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Detection of Tumours with Nuclear Magnetic Resonance Spectroscopy of Plasma

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Abstract—*A systematic investigation of cancer detection by water-suppressed nuclear magnetic resonance (NMR) of plasma is reported. With additional suppression of lactate, a statistically significant difference between the linewidths of the methylene group signal of patients with untreated cancer (average linewidth 26.9 ± 3.9 Hz) and normal controls (average linewidth 31.1 ± 4.9 Hz) has been found. However, overlap was found between these two groups. It is shown that recognition of malignancy could be improved by consideration of the different relations of the linewidths on the content of serum triglycerides and the observation of a shoulder at the high field side of the methylene signal. Preliminary investigations on lipid fractions separated by ultracentrifugation (UC) indicate a connection of the appearance of the high field shoulder and the HDL lipoprotein.*

INTRODUCTION

Fossel *et al.* [1] recently published a new method of detecting cancer by water-suppressed proton NMR of plasma. The average linewidths of the methyl and methylene resonances, mainly originating from lipids in the plasma, were found to be significantly smaller in patients with untreated cancer (29.9 ± 2.5 Hz) than in normal controls (39.5 ± 1.6 Hz). The findings of Fossel *et al.* [1] have been questioned by many groups [2–10], although Fossel *et al.* [1] confirm their findings in recent contributions [11–14]. The importance of a simple cancer test for cancer screening programmes, diagnosis and for monitoring of therapy and follow-up prompted us to start a systematic evaluation of this method to find additional criteria which would eventually improve the NMR test.

PATIENTS AND METHODS

Patients and blood samples

Blood (5 or 10 ml) was collected in vacutainer tubes containing citrate as an anticoagulant. Plasma from each sample was prepared by centrifugation within 2 h and was stored at −20°C for later analysis. We analysed plasma from 284 patients, whose characteristics were as follows: 56 patients with untreated malignancy, 120 patients being treated for a malignant disease, 50 patients with non-malignant diseases and 58 normal controls. In addition to NMR analysis, routine clinical and biochemical parameters were evaluated from each patient and correlated with the NMR results. Diagnoses of patients with malignancies included: haematological malignancies, cancer of the lung, prostate, gastrointestinal tract, breast, genito-urinary tract, head and neck and malignant melanoma. The non-malignant diseases were mainly rheumatism and chronic heart diseases.

NMR analysis

Plasma (0.6 ml) was mixed with 0.05 ml of D_2O (to provide a lock signal for the NMR spectrometer) and transferred to a NMR tube with an outside diameter of 5 mm. The measurements were done with Bruker AC-300, Bruker AM-400 and Bruker AM-600 spectrometers equipped with an Aspect 3000 computer and automatic sample changer for the 300 MHz spectrometer. The proton spectra were obtained after 6 s presaturation of the proton signal from water and suppression of the lactate signals with a 180°–τ–90°–AQ inversion–recovery sequence. After two dummy scans, eight FIDs were accumulated and a 2 Hz line broadening function was applied to the FID to improve the signal-to-noise ratio. After Fourier transformation the spectra were plotted in the region 0.5–1.5 ppm

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on a scale of 10 Hz/cm and the linewidths at half-height were determined according to the procedure described by Fossel *et al.* [1].

Standard clinical parameters

Triglycerides were automatically analysed on a BM/Hitachi System 737 by the use of enzymatic cleavage of the triglycerides and indirect determination of glycerol by a colour reaction [15]. Cholesterol was determined automatically on a BM/Hitachi System 737 by enzymatic cleavage of cholesterolesters and determination of H_2O_2 after enzymatic oxidation of cholesterol [16]. Immunoglobulins were determined by electrophoresis on an Olympus HITE system 200 using acetate foil [17].

Separation of lipoproteins by ultracentrifugation

Plasma was subjected to gradient ultracentrifugation using a Beckmann L8/55M centrifuge at 4°C. After ultracentrifugation for 24 h at 39,000 rpm the fractions were pooled and dialysed against a buffer containing 0.15 mol/l NaCl, 10 mmol/l Tris–hydroxymethylaminomethane and 1 mmol/l EDTA at pH 7.4. Cholesterol content was evaluated by standard procedures, NMR spectra were measured as described above.

Contents of immunoglobulins were determined by electrophoresis.

Comparisons between group means were performed by using Student's *t*-test using the Statgraf program package [18] on an IBM PS/2 model 60. Regression analysis was performed using the same program package and the method of least squares.

RESULTS

Influence of field strength, lactate signals and selected signals

For technical reasons (our automatic sample changer was installed in the 300 MHz spectrometer) we first had to check the influence of the strength of the magnetic field on the linewidths and the selectivity of the method. Thirty-seven samples were measured with suppression of the lactate signals on both the 300 MHz and 400 MHz spectrometers. The average linewidth of the methylene signal measured at 400 MHz exceeds the average linewidth of the methylene signal measured at 300 MHz by 6.4 Hz. Measurements at 600 MHz resulted in an average increase in the methylene linewidth of 20.4 Hz compared with the 300 MHz results.

