Some Factors That Influence the Plasma Lipoprotein $^1$H NMR Spectra of Normal and Cancer Patients: An Oncolipid Test?

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Received May 16, 1990

Selected factors have been evaluated in order to determine their influences on the plasma lipoprotein proton NMR spectra of normal and cancer patients. The variables were donor’s diet (fasting/non-fasting), temperature and time of sample storage, processing procedure, centrifugation speed, and water pre-saturation time. Plasma samples from fasting individuals that were placed immediately on ice, spun at 1,000 and 3,000 g for 15 minutes, and the proton NMR spectrum acquired with the Carr-Purcell Meiboom-Gill (CPMG) pulse sequence, using a two-second water pre-saturation time, consistently gave reproducible results. Resonances attributed to lactate were minimized under these processing conditions. Centrifugation speed and pre-saturation time did not affect the average line width; however, donor fasting state, processing temperature, and storage time did alter the line width. Most important, blood chemistry analysis revealed an inverse correlation between triglyceride levels and average methyl and methylene line widths. Thus, these factors alone caution against the indiscriminate use of proton NMR spectra to differentiate plasma from normal and cancer patients.

INTRODUCTION

An easy, accurate, and non-invasive test for screening early malignancy has long been sought in vain. Fossel et al. reported that the average line widths of plasma lipoprotein lipid methyl and methylene resonances in water-suppressed proton nuclear magnetic resonance (NMR) spectra were narrower in cancer patients than in healthy controls [1]. They initially reported that the mean line width for 44 normal controls was 39.5 ± 1.6 Hz, while that of 81 untreated cancer patients was 29.2 ± 2.5 Hz with no overlap ($p < 0.0001$) [1]. Thus a line width of 33 Hz was set as a line of demarcation in detecting cancer.

This use of high-resolution proton NMR spectra of plasma as a means of detecting malignancy has been received with guarded optimism. The cause of the line width narrowing phenomenon was not elucidated, although Fossel et al. postulated that...

**Abbreviations:** BPH: benign prostatic hypertrophy  CPMG: Carr-Purcell Meiboom-Gill  D$_2$O: deuterium oxide  EDTA: ethylenediamine tetraacetate  FID: free induction decay  HDL: high-density lipoprotein  J: scalar through bond coupling constant  LDL: low-density lipoprotein  NMR: nuclear magnetic resonance  $^1$H: proton  RBC: red blood cell  RI: ratio of intensities  VLDL: very-low-density lipoprotein

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the decreased line widths observed in cancer patients may be due to the increased transverse relaxation ($T_2$) time of these lipid resonances as a result of an undefined host response reflected in an increased lipoprotein lipid mobility. Since line width and transverse relaxation time are inversely related, increased lipid mobility would thus give rise to narrow line widths. Subsequently, Otvos et al. reported that the ratio of intensities (RI) of the lipoprotein lipid methyl resonance calculated from spectra acquired with short (2.4 msec) and long (120 msec) variable delay ($\tau$) in the Carr-Purcell Meiboom-Gill (CGMG) pulse sequence is a more sensitive means of differentiating between narrow and broad line width groups [2].

In related earlier studies, Mountford et al. noted high levels of triglycerides and cholesterol esters in the plasma membranes of a metastatic rat mammary adenocarcinoma cell line [3]. They suggested that these high levels could be responsible for the observed long $T_2$ values. In low-density lipoproteins (LDLs), phase transition temperatures decrease as the triglyceride/cholesterol ester ratio increases [4]. A change in phase to a less ordered state would thus result in increased lipid mobility in LDLs, thereby producing narrower line widths. In subsequent studies, Mountford et al. also reported the presence of a distinct proteolipid (lipoprotein) complex containing a 20 percent glycolipid component (fucogangliosides) in the plasma of a patient with malignancy [5].

These studies have generated much interest and skepticism as to the validity of the so-called “Fossel” test. Many groups who repeated Fossel’s protocol have found unacceptable overlap of line width measurements between cancer and normal patients [6–12]. Such inability to reproduce Fossel’s results could have been due to the possibility that many variables were not identically controlled, compared to those of the Fossel protocol, and the result has raised important questions regarding the reproducibility and validity of proton NMR of plasma as a means of distinguishing malignancy from normal state.

