxidant. The triglyceride and cholesterol ester fractions were isolated by thin-layer chromatography on Silica gel 60 chromatoplates (Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (70:30:2) as solvent. Lipid fractions were identified under UV-light after spraying with 0.01% dichlorofluorescein in ethanol, and the appropriate fractions were transmethylated with 0.5 mol/L methanolic sodium (Supelco Inc, Bellefonte, PA). The fatty acid methyl esters were extracted into hexane, and dried over anhydrous sodium sulphate before GLC analysis.

The fatty acid analysis was performed by analyzing fatty acid methyl esters on a SP2330 fused silica column (Supelco Inc, Bellefonte, PA) using a Hewlett Packard 5890 gas liquid chromatograph equipped with flame ionization detector and a model 7673A

### Table 3. Fatty Acid Composition of VLDL Lipoprotein Lipids, Grouped According to the Presence or the Absence of Cancer

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
<th>Esterified Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 5)</td>
<td>Cancer (n = 7)</td>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>35.3 ± 2.2</td>
<td>46.3 ± 3.6</td>
<td>9.4 ± 0.2</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>20.1 ± 1.7</td>
<td>13.5 ± 4.2</td>
<td>21.1 ± 0.6</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>2.2 ± 0.6</td>
<td>2.2 ± 0.7</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>32.0 ± 3.1</td>
<td>30.5 ± 2.4</td>
<td>48.0 ± 1.6</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>39.5 ± 3.4</td>
<td>50.0 ± 3.6</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>24.2 ± 4.1</td>
<td>16.4 ± 4.5</td>
<td>30.7 ± 1.7</td>
</tr>
<tr>
<td>(n-6) acids</td>
<td>4.1 ± 1.7</td>
<td>3.0 ± 1.6</td>
<td>9.4 ± 2.9</td>
</tr>
<tr>
<td>(n-3) acids</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The nomenclature of fatty acids is explained in Table 2.

* Significantly different from controls (P ≤ .01).
† Significantly different from controls (P ≤ .05).

### Table 4. Fatty Acid Composition of LDL Lipoprotein Lipids, Grouped According to the Presence or the Absence of Cancer

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
<th>Esterified Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 5)</td>
<td>Cancer (n = 6)</td>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>35.9 ± 2.5</td>
<td>46.3 ± 3.6</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>20.9 ± 4.3</td>
<td>13.5 ± 4.2</td>
<td>21.1 ± 0.6</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>2.2 ± 0.6</td>
<td>2.2 ± 0.7</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>32.0 ± 3.1</td>
<td>30.5 ± 2.4</td>
<td>48.0 ± 1.6</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>39.5 ± 3.4</td>
<td>50.0 ± 3.6</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>24.2 ± 4.1</td>
<td>16.4 ± 4.5</td>
<td>30.7 ± 1.7</td>
</tr>
<tr>
<td>(n-6) acids</td>
<td>4.1 ± 1.7</td>
<td>3.0 ± 1.6</td>
<td>9.4 ± 2.9</td>
</tr>
<tr>
<td>(n-3) acids</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The nomenclature of fatty acids is explained in Table 2.

* Significantly different from controls (P ≤ .01).
† Significantly different from controls (P ≤ .05).
Magnetic Resonance Spectroscopy Methods

A few samples were lost during processing, and there was not sufficient material for reanalysis.

### Polyunsaturated fatty acids

- 20:4 (n-6)
- 20:5 (n-3)
- 20:6 (n-3)

### Monounsaturated fatty acids

- 18:1 (n-9)
- 18:1 (n-7)
- 18:1 (n-11)

### Saturated fatty acids

- 16:0
- 18:0
- 20:0

### Fatty acid nomenclature

- (n-3) acids
- (n-6) acids
- (n-9) acids

The reproducibility was found to be adequate and the proton decoupling was consistent. By using a spectral window of 234 ppm for acquisition, reproducible phase and baseline corrections were obtained. The baseline roll was corrected with a fifth-grade polynomial correction routine. To prevent erroneous ratios for the peaks at 128 and 130 ppm, special care was taken to get adequate signal to noise for the hydrogen attached carbons, with special attention to pulse length and acquisition repetition time. The 0 to 180 ppm spectra were plotted, and resonances in the unsaturated (127 to 132 ppm) and saturated (10 to 45 ppm) aliphatic regions were identified and quantified for statistical intergroup comparisons.