Similar to others [19], we found that the lactate signals vary in intensity and interfere with the linewidth measurements. We therefore developed a method to suppress the lactate signals by partially relaxed NMR spectroscopy [20]. This was achieved by applying a delay of 0.8 s after the initial 180° pulse which results in zero magnetization of the lactate protons. Suppression of the lactate signals results in better selectivity in identification of malignancy. This was checked by comparing 20 samples of untreated cancer patients and eight samples of normal controls after measurement with and without suppression of the lactate signals.

In contrast to the instructions of Fossel *et al.* [1] we only used the linewidth of the methylene signal as the discrimination parameter. A comparison between the values of normal controls and patients with untreated malignancies based on the average linewidth for the methylene and the methyl groups according to Fossel *et al.* [1] (Fig. 1) with the linewidth of the methylene signal alone (Fig. 2) resulted in increased selectivity for the detection of cancer.

As illustrated in Fig. 2, the values of the linewidth of the methylene signal of patients with malignant diseases (mean 26.9 ± 3.9 Hz) are lower than those of the controls (mean 31.1 ± 4.9 Hz). This difference is statistically highly significant ($P < 0.000002$). Using the average linewidth of the methylene and the methyl peak (Fig. 1) the difference between the two groups is smaller as indicated by $P < 0.0017$ (mean malignant diseases 30.2 ± 4.6 Hz, normal controls 33.1 ± 4.7 Hz). Thus we conclude that the Fossel test as described [1] does not work in our hands. However, suppression of the lactate and consideration of only the methylene signal results in a statistically significant differentiation between the two groups (Fig. 2).

Influence of instrumentation, operator, storage and food intake

The reproducibility of the measurements was found to be excellent (within 1 Hz), especially since all samples were measured completely automatically with the automatic sample changer so that operator errors could be excluded. The reproducibility of the evaluation of the linewidths at half height was checked by measuring the linewidths of 10 spectra by five different persons. For the methylene signal an average variation of 0.8 Hz, for the methyl signal an average of 1.2 Hz as interobserver variability was found. Measuring every sample of 12 samples twice resulted in an average error of instrumentation and linewidth evaluation of 0.8 Hz.

The influence of storage on the linewidths was checked by measuring five samples originating from different individuals after varying time intervals: within 1, 2.5, 6.5, 24 and 48 h of storage at room temperature. A significant increase in linewidth of the methylene signal of 3.0 Hz was observed after storage for 24 h at room temperature. Aliquots of the same samples were refrigerated at −20°C within 1 h after sampling. These samples were measured 2 weeks later and no significant changes in linewidth could be observed as compared with the fractions

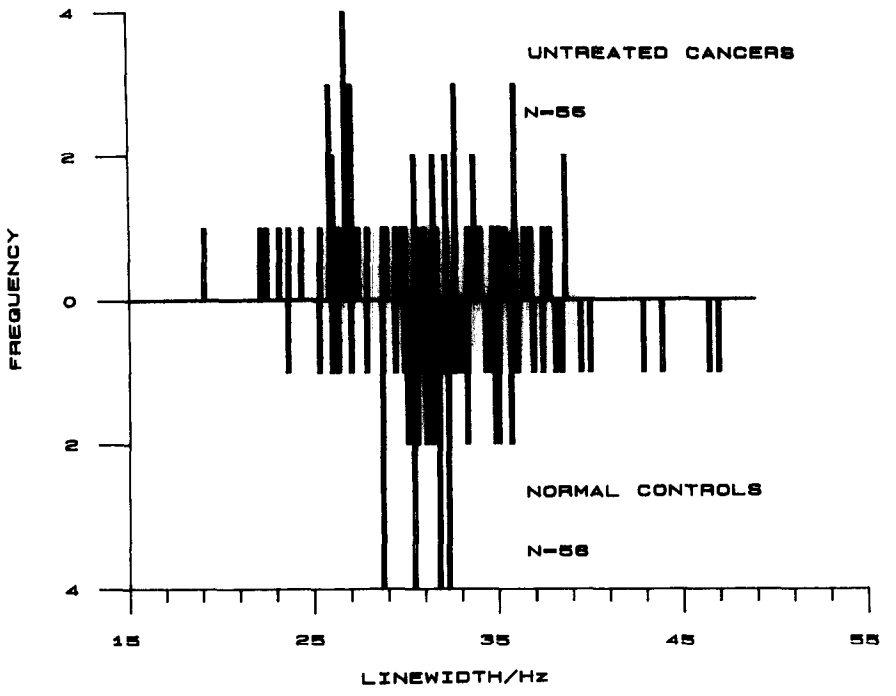

Fig. 1. Comparison between the groups of patients with untreated cancer and normal controls at 300 MHz *based on measurement of the average linewidth of the methylene and methyl peaks as described by Fossel* et al. [1].