It seemed clear, therefore, that in order to compare data between laboratories and to optimize the sensitivity of such measurements, the variables affecting proton NMR line width needed to be identified and controlled. In our study, we investigated the effects of several factors which may affect such measurements. These were sample handling time, sample handling temperature, probe temperature, centripu-ration speed, water pre-saturation time, and patient dietary status (fasting or non-fasting). We also analyzed various plasma lipid components of cancer and healthy controls, to understand better the underlying biochemical basis for the line width narrowing phenomenon.

**MATERIALS AND METHODS**

**Subjects**

Plasma samples were obtained from eight patients with metastatic malignancies, including carcinoma of the colon, stomach, and breast, diffuse non-Hodgkin’s lymphoma, and liposarcoma. All patients were undergoing chemotherapy. Nine normal samples were obtained from apparently healthy male and female volunteers ranging in age from 23 to 53 years. One normal subject was subsequently diagnosed as having benign prostatic hypertrophy (BPH), a condition reported to affect average line width [1].
Sample Collection

Plasma samples for the dietary study were obtained from a normal (BPH) donor in the fasting state, and 30, 60, and 120 minutes postprandially. Plasma samples from all normal donors not in the dietary study were taken after a 12-hour fast. The dietary status of the cancer patients was not controlled, and the samples were obtained in an outpatient clinic.

Sample Preparation

Blood plasma samples was collected in ethylenediamine tetraacetate (EDTA) Vacutainers (Becton Dickinson). Except for changes in the variable under study, all specimens were handled by the following procedure: samples were placed on ice immediately upon collection and all subsequent processing was done in a cold room at 4°C. Samples were first centrifuged in their collection tubes at 1,000 g for 15 minutes; plasma supernatant was transferred to a new Vacutainer and again centrifuged at 3,000 g for 15 minutes. Following the centrifugation, 0.4 ml of plasma and 0.1 ml of deuterium oxide (D₂O) were placed in 5 mm NMR tubes and stored at 4°C until proton NMR spectra were recorded. Times from collection until spin were typically less than one hour. Effects of temperature were studied by preparing samples as above, at room temperature (27°C) and in a cold room (4°C).

Plasma Chemistry

Some of the components of the plasma lipoprotein were determined, using an EPOS Analyzer 5060 (EM Diagnostics) with the following diagnostic reagent kits: Fastchem cholesterol (Boehringer-Mannheim Diagnostics), HDL-cholesterol No. 351 (Sigma Diagnostics), and Triglyceride No. 338 (Sigma Diagnostics). Total protein was determined on a Gemini centrifugal analyzer, using a Gemini Biuret Reagent kit (ElectroNucleonics, Inc.).

NMR Spectroscopy

High-resolution ¹H NMR measurements were performed on a Bruker AM 500 MHz (11.7 T) spectrometer. All measurements were made on spinning 0.5 ml samples in 5 mm tubes at 25°C with D₂O as an internal lock. Spectra at 500 MHz were acquired with eight transients preceded by four dummy scans, using the CPMG method with 8 K data points and an acquisition time of 0.95 second. Variable (τ) delays used in the CPMG sequence were 3.2, 12, 100, and 120 msec. The water pre-saturation pulse time was two seconds, except in a study of this variable. All free induction decays (FIDs) were processed with a 1 Hz line broadening. Line widths for the plasma lipoprotein lipid methyl and methylene resonances at 0.9 ppm and 1.3 ppm, respectively, were calculated at half the distance from the baseline to the apex of the peak in a manner similar to the procedure used by Fossel et al. (Fig. 1). The optimal phasing of these spectra was achieved by setting the phase correction in any particular CPMG data set equal to that determined for the longest (τ) delay where the broad underlying background signal is absent. The spectra used to calculate the ratio of intensities (RI) data for the plasma lipoprotein lipid methyl peaks were collected with either variable (τ) delays of 3.2 and 120 msec, as reported by Otvos et al. [2], or 12 and 100 msec for the earlier runs in the CPMG pulse sequence.
Temperature-dependent NMR studies were performed on a Bruker MSL 200 MHz (4.7 T) spectrometer at temperatures ranging from 10–45°C.