### Results

- The proton-decoupled ¹³C NMR spectra of unfrozen human blood plasma were recorded at 125.75 MHz in 5-mm tubes on a Bruker AM500 NMR spectrometer system operating in the Fourier transform mode with quadrature detection. The observation temperature was kept constant at 25°C. Extensive signal averaging was required. The spectra were acquired with 30,000 to 60,000 scans. A spectral window of 29411 Hz was accumulated in 65536 data points in an acquisition time of 1.114 seconds after a 30°C pulse. A pulse delay of 1 second was used. The data were zero-filled to 131072 data points before Fourier transform. A line-broadening factor of 30 Hz was used on the time-domain data. To minimize sample heating, a bi-level WALTZ-16 decoupling scheme was used with a power level of 1 W applied during the acquisition time and a level of 0.4 W applied during interpulse delay. The O₂ frequency was placed close to the water resonance at about 4.8 ppm. When samples were run more than once, the reproducibility was found to be adequate and the peak height measurements were done by two observers and the average between-observer variation was less than 0.5%. Examples of spectra with the measured peaks identified are shown in Fig 1.

### Table 5. Fatty Acid Composition of HDL Lipoprotein Lipids, Grouped According to the Presence or the Absence of Cancer

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
<th>Esterified Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 4)</td>
<td>Cancer (n = 6)</td>
<td>Control (n = 2)</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>36.7 ± 1.6</td>
<td>43.8 ± 6.8</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>20.9 ± 2.0</td>
<td>15.9 ± 5.4</td>
<td>21.9 ± 0.9</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>1.8 ± 0.6</td>
<td>2.5 ± 2.3</td>
<td>6.6 ± 1.4</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>33.0 ± 4.0</td>
<td>31.1 ± 3.0</td>
<td>42.7 ± 0.6</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>40.2 ± 1.7</td>
<td>47.4 ± 7.0</td>
<td>12.6 ± 0.1</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>(n-6) acids</td>
<td>25.0 ± 2.7</td>
<td>20.1 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>3.4 ± 0.8</td>
<td>2.7 ± 1.9</td>
<td>11.0 ± 3.5</td>
</tr>
</tbody>
</table>

* Statistically different from controls (P < .01).
† Significantly different from controls (P < .05).

The nomenclature of fatty acids is explained in Table 2.

**Fig 1.** ¹³C NMR spectra of plasma. (A), patient with malignant disease. (B), healthy subject. The 128 to 130 ppm, special care was taken to get adequate signal to noise for the hydrogen attached carbons, with special attention to pulse length and acquisition repetition time. The 0 to 180 ppm spectra were plotted, and resonances in the unsaturated (127 to 132 ppm) and saturated (10 to 45 ppm) aliphatic regions were identified and quantified for statistical intergroup comparisons. The quantification of resonances was made by drawing the base line and measuring the peak height manually and blinded to clinical data. The peak height measurements were done by two observers and the average between-observer variation was less than 0.5%. Examples of spectra with the measured peaks identified are shown in Fig 1.

Using pure fatty acid extracts, the shifts of unsaturated fatty acid carbons in the 127 to 132 ppm part of the spectrum were determined (J. Krane et al, manuscript in preparation). The monounsaturated fatty acids, of which oleate is the most abundant in plasma lipids, showed in this region resonances around 130, but not at 128 ppm. The polyunsaturated (ie, two or more double bonds) acids showed resonances at both 128 and 130 ppm. Regarding fatty acids with
two double bonds, the (n-6) fatty acids linoleate, which quantitatively is the most important of the polyunsaturated fatty acids of plasma lipids, and the much less abundant eicosadienoate had two resonances each at 128, and further two resonances at 130 ppm. The other polyunsaturated fatty acids had more resonances in the 128 than in the 130 ppm part of the spectrum. For resonances appearing between the 128- and 130-ppm peaks, the fatty acids responsible for most of these signals were arachidonate (20:4, n-6), and the (n-3) acids eicosapentaenoate and docosahexaenoate. Therefore, the composite peaks at 128 and 130 ppm are made up of signals from different fatty acids. The intensity of the peaks (height and area under curve) is determined by the amount of each fatty acid, but also by the number of double bonds within the fatty acid. The saturated fatty acid carbons showed resonances in the 10- to 45-ppm part of the spectrum.