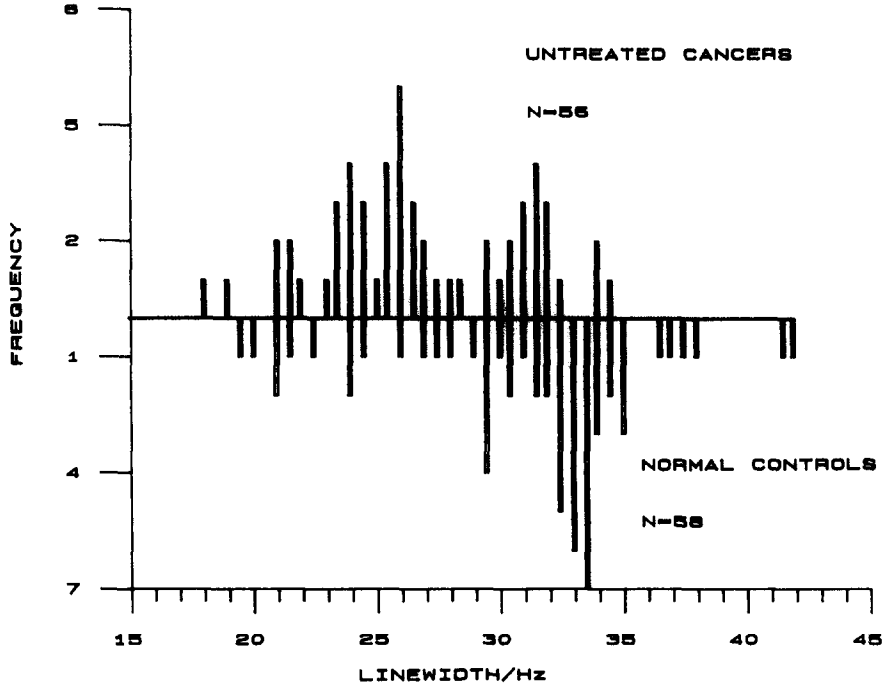

Fig. 2. Comparison between the groups of patients with untreated cancer and normal controls at 300 MHz *based on measurement of the linewidth of the methylene group only.*

measured within the first hour after collection. Storage of the plasma at 0°C resulted in a significant increase in the linewidth of the methylene signal of 3.0 Hz after 1 week as compared to aliquots of the same samples which were stored at −20°C or measured within 1 h after sampling. Thus we conclude that refrigeration of the samples at −20°C soon after sampling is essential to achieve reproducible linewidths.

In six normal volunteers undergoing a day profile we checked the influence of daytime and food intake on the linewidths. We found a significant influence

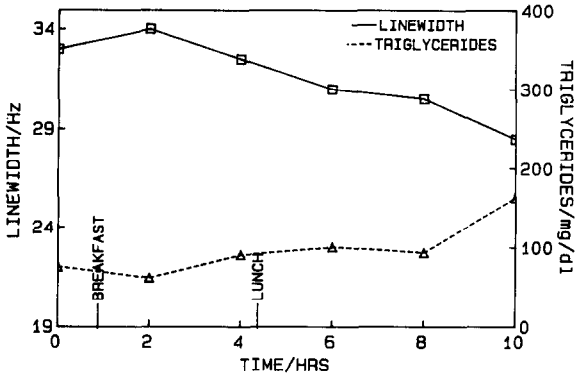

Fig. 3. Day profile of a normal volunteer showing the influence of food intake on triglyceride content and linewidth of the methylene signal.

of food intake on the linewidths of the methylene signal only for persons with fasting triglyceride levels smaller than 160 mg/dl. Persons with higher fasting levels of triglyceride did not exhibit alterations of the linewidths although the triglyceride level altered during the day. Apparently healthy persons showing broad linewidths after 12 h of fasting were observed to show postprandial narrow linewidths (Fig. 3).

Correlation with clinical parameters

After correlation of a great number of different clinical parameters, which were collected for every sample of plasma as far as possible with the linewidths, only the triglycerides resulted in a correlation with the linewidths of the methylene signal [$Y = -0.043*X + 33.7$; $r = 0.629$; X = triglycerides (mg/dl); Fig. 4]. The same was true for the ratio of the integrals of the methylene and methyl signal [$Y = 0.0053*X + 1.05$; $r = 0.812$; X = triglycerides (mg/dl)]. For non-malignant diseases a weak correlation with the γ-globulins [$Y = 0.635*X + 19.664$; $r = 0.605$; X = γ-globulins (% of the whole protein)] was found. No correlation was found with the blood sedimentation rate, the albumin content or the haematocrit as indicated by Schuhmacher *et al.* [21]. A correlation with the fractions of lipid electrophoresis of plasma resulted in only a very weak dependence of the VLDL fraction on the linewidth of the methylene signal [$Y = -0.233*X + 12.8$; $r = 0.591$; X = VLDL (%)].