Statistical Methods

The significance of differences in means was calculated by the *t* test, and the linear correlation of two variables, *r*, by the Pearson product-moment formula.

RESULTS

The mean average line width observed for the nine normal controls was 41.0 ± 7.0 Hz (range, 27.9–51.7 Hz) while that of the eight treated cancer patients was 35.2 ± 5.5 Hz (range, 24.1–42.5 Hz). The mean methyl RI of the control group was 5.22 ± 2.37 (range, 1.66–8.96) while that of the cancer group was 2.10 ± 0.90 (range, 0.66–3.64)(Fig. 2). For the average line width measurements, there was a considerable overlap between the cancer and normal groups, and the differences between control and cancer group means were not significant (*p* > 0.3). For the ratio of intensities measurements, the difference between the means was significant (*p* < 0.005), even though the range of values had some overlap. In comparison, Fossel et al. reported that the mean line width for 44 normal controls was 39.5 ± 1.6 Hz, while that of the 81 untreated cancer patients was 29.2 ± 2.5 Hz with no overlap (*p* < 0.0001) [1].

The effect of diet (fasting versus non-fasting) on the plasma lipid line widths and RI was studied by obtaining blood samples from one fasting normal donor, one-half,
one, and two hours after a high-fat meal. As anticipated, plasma samples obtained postprandially showed increased lipid resonance intensities when compared with those of the same donor in the fasting state. There were no changes in the methyl RI, but a small decrease (3.6–2.9 Hz) in the average line width was noted between fasting and postprandial samples (Fig. 3).

The effect of varying centrifugation speed was studied at 1,000, 2,000, 3,000, 5,000, and 10,000 g to establish whether high-speed spins may separate out some plasma components such as fibrin, a process which may change the line width or RI results. No significant changes in the average line widths (maximum variation, ±2.1 Hz) or RI (maximum variation, ±0.29) were noted, however, in several combinations of spin speeds tested (Fig. 4).

The effect of sample storage time at 4°C was studied on three control samples for 19 days because reproducibility of data was important for samples which were allowed to stand for prolonged periods of time in storage. Our study showed that there was no change in the average line width up to 19 days of storage; however, it was interesting to note that the RI increased as a function of time except for one time point in one sample (Fig. 5). Samples stored beyond 19 days were not studied. We observed that some samples which were stored for several days had white precipitates at the bottom of the NMR tubes. Furthermore, plasma samples stored at room temperature for over 24 hours showed a large increase in lactate methyl doublet at
1.35 ppm (J = 7.35 Hz) whereas samples stored at 4°C showed minimal increase in the lactate doublet (Fig. 1).

The effect of various water pre-saturation times on the average line widths and RI were studied because Fossel et al. [1] used six-second pre-saturation time, whereas the protocol prepared by G.N. Chmurny at NIH [personal communication] recommended a two-second pre-saturation time. Three random plasma samples (two cancer, one control) were analyzed at pre-saturation times of 0.5, 1.5, 2.0, 4.0, and 5.0 seconds. Over this range of pre-saturation times, no significant changes in line width or RI were observed (Fig. 6).

The effect of varying NMR probe temperatures on the plasma line width was studied on two normal samples, since increasing sample temperatures may increase the mobility of methyl and methylene groups of the lipids of lipoproteins, thus causing a decrease in the lipid proton line width. This study was done on a 200 MHz NMR spectrometer, acquiring data at temperatures ranging from 10–45°C. A sample with low triglyceride level of 42 mg/dl showed a sigmoidal change in the average line


width when plotted as a function of temperatures (Fig. 7, sample A). The part of the curve with highest slope (temperature, 15–25°C) probably indicates the phase transition of cholesterol ester in the plasma lipoproteins from solid to liquid phase, since liquid phase has increased methyl and methylene mobility, resulting in lower lipid line width. On the contrary, sample B, with a relatively high triglyceride level of 129 mg/dl, exhibited no significant line width changes; the average line widths were nearly constant at 16–17 Hz over the temperature range examined. Such an observation is most likely due to a decreased phase transition temperature of the lipid lipoproteins with high triglyceride levels [13]. This sample may have been in a liquid phase at temperatures above 10°C, thus resulting in almost constant average line width at higher temperatures.

