Eur J Cancer, Vol. 26, No. 5, pp. 611–615, 1990.
Printed in Great Britain

0277-5379/90$3.00 + 0.00

Proton Nuclear Magnetic Resonance Spectroscopy of Serum Lipoproteins in Rabbits with Implanted VX-2 Carcinoma

Gro Gadeholt, Svetlana Kruse, Line Halsteinslid and Einar Sletten

Serum from two groups of rabbits, all offspring from the same parents, was subjected to NMR spectroscopy in order to monitor the progress of malignant disease. One group had VX-2 carcinoma implanted in the kidney while the control group were sham-operated with injection of physiological saline. Later, the control group was subjected to dietary restrictions to produce a weight loss equivalent to that of the rabbits with tumor. Progressive cancerous growth with cachexia produced characteristic changes in the lipoprotein spectra distinctly different from those induced by weight loss induced by food intake restrictions. A shoulder on the high-field side of the methylene resonance observed in the control spectra disappeared during the progress of cancerous growth. These spectral changes, however, are not adequately described by line width measurements at half-height as suggested for the original Fossel test.
Eur J Cancer, Vol. 26, No. 5, pp. 611—615, 1990.

INTRODUCTION

INTEREST in using proton nuclear magnetic resonance (NMR) as a diagnostic tool in malignant disease has been revived recently following a paper by Fossel *et al.* [1] on detection of malignant tumors based on NMR spectra of plasma. The mean line widths of the methyl and methylene resonances of the lipoprotein lipids in plasma were shown to correlate with the presence or absence of cancer. Values for the average line width were lower in patients with malignant tumors.

Encouraged by the work of Fossel *et al.* [1], several research groups have initiated similar studies, but in most cases have failed to produce the same excellent discrimination between cancerous and non-cancerous samples [2–4]. In a recent commentary Fossel [5] pointed out several methodological aspects which in his opinion may account for this apparent discrepancy. Experimental factors mentioned to be of special importance were: field strength, recording temperature, field homogeneity and storage conditions. Fossel admits that hypertriglyceridemia [2, 6] still causes false positives.

The number of variables affecting the lipoprotein NMR spectra has been shown to be quite large. The studies published on the Fossel test so far comprise different types and stages of malignant disease, different ages and sexes, and a variety of experimental procedures. Assessment of the validity of the method has proved to be difficult and is still a matter of debate.

Preliminary studies of the NMR spectra from rabbit serum resulted in spectra quite similar to those obtained from human serum (Fig. 1). This fact encouraged us to carry out a controlled NMR study on rabbits. In the present work we have investigated the variation in lipoprotein spectra of rabbits following implantation of VX-2 carcinoma or saline injection into one kidney. Our aims were to study: (1) whether the same changes in the serum lipoprotein spectrum take place in rabbit VX-2 carcinoma, as have been seen in human malignant disease, (2) whether a surgical procedure may produce changes in the serum lipoprotein spectrum and (3) whether weight loss as a result of food intake restrictions may produce changes in the spectral pattern similar to those seen in cancer cachexia.

Correspondence to S. Kruse.
G. Gadeholt is at the Oncological Research Laboratory, Department of Diagnostic Radiology and S. Kruse, L. Halsteinslid and E. Sletten are at the Institute of Chemistry, University of Bergen, N-5007 Bergen, Norway.

MATERIAL AND METHODS

Animals

The study was performed on nine male and 15 female French Burgundy/Chinchilla hybrid rabbits, all offspring from the same parents, but from three litters with 1 month age interval. At the time of tumor implantation the median age was 103 days and the average weights 2.7 and 3.2 kg, respectively, for males and females. There were four males and eight females with tumor, and five male and seven female control rabbits.

The rabbits were housed individually in stainless steel cages and provided with standard laboratory rabbit pellets and water *ad libitum* with electrolytes 22 g/l added (Ewolyt, Ewos A.S., Skårer, Norway). Ewolyt consists of glucose 62.6%, sodium glutamate 2.7%, citric acid 9.1%, NaCl 21.6%, potassium citrate 3.0%, sodium-methyl-*p*-hydroxinbenzoate 0.4% and vaseline oil 0.6%. We have observed that the rabbits recover faster when Ewolyt is added to the drinking water postoperatively. In addition 1 ml of a vitamin supplement consisting of equal parts of colloidal ferridextran complex (Idofer 12% vet., Ferrosan, Copenhagen Denmark) and B vitamins (Becoplex vet., Ferrosan, Copenhagen, Denmark) was injected intramuscularly in alternating hindlimbs after blood sampling every 12 days in an attempt to compensate for a slight anemia due to frequent blood sampling.

Tumor

The VX-2 tumor is a highly malignant anaplastic squamous cell carcinoma characterized by rapid and predictable metastases to regional lymph nodes and lungs. Its growth characteristics

and histology have been well described [7, 8]. The VX-2 carcinoma was maintained by serial passage of approximately 10^6 cells into the hindlimbs of a donor rabbit. Two weeks later the donor rabbit was anesthetized, and the skin shaved and swabbed with 70% alcohol. Using sterile techniques, the skin and subcutaneous tissues were deflected and the tumor (1–2 cm in diameter) was dissected free from surrounding muscles and fasciae. Small pieces of tumor were removed from the peripheral, less necrotic areas, and were finely chopped using a MacIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, U.K.). The tumor tissue was suspended in Hank's balanced salt solution (Flow Laboratories, U.K.) and gently homogenized with a Downe's homogenizer before the tissue was forced through a cytosieve into a sterile Petri dish. The cell viability was checked with trypan blue stain (Sigma Chemical Company, U.S.A.), and the cell concentration adjusted to approximately 10^8 cells/ml.