Detection of malignancy

Despite the statistically significant differentiation between the samples from patients with untreated malignancies and normal controls by considering the linewidth of the methylene signal of lactate suppressed spectra (Fig. 2), there is an overlap between the groups. Taking 29 Hz as the discrimination limit between the groups (average linewidth of samples from patients with untreated cancers 26.9 Hz ± 3.9 Hz, normal controls 31.3 Hz ± 5.3 Hz) 33.9% of patients with untreated cancers (19 samples) had linewidths greater than 29 Hz and 24.1% of the normal controls (14 samples) had linewidths smaller than 29 Hz. Figure 2 shows the distribution of the linewidths of the group of patients with untreated cancers around two different linewidths (around 26 Hz and around 31.5 Hz). This indicates that there is a group of malignant diseases which systematically are not recognized by this method. Concerning the content of triglycerides in the plasma of the groups of persons with untreated cancers and normal controls, we found a high dependence of the linewidth of the methylene signal on the content of triglycerides in the plasma for normal controls and persons with untreated cancers showing linewidths greater than 29 Hz. In contrast, for the group of patients with untreated cancers showing linewidths smaller than 29 Hz, a broad range of contents of triglycerides was found showing no more correlation of the linewidth with the content of triglycerides. Therefore we conclude that, especially in the group of persons with untreated malignancies showing linewidths smaller than 29 Hz, the linewidth is not primarily modulated by the triglyceride content as indicated in the other groups. In this group subtances other than triglycerides seem to influence the linewidth of the methylene signal.

We found no difference between patients with untreated malignancies and the other patient groups (Fig. 5).

However, several experiments were performed to find out additional factors which could enhance the specificity of the method.

The first parameter found was the observation of a shoulder at the high-field side of the methylene signal (Fig. 6). We observed this high-field shoulder in 84.4% of the normal controls and only in 42.8% of the patients suffering from malignant diseases.

Samples from patients with untreated malignancies showing linewidths of the methylene signal greater than 29 Hz, in particular, exhibited such a shoulder (78.9%), whereas linewidths smaller than 29 Hz rarely showed such a shoulder (24.3%). In contrast, normal controls with linewidths smaller than 29 Hz showed such a shoulder (78.5%) and nearly all of this group with linewidths equal to or greater than 29 Hz showed such a shoulder (90.3%). This suggests that there is a possibility of distinguishing normal controls with linewidths smaller than 29 Hz from untreated cancers, but this method is unable to distinguish untreated malignant diseases with linewidths equal to or greater than 29 Hz from normal controls. The appearance of such a high-field shoulder for 300 MHz measurements is sometimes a subjective criterion. It probably originates from different compositions of lipids in the plasma. We tried therefore to separate the

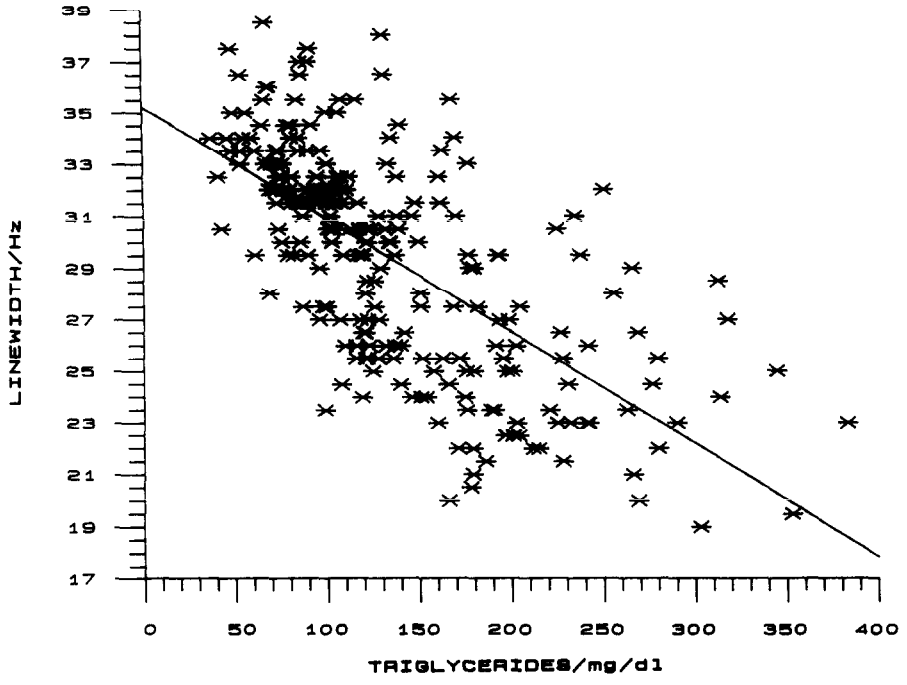

Fig. 4. Correlation of the linewidth of the methylene signal and the content of triglycerides in plasma.