Finally, the effect of various plasma lipoprotein components on the average line width was studied by comparing the plasma levels of cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and total protein with the average line width. We assumed that, of the many components of plasma, the substances which are most likely to affect the proton NMR spectra of the plasma lipids were the individual plasma lipid components and the total plasma proteins. The latter might
be expected to exert a concentration-dependent effect in its role as the principal determinant of plasma viscosity. Five available normal and eight cancer patients were studied. The comparisons revealed a significant linear inverse correlation between plasma triglyceride level and the lipid line width (Fig. 8, \( r = -0.59, p < 0.05 \)). Other plasma components showed no significant correlations.

**DISCUSSION**

We have investigated various experimental and technical factors which can affect the proton NMR spectra of plasma lipids and tried to elucidate the biochemical mechanism behind Fossel's line width narrowing phenomenon. Such a study was necessary in order to compare data among independent researchers and to enhance reproducibility. Among the factors that were tested, triglycerides in plasma had the most significant line width narrowing property, creating "Fossel-positive" results. Such a finding was not surprising since Deckelbaum et al. [4,13] had shown that triglycerides in LDL can interact with cholesterol esters and lower phase transition temperature of plasma lipids.
Since the completion of our investigation, others have also shown that a positive Fossel test is secondary to a hyperlipidemic condition [15–18,22,23], and that a complete separation of malignant (line width < 33 Hz) from normal (line width > 33 Hz) state is not reproducible [7,10–12,14,15,24–26]. Thorough studies done by Wilding et al. [15] and most recently by Chmurny et al. [27] demonstrated, however, a consistent pattern of a lower mean average line width for cancer patients compared to healthy controls. We believe that this significantly lower average line width in cancer patients may be due to the production of tumor necrosis factor in the host response to malignant tumor, which can suppress lipoprotein lipase and result in elevated triglyceride levels in plasma. The exact biochemical mechanism of this effect of tumor necrosis factor is still not fully understood and deserves further study.

In this study, the average line width measurements exhibited mean values that were not significantly different between cancer and normal patients, in contrast to those reported by Fossel et al. Furthermore, there was extensive overlap in the ranges of these values for the two groups, causing the results for any given individual to be of minimal diagnostic importance. The ratio of intensities measurements did, however, show a significant difference between the group means but with some
overlap. Several other laboratories also reported significant overlap in the values of line width measurements between cancer and control groups [7,10–12,14,15]. It should be noted that all cancer patients in our study were concurrently undergoing chemotherapy. Even though Fossel studied only patients with untreated malignant tumors, Wilding et al. reported that there were no significant differences in average line width measurements between treated and untreated populations [15]. Furthermore, there were no apparent differences among the line width measurements in three patients on whom both pre-treatment and follow-up samples were obtained.

We observed that some sample processing variables had no effect on line width and RI measurements. Centrifugation speed (1,000 to 10,000 g) did not appear to be of any importance as long as the samples were spun at low speed initially to prevent hemolysis. Similarly, there were no changes in the proton NMR spectra of plasma samples accumulated with varying water pre-saturation times of 0.5 to 5.0 seconds. We followed the protocol prepared by G.N Chmurny at NIH [personal communication] because two-second water pre-saturation time can adequately suppress the water proton signal while avoiding problems associated with sample heating and possible disruption of plasma lipoproteins.
Sample processing and storage temperature, on the other hand, had line width broadening effects on the measurements of the plasma lipoprotein methylene resonance line width. When samples were processed at room temperature or were allowed to stand at room temperature overnight before processing, a significant increase in lactate doublet was observed at 1.35 ppm. The effect of lactate doublet on line width measurement is clearly shown in Fig. 1. When samples were placed on ice immediately after collections and processed at 4°C, the growth of lactate doublet was minimized, most likely by slowing down red blood cell (RBC) glycolysis. Lactate signal was still present to some degree, however, even when samples were processed at optimal conditions, probably because normal plasma contains 0.5–2.2 mmol/liter of lactic acid, and lactate levels can be elevated from hypoxia, hemorrhage, shock, sepsis, cirrhosis, or exercise. Ingrowth of lactate in samples was minimized for at least 20 days by storing samples at 4°C, and the average line width did not change significantly. Furthermore, some precipitates were noted in all samples stored up to 20 days, but the line width measurements did not change. Others have reported, however, that samples kept at 4°C for two weeks had a marked increase in line width measurement (three out of four samples) [16].