Statistical Methods

The results are presented as the mean ± 1 SD. The comparisons between two different groups were analyzed by nonparametric tests on unpaired data (Mann-Whitney). All P values are two-tailed and regarded statistically significant if P < .05.

RESULTS

$^{13}$C NMR spectra. The ratio of the intensity of the resonance at 31.6 ppm (the chemical shift for the C$_{12}$ carbon of linoleic acid and arachidonic acid) to that at 32.1 ppm (the shift for the C$_{12}$ carbon of oleic acid and the saturated acids hexadecanoate and octadecanoate) was lower in the group of cancer patients compared with healthy subjects (P = .02) (Table 6). This corresponds to the decreased linoleic acid and increased oleic acid found in fatty acid analyses reported below.

The ratio of the intensity of the resonance at 24.4 ppm (the shift for the C$_{1}$ carbon of mainly hexadecanoic acid and octadecanoic acid) to that at 24.9 ppm (the shift for the C$_{3}$ carbon of both oleic acid and linoleic acid) was not different from that of controls (P = .44) (data not shown). When these analyses were repeated excluding the pregnant women from the control group, the 24.4/24.9-ppm intensity ratio for the control group was 0.99 ± 0.062 compared with 0.92 ± 0.10 for cancer patients (P = .10). For two of the pregnant women, the 24.0/24.9-ppm intensity ratio was below 0.9. The subject with the lowest 24.0/24.9-ppm intensity ratio (cancer patient, ratio = 0.60) had the highest triglyceride value (5.3 mmol/L), whereas the subject with the highest ratio (healthy woman, ratio = 1.23) had the lowest triglyceride value (0.6 mmol/L) observed.

In a linear-regression analysis including all participants, there were no significant correlations between age or body-mass index and the above-mentioned $^{13}$C NMR peak intensity ratios.

Fatty Acid Analyses by GLC

For simplicity, the fatty acid data are presented including the values of pregnant women in the control group because the main differences in fatty acid distribution between the groups were not materially different depending on whether the pregnant controls were included or excluded.

Unfractionated plasma, fatty acids in triacylglycerols. In quantitative terms, the most important fatty acids detectable by GLC in the triglyceride fraction of plasma lipoproteins were oleate; hexadecanoate; linoleate; octadecanoate; hexadecenoate; arachidonate; and docosahexaenoate. Together, they constituted more than 94% of the fatty acids in this fraction of plasma lipids (healthy controls). Total plasma triglycerides showed higher amounts of oleic acid in the cancer patient group than in controls (P < .05) (Table 2), and the amount of total (n-6) acids was lower in the cancer group, but the difference was not statistically significant (P = .09). The amount of (n-3) fatty acids did not differ between the groups.

Fatty acids in esterified cholesterol. Linoleate was the most abundant fatty acid in the cholesterol ester fraction. The level of this acid was lower in cancer patients than in healthy subjects (P < .01). Oleic acid was higher in cancer patients than in controls (P < .05). The amount of saturated fatty acids in cancer patients did not differ from that of healthy subjects. For both unfractionated plasma and lipoprotein fractions, there was a tendency for arachidonic acid to be higher in the cancer group. The values for (n-3) fatty acids did not differ between the two groups (Table 2).

Fatty acids in phospholipids. Within phospholipids, the total saturated fatty acids constituted 43.8% ± 0.7% of the fatty acids measured. By comparing the relative distribution of individual fatty acids in cancer patients and healthy subjects, no differences were found (Table 2).

Lipoprotein fractions. In addition to fatty acid analyses of total plasma lipids, fatty acid composition was analyzed in isolated VLDL, LDL, and HDL fractions. The relative fatty acid composition of triglyceride, phospholipid, and cholesterol ester for each of these lipoprotein fractions (Tables 3, 4, and 5) varied only slightly when compared with unfractionated plasma (Table 2). After stratifying into patient and control groups, the fatty acid data for each lipoprotein class given in Tables 3 through 5 showed nearly identical changes in relative fatty acid distribution between groups as those observed in analyses of unfractionated plasma (Table 2).
Mainly, the analyses showed an increase in oleic acid and a decrease in linoleic acid levels in the cancer patients. Because of small numbers in patient and control groups, statistical tests were not done for the phospholipid data.