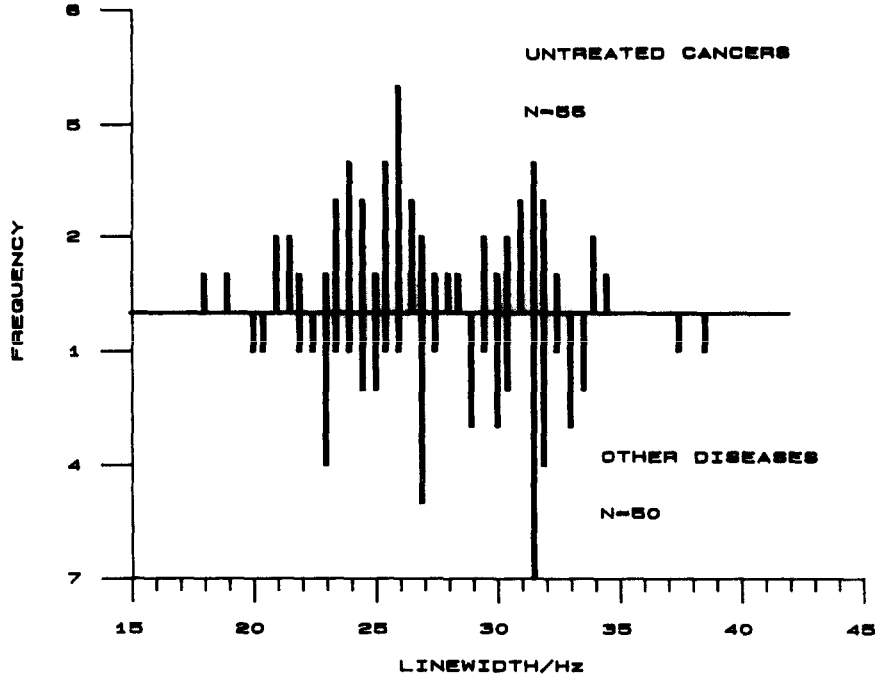

Fig. 5. Comparison between the groups of patients with untreated cancers and patients with non-tumour diseases at 300 MHz.

lipids from the plasma to get narrower and more readily distinguishable signals.

A method of separating components contributing to the signals in plasma is ultracentrifugation of the plasma to separate different lipoprotein fractions. All four fractions yielded broad signals in the methylene and the methyl region typical for normal plasma. The VLDL fraction (linewidths in the range of 20 Hz) yielded narrow lines as compared to the original plasma, whereas the other fractions had linewidths in the range of the linewidth of the corresponding plasma. The linewidths of the signal in the methylene region of the VLDL and the HDL fractions seemed to be unspecific, whereas the linewidth of the LDL fraction in 50% of the samples showed linewidths corresponding to the methylene linewidth of the original plasma. Another important observation after ultracentrifugation was that the