Although the prolonged storage of plasma samples at 4°C had no significant effect on the average line width of plasma lipid resonances, the RI increased with storage time and exhibited a difference after only three days. The large increase in methyl peak intensity at short variable delay suggested that the precipitation or decomposition of a plasma component initially present in the broad resonances beneath the methyl envelope may be responsible for the observed rise in RI as a function of time. While the mechanism responsible for these increasing RI values is not clear and warrants further study, it could be related to the precipitates formed in samples after prolonged storage.

The effect of diet was studied in one normal subject after a heavy meal. The proton NMR acquisition and processing conditions were held constant so that the area of the resonance envelopes could be used to reflect lipid concentrations accurately.
Even though the lipid resonances were increased in intensity as expected, the average line width decreased 3.6 Hz (10 percent decrease). Mims et al. also reported 5 to 15 percent decrease in the average line width of plasma samples taken from four subjects after eating [16]. Since the major effect of a meal on plasma is an increase in the total triglyceride level, it was plausible to assume that the decrease in average line width may be related to the rise in triglyceride level. When the average line widths of several normal and cancer patients were plotted against the total triglyceride level, a significant inverse linear correlation was noted between line widths and triglyceride concentrations.

A correlation between plasma triglyceride level and the average line width of lipoprotein lipid resonances has been demonstrated by Wilding et al. [15] and Mims et al. [16]. Wilding reported a logarithmic correlation of \( y = 110(X^{-0.27}) \) with coefficient of correlation of \(-.91\), and Mims reported an inverse linear correlation below triglyceride level of 120 mg/dl with \( r = -.643\).

The mechanism of the inverse correlation between line width and triglyceride level in plasma could be due to several factors. First we must consider that the methylene and methyl proton NMR peaks of plasma lipoproteins are composite peaks of various plasma lipoprotein species: chylomicrons, very-low-density lipoprotein (VLDL), LDL, and HDL. In normal fasting plasma, lipid molar components are as follows: phospholipids 35 percent, cholesterol esters 36 percent, triacylglycerols 13 percent, cholesterol 15 percent, and free fatty acids 6 percent [9]. Since it has been reported that line width can vary in different lipid classes—the narrowest in VLDL, the primary carrier of triglyceride, and broadest in LDL, the primary carrier of cholesterol and phospholipids [17]—it is logical to assume that plasma lipid with a high triglyceride level will show narrower line width. Mountford et al. have shown that if plasma lipid NMR spectra are dominated by VLDL (triglyceride) peaks, then the average line width is narrower [18].

Second, since the plasma lipoprotein species can exist in either crystalline or liquid-like states [7], the proton resonance line widths will depend on the physical state of the lipid, with narrow peaks arising from liquid components and broad peaks arising from crystalline components [17]. Line width of lipids in liquid-like states are narrower because the mobility of methyl and methylene groups of the lipids is increased. We have shown that when a plasma sample with low triglyceride level (42 mg/dl) is heated from 10 to 40°C, lipid line width can decrease from 30 to 20 Hz (33 percent) with a transition temperature between 15–25°C. This observation is in reasonable agreement with that of Deckelbaum et al., who reported the phase transition temperature of LDL to be 28.3°C [4]. The second sample with a relatively high triglyceride level (129 mg/dl) did not show any narrowing in lipid line width (17 to 16 Hz) because increasing triglyceride content lowers the transition temperature in intact LDL, presumably through the effect of triglyceride on the cholesterol esters in LDL [18].