**DISCUSSION**

By comparing cancer patients and controls, we found differences in the $^{13}$C NMR spectrum. These differences were consistent with variations in fatty acid composition of lipoprotein lipids observed by GLC analyses. The observed patterns in lipoprotein lipids suggest that cancer patients have a relative increase in oleic acid and a decrease in linoleic acid. The major lipids within each of the VLDL, LDL, and HDL lipoproteins are triglycerides, phospholipids, and esterified cholesterol, and in general, the relative distribution of fatty acyl chains varies among these lipids. For example, in esterified cholesterol, we found a higher percentage of polyunsaturated acids than in triglycerides, and this observation was consistent within all three lipoprotein classes. Therefore, the distribution of total fatty acids in plasma is expected to vary as an effect of differences in relative concentrations of VLDL, LDL, and HDL per se. However, our analyses were suggestive of differences in lipids within each separate class of lipoproteins, with respect to relative fatty acid composition. In LDL, eg, the composition of fatty acids of esterified cholesterol differed between the cancer and control group. If representative, these findings may indicate that the metabolism of fatty acids differed between cancer patients and healthy subjects or that the groups had different dietary intake of fatty acids before blood sampling.

In another study, the fatty acid composition of circulating lipoprotein lipids was similar in 10 cancer patients and five healthy women. A case-control study that analyzed fatty acids in serum phospholipids found a lower level of linoleic acid in breast cancer patients than in controls. The triglycerides and cholesterol esters were not analyzed in that study. In our study, we did not find differences in fatty acid composition of phospholipids between cancer patients and controls. Considering the 128/130-ppm intensity ratio of the $^{13}$C NMR spectrum, a trend of lower ratios was observed in patients with malignant disease. Very few reports have been published on this matter. In patients referred for breast biopsy, little overlap was reported for the values of this ratio when comparing patients with malignant breast tumor and those with either a benign tumor or no tumor. The patients differed between the studies because all our patients had metastases, and included various malignant diseases, whereas the other study was confined to breast cancer, but included patients at various stages. Still, reanalysis of our data showed that inclusion or exclusion of breast cancer patients did not materially alter our result for the 128/130-ppm intensity ratio and had no influence on the differences between cancer patients and healthy controls in our study.

In a study of plasma from patients with intracranial neoplasms, the 128/130-ppm intensity ratio was suggested as an indicator that could measure the amount of linoleic acid relative to oleic acid, as suggested by others. To us, this matter seems to be more complicated. Oleic acid is visible at 130 ppm, whereas linoleic acid is visible both at 128 and 130 ppm. In addition, carbons from other polyunsaturated fatty acids seem to affect the peak at 128 ppm, but also at 130 ppm. In our data, samples from cancer patients tended to have higher levels of arachidonic acid, and signals from this fatty acid might have interfered with the composite resonances at 128 to 129 ppm. The 31.6/32.1-ppm intensity ratio varied more consistently between the groups, reflecting increased oleic acid and decreased linoleic acid in the cancer group.

From these analyses, we cannot suggest mechanisms that may cause changes in fatty acid metabolism associated with malignancy. Peroxidation of fatty acids by oxygen-free radicals has been proposed to induce specific changes of lipids in patients with malignant disease. The observed differences in fatty acid distribution in our study were most pronounced for esterified cholesterol, of which linoleate is the quantitatively dominating fatty acid. If present, the peroxidation of fatty acids might be selective, as suggested by the observation that arachidonate was not decreased in our group of cancer patients.

Cancer cells of some tumors probably have increased amounts of unsaturated fatty acids in their cell membrane, compared with noncancer cells. An increased unsaturation, estimated as decreased ratio of stearic to oleic acid, has been found in circulating erythrocytes from patients with different solid tumors and in tissue from malignant prostatic disease. Significant reduction in hexadecenoic and oleic acids and elevation of the relative proportions of hexadecanoic, octadecanoic, and arachidonic acids in samples from oral squamous cell carcinoma have been found. Decreased levels of both linoleic and oleic acids in phospholipids of erythrocyte membranes in postmenopausal breast cancer patients have been reported. Utilization of polyunsaturated acids by proliferating tumor cells might lead to a decreased pool of these fatty acids, thereby affecting the incorporation into lipoproteins. Other mechanisms that might possibly interfere with fatty acid metabolism in malignant disease are changes in the activity of specific liver and plasma enzymes or in circulating hormones.