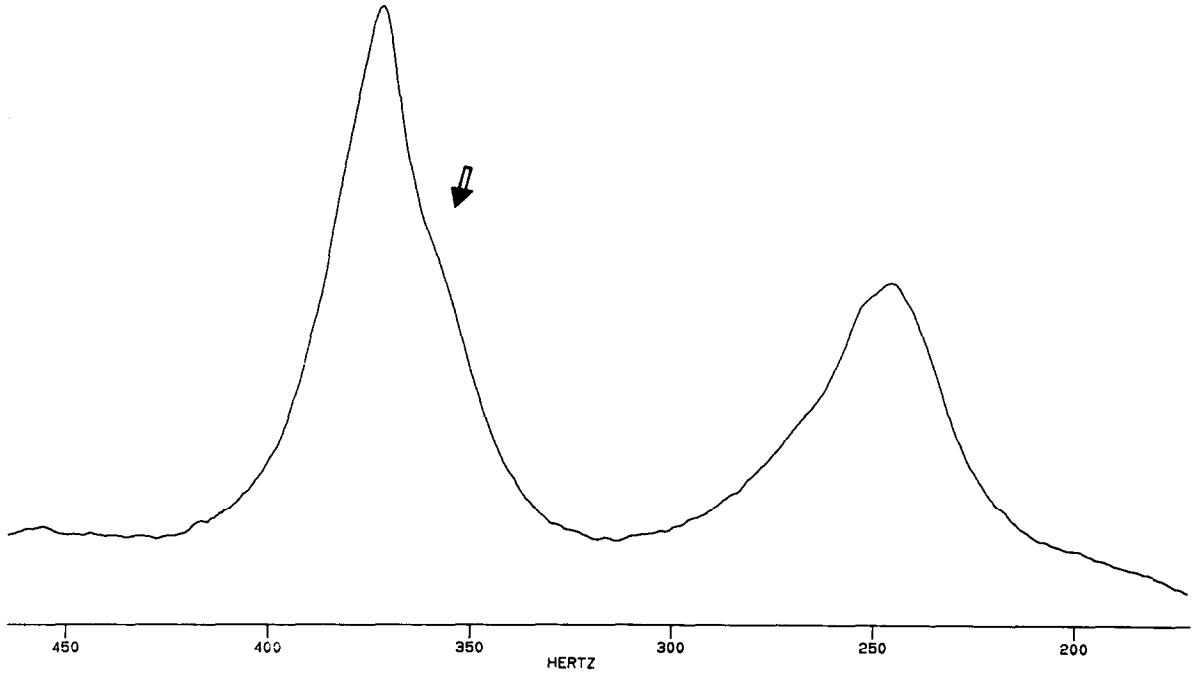

Fig. 6. Typical 300 MHz NMR spectrum showing a shoulder at the high-field side of the methylene signal.

signal in the methylene region of the LDL fraction was 6 Hz and the signal of the HDL fraction 11 Hz upfield from the signal of the VLDL fraction. This indicated that the shoulder at the high-field side of the methylene signal originates from the HDL fraction of the plasma.

Considering the dependence of the specificity of recognition of malignancy on subgroups of tumours, we found that 80% of the haematologic malignancies and 86% of the small-cell lung cancers gave linewidths smaller than 29 Hz. Other species of cancers did not show such high specificites (Table 1).

We performed measurements at 600 MHz because we hoped to get better observation of the high-field shoulder and better differentiation of the groups. We compared the linewidths of the methylene group signal of the groups of 20 persons with untreated cancers and 18 normal controls measured at 300 and 600 MHz. Measurement at 300 MHz resulted in three normal controls with linewidths smaller than 29 Hz and five patients with untreated cancers with linewidths greater than 29 Hz (Fig. 7). Measurement of the same samples at 600 MHz resulted in three normal controls with linewidths smaller than 50 Hz (the discrimination limit at 600 MHz), two plasma samples from persons with untreated cancers with a linewidth of 50 Hz and four persons with untreated malignancies with linewidths greater than 50 Hz (Fig. 8).

Table 1. Specificity of tumour recognition in different tumour groups

Cancer type	Linewidth >29 Hz	Linewidth ≤29 Hz
Gastrointestinal	5 (50%)	5 (50%)
Urogenital	4 (40%)	6 (60%)
Head and neck	2 (67%)	1 (33%)
Small-cell bronchial	1 (14%)	6 (86%)
Other bronchial	2 (67%)	1 (33%)
Haematologic	2 (20%)	8 (80%)
Other cancers	6 (60%)	4 (40%)

Measurement at 600 MHz resulted in a very clear observation of the high-field shoulder as compared to measurements at 300 MHz (Fig. 9). However, it was not possible to use it as an additional discrimination criterion. Sixty-five per cent of the group of persons with untreated cancers and 89% of the group of normal controls showed this shoulder.

Therefore, we conclude that even at very high magnetic fields there is severe overlap between the groups of patients with untreated cancers and normal controls.

DISCUSSION

We have found the method described by Fossel *et al.* [1] to be unsuitable for detecting cancer in a screening test. Introduction of suppression of the signals from the lactate protons regarding only the linewidth of the methylene signal resulted in a statistically significant distinction between the groups of untreated cancer (average linewidth of the methylene signal 26.9 ± 3.9 Hz) and normal controls (average linewidth 31.1 ± 4.9 Hz). Nevertheless there was much overlap between the groups of normal controls and untreated cancers (Fig. 2) and therefore we studied the influences of other parameters on the linewidths. The only relatively