Third, the plasma lipid line width can also be influenced by intermolecular interactions within the lipoproteins [1,6,16,18]. The long-chain aliphatic groups of plasma lipids can interact through hydrophobic attractions, resulting in decreased molecular mobility and flexibility. The reduced motion can result in broader line widths and decreased values for \( T_2 \).

Fossel et al. stated that the decreased intermolecular interactions via an unknown mechanism in the plasma of patients with malignant tumors is the basis for the
narrowing of methyl and methylene resonances in the proton NMR spectra [1]. They proposed that host response to malignant tumors may affect the plasma lipid line width either by eliciting structural differences in lipids or through the presence of additional components in the lipoproteins. Soon after Fossel's publication, Montford et al. demonstrated the presence of a distinct proteolipid (lipoprotein) complex containing a 20 percent glycolipid component (fucogangliosides) in the plasma of a patient with ovarian cancer [5]. Even though the authors claimed that the fucoganglioside is responsible for the narrowing of the line width, others reported that the concentration of the "oncolipid" is much smaller than that of VLDL and that it would not be detectable by Fossel's method [14]. Furthermore, addition of a new glycolipid resonance to the plasma lipoprotein lipid resonances envelope would not be expected to decrease the line width but rather to increase the line width or to leave it unchanged.

Our study and others have shown that the plasma triglyceride level has a profound effect on the average plasma lipid line width measurement. Hypertriglyceridemia has been observed in humans with cancer and in animals in response to tumors. Rouzer and Cerami observed that serum of rabbits infected with Trypanosoma brucei became remarkably lipemic, with a major component being triglycerides [19]. Spiegel et al. reported that patients with acute leukemia and non-Hodgkin's lymphoma have low levels of HDL and elevated VLDL and triglycerides in their serum [20]. In both cases, it was thought that the rise in triglyceride is probably due to an abnormality in systemic triglyceride clearance metabolism. Later, a unique macrophage protein also known as cachectin, which is responsible for suppression of lipoprotein lipase activity, was isolated by Beutler et al. [21]. It is suggested that, in response to the growth of cancer cells, activated macrophages release cachectin, a multipotent cytokine which can cause tumor necrosis and cachexia.

It is possible that the effect of line width narrowing seen by Fossel et al. in cancer patients could be due to the rise in triglyceride in serum by the action of cachectin on lipoprotein lipase. It is then clear that the "Fossel test" is not a specific test in screening cancer because any disease process which can cause a rise in serum triglyceride level would give a false-positive result. One may, however, be able to utilize this technique in following progression of cancer, once it has been established that a patient has malignancy. Even though one could achieve the same goal by measuring serum triglyceride levels or lipoprotein lipase activity, NMR may be a more sensitive, faster, and easier method of studying plasma lipoproteins. Even though NMR is not very cost-effective at this time, it may be possible to reduce the cost by tailoring a spectrometer specifically aimed at studying plasma lipoproteins. Research is already in progress by Otvos et al. to study selectively plasma lipoprotein components using NMR spectroscopy [personal communication].

Recently, Schuhmacher et al. have demonstrated that T2 measurements in combination with apolipoprotein-A level in plasma can discriminate tumor patients from healthy controls with specificity and sensitivity of 96.5 percent and 80 percent [28]. Such measurements were shown to be a reflection of general inflammatory response of the host to tumor, since T2 correlated inversely with plasma fibrinogen levels and erythrocyte sedimentation rate. Other hematologic parameters as well as NMR T1 and T2 measurements failed to differentiate between malignant and non-malignant disease processes, thus again arguing against the use of NMR T1 and T2 measurements to screen for cancer. Nevertheless, the usefulness of NMR in following the
progression of malignant tumors or regression after a therapy may need further investigation and may prove to be a useful tool in the management of cancer patients. A recent commentary on NMR spectroscopy in cancer patients concludes that the weight of the evidence does not allow the use of this technique for diagnostic purposes [29].

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (DK 18778).

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