Analyzing patients under treatment might be problematic because cancer therapy procedures themselves might affect the outcome of the analyses. Surgical treatment may result in the removal of all tumor cells in patients before obtaining samples. All patients in this study suffered from metastatic disease, and had not been subjected to surgical resection of the tumor before sampling. Among patients who were under treatment with radiation or hormonal agents at the time of sampling, none were in complete remission at the time of obtaining samples, and all were regarded to have active tumor disease at the time of inclusion. The effects of various chemotherapeutic agents and radiation on the blood lipids are not known in detail. A study on renal tumors in rabbits could show that those successfully treated with chemotherapy (doxorubicin) had changes in the serum lipoproteins, whereas those unsuccessfully treated did not. Studies on humans suggest that patients who responded favorably to chemotherapy had changes in their serum lipids and lipoproteins, but the data were too sparse to conclude for those who did not respond. Nearly half of our patients were ob-
served with no specific antitumor treatment at the time of blood sampling, and none of the patients had been treated by cytostatics or subjected to surgical procedures within the last 3 weeks before blood sampling. Only 5 of the patients were receiving radiation therapy, and 2 of these had just started treatment. All had radiation against small, local fields. When the groups were analyzed excluding the patients undergoing hormonal or radiation treatment, the results were not materially changed. This indicates that the results of the NMR or GLC fatty acid analyses were not affected by the treatment regimes themselves. The possible effects of cancer treatment regimens on the fatty acid composition need further investigation.

The outcome of the analyses may be affected by differences in the distribution of sex and age between patient and control groups. However, the statistical analyses did not show any significant correlations between these factors and the $^{13}$C NMR ratios of interest. We reviewed raw data from the fatty acid analyses, and could not find that the observed changes in fatty acid distribution in cancer patients were restricted to older or male patients. Still, owing to the relatively small number of participants, possible influences of these factors on the outcome of $^{13}$C NMR and fatty acid analyses should be examined in larger series of patients.

Another possible explanation for the observed differences in fatty acid distribution between cancer patients and control subjects is that the diet of patients may have differed from that of controls, and this may have led to changes in their lipoprotein lipids. Anorexia and low caloric intake may be prevalent in cancer patients, especially in those with advanced disease. Therefore, the observed changes in fatty acid distribution could be related to changes in food intake before blood sampling. However, the medical recordings did not indicate any substantial dietary changes. Also, the patient and control groups were comparable with respect to weight and body-mass index. It seems unlikely that the substantial difference in linoleic acid levels between the groups could be caused by differences in diet alone. Furthermore, the levels of (n-3) acids did not differ between the groups, and this does not support any major differences in diet between them. Concomitant conditions (eg, liver disease) that could influence the metabolism of fatty acids were not recorded in our study.

Changes in fatty acid profile may be related to nonspecific stress among patients with cancer and patients with inflammatory disease. Fatty acid measurements with GLC on patients with rheumatoid arthritis and control subjects reported differences in fatty acid profile.20,21 Most studies used measurements in phospholipids only, but we did not observe fatty acid differences in phospholipids between cancer patients and healthy controls. One study reported measurements both on serum phospholipids and adipose tissue in patients with rheumatoid arthritis.29 Findings in serum phospholipids were not observed in their analyses on adipose tissue, and this may contradict our findings in triglycerides and esterified cholesterol among cancer patients. This may indicate that patients with inflammatory diseases have changes in fatty acid profile that are different from those with malignant disease. From our study, we cannot exclude that the differences in fatty acid profile could be influenced by nonspecific inflammatory processes caused by the tumor or the host. This question needs further investigation.

It should be emphasized that the results of our study need to be regarded with caution. The study is small, and the groups are heterogeneous because different types of cancer with various treatment regimens are included, and the control group includes some pregnant women. We have found certain differences in the $^{13}$C NMR spectra and fatty acid profiles between a group of cancer patients who have received their initial treatment and a group of healthy controls. If we assume that the diet does not substantially differ between patients and controls, these findings may indicate that patients with malignant disease could have a different fatty acid metabolism. We suggest that $^{13}$C NMR spectroscopy could be useful in evaluating differences in lipid profiles. As a result of these encouraging but preliminary findings, we are attempting to refine this technique to enable characterization of plasma lipids.

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Characterization of plasma lipids in patients with malignant disease by 13C nuclear magnetic resonance spectroscopy and gas liquid chromatography

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