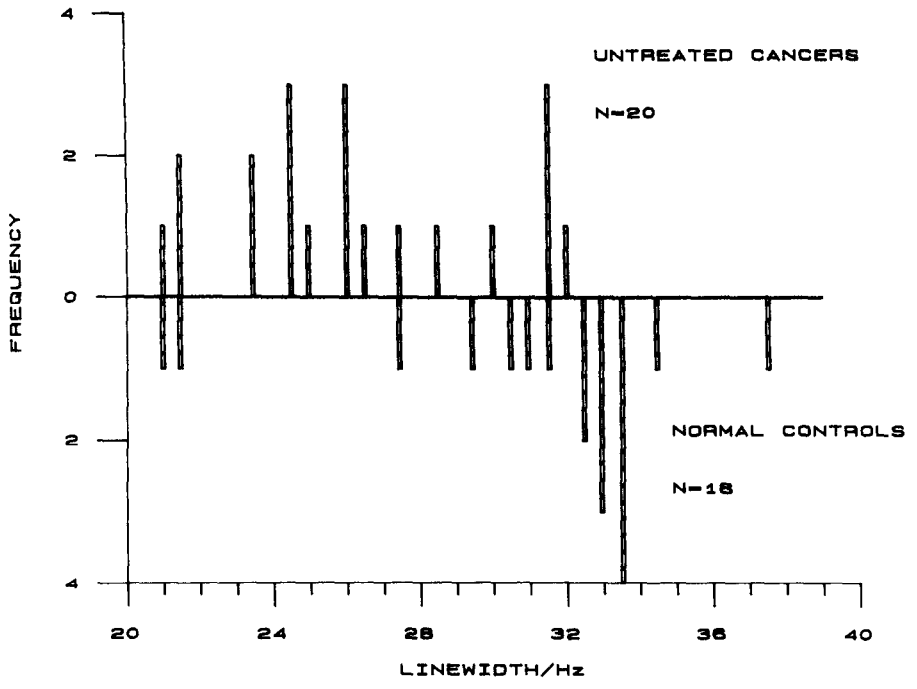

Fig. 7. Comparison of the distributions of the linewidths of the methylene signal for patients with untreated cancers and normal controls, measurements at 300 MHz.

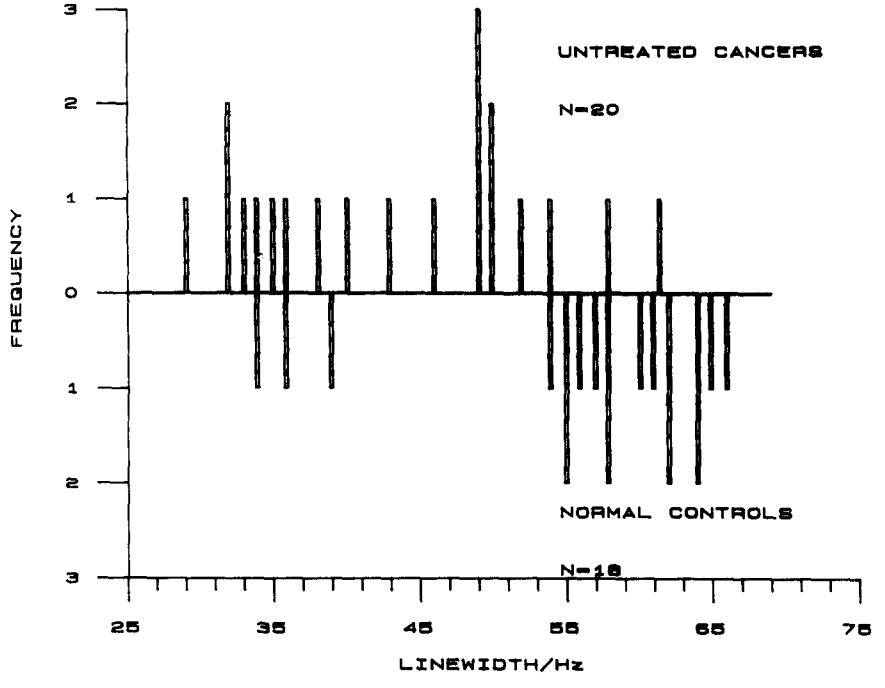

Fig. 8. Comparison of the disributions of the linewidths of the methylene signal for patients with untreated cancers and normal controls, measurements at 600 MHz.

strong correlation found was with the content of triglycerides in the plasma. The statistically significant differentiation of the groups of patients with untreated malignancies and normal controls therefore is caused by the higher average triglyceride content of the group of patients with untreated malignancies (in average 66 mg/dl higher triglyceride content than the group of normal controls). These findings are in full agreement with a recently published article by Wilding *et al.* [22]. Higher levels of triglycerides in the patients with malignant diseases were found by Dilman *et al.* [23] and Spiegel *et al.* [24], too.

For cancers leading to linewidths smaller than 29 Hz, other factors besides the content of triglycerides seem to have a strong influence on the linewidths whereas this was not found in the other groups. This observation, the distribution of the

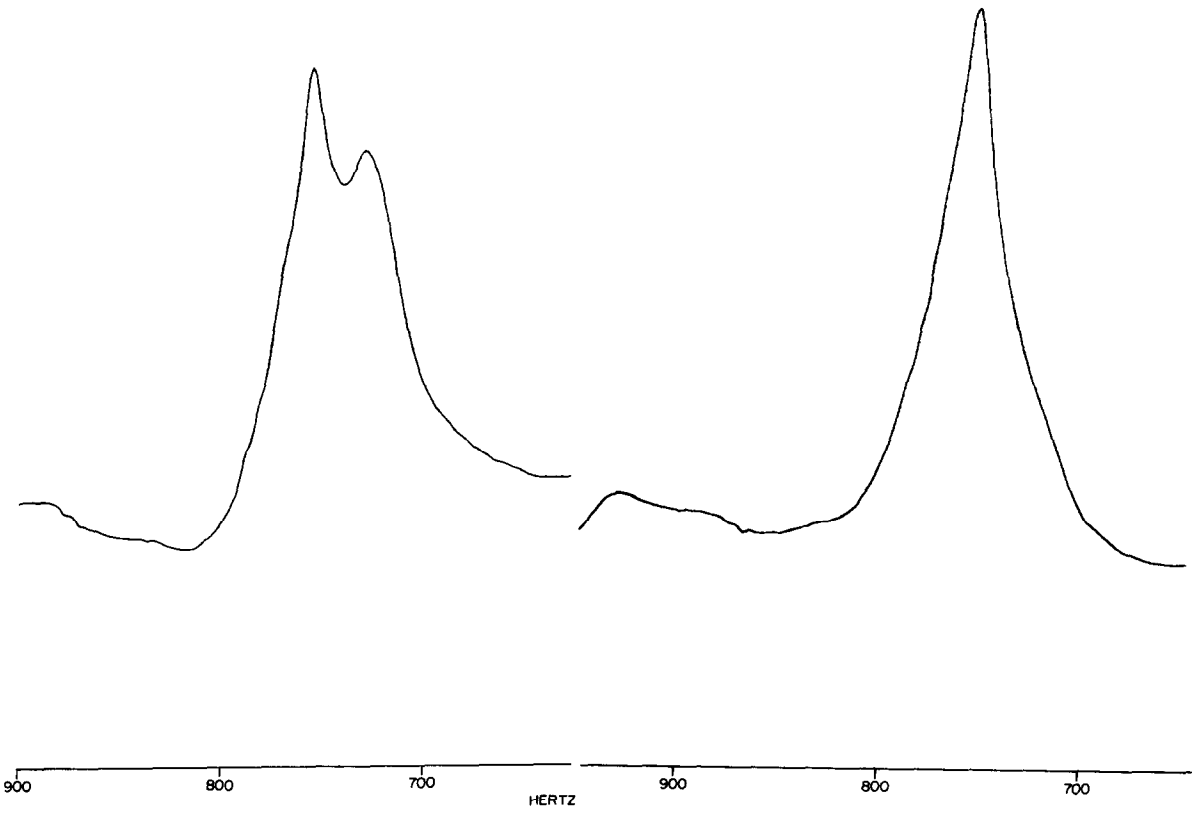

Fig. 9. Comparison of methylene signals with and without high-field shoulder, measured at 600 MHz.

linewidths of the group of untreated cancers, and the different specificity of tumour recognition in the different tumour groups indicated that there are tumours which systematically could not be detected by this test. Considering the groups of small-cell lung cancers and haematologic malignancies only, 83% of these cancers could be detected by the method whereas only 47% of all other malignancies could be detected by this method. Therefore the Fossel test would be of value for these subgroups only.*

Despite the relatively good differentiation achieved between the groups of untreated cancers and normal controls it is still not possible to distinguish cancers in a clear-cut manner from other diseases (Fig. 4) with this method.

A study of the possibility of checking the recurrence during therapy with this method is still in progress. Measurements at 300 MHz indicated that the recognition of cancer is connected with the non-appearance of the high-field shoulder. Therefore we started work to check on the molecular origin of this shoulder. Ultracentrifugation indicates that it could stem from the HDL and/or the LDL fraction because of its chemical shift.

Direct measurements of the relaxation rates of the signals are in progress to check whether the linewidths are mainly influenced by relaxation mechanisms and/or effects of different compositions of the plasma. First results indicate that the linewidth of the methylene signal correlates with the transverse relaxation time of this signal whereas this could not be found for the methyl signal. The longitudinal relaxation time hardly varied from sample to sample. Measurements of the transverse relaxation times of 10 samples from patients with untreated cancers and 10 samples from normal controls indicated that measurement of T_2 could not enhance the selectivity of the recognition of the groups.

The correlation of the linewidths with triglycerides and the origin of the lines from lipids, confirms the connection of cancer and changed lipid metabolism which has been referred to relatively frequently in recent years [22–27].

*We recently performed a double blind study on 131 persons, 32 normal controls, 67 patients with treated non-seminomatous testicular cancer with no evidence of disease and 32 patients with non-seminomatous testicular cancer in collaboration with Dr. M.E. Scheulen and Dr. H. Höfeler of the Westdeutsches Tumorzentrum, Essen. This study yielded nearly exactly the same results as given in this paper, although experimentally we followed in detail the most recent NMR protocol issued by E.T. Fossel.

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