Surgical procedure

For tumor implantation or sham operation in the kidney the rabbits were anesthetized with fentanyl 0.2 mg/fluanison 10 mg (Hypnorm, Janssen Pharmaceutica, Belgium) 0.3 ml/kg intramuscularly and 5 min later midazolam (Dormicum, Roche, Switzerland) 2 mg/kg intraperitoneally. Additional anesthesia was given regularly every hour or when required. Using sterile technique the left kidney was exteriorized through a left flank incision. Tumor cells suspended in 0.02 ml Hank's solution were injected lateroposteriorly into the cortex at the midportion of the left kidney. In order to seal the needle track and prevent retrograde seeding of tumor cells a drop of cyanoacrylate adhesive (Sicomet, Henkel, F.R.G.) [9] was placed on the kidney surface at the injection point as the syringe was withdrawn. The incision was sutured in three layers. The control group was subjected to the same surgical procedure, however, with injection of 0.02 ml of physiological saline. For postoperative analgesia 0.1 ml/kg buprenorphinum (Temgesic, Reckitt & Colman, U.K.) was given subcutaneously.

Blood samples

Under light sedation with Hypnorm 0.1 ml/kg intramuscularly blood samples from one of the auricular arteries were acquired from all rabbits on 2–3 occasions from 6 weeks of age up until the time of surgery. Additional blood samples were obtained immediately preoperatively and 1, 24, 48 and 96 h postoperatively. Thereafter the rabbits were checked daily for signs of disease with palpation of the abdomen and kidneys. Every 4 days blood samples for NMR spectroscopy, hematocrit and leucocyte counts were obtained and the weight was registered. Approximately 1 ml blood was sampled into a vacutainer tube with no additive (Becton Dickinson, Rutherford, NJ, U.S.A.) in order to secure enough serum for lipoprotein NMR spectroscopy. After the blood sample had been taken, buprenorphinum was administered subcutaneously to counteract the sedative effect of the anesthetic.

Sick-looking rabbits with progressive weight loss approximating 15–20% of the peak weight were killed with an overdose of pentobarbital and autopsied 21–69 days after tumor implantation. Representative tissue was examined histologically for evidence of malignancy.

Six of the control rabbits were killed and autopsied 70 days after sham implantation. The remaining six control rabbits were weighed and blood samples were taken for NMR spectroscopy every 16 days. One rabbit had to be excluded after it had accidentally fractured the spine.

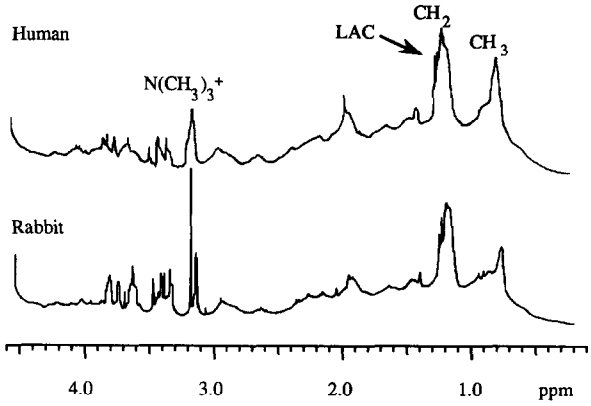

Fig. 1. Proton NMR spectra at 400 MHz of serum from healthy humans and rabbits.

At 33 weeks age the remaining five control rabbits were put on a restricted dietary scheme consisting of approximately 30 g pellets twice daily and plain water *ad libitum* in order to reduce body weight. After a weight reduction of approximately 17%, equivalent to that of rabbits with tumor, they were again given a full daily supply of pellets (100–120 g).

NMR analysis

All spectra were acquired 1–7 h after sampling, using a Bruker AM-400 spectrometer operating in quadrature detection mode with the sample spinning. In order to study the effects of deuterium oxide (D_2O) and tetramethyl ammonium nitrate (TMA) on the NMR spectra we measured the same sample with and without D_2O and TMA added. Serum samples of approximately 0.6 ml containing 10% D_2O were placed in 5 mm NMR tubes. The sample temperature during the NMR measurements was 24°C. We used the signal from D_2O as a lock substance and to optimize the homogeneity of the magnetic field during data acquisition.

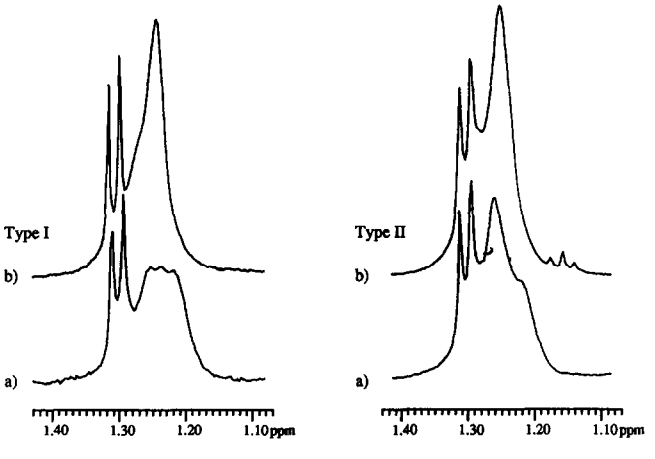

Fig. 2. Two typical peak shapes of the methylene resonance of serum from healthy rabbits before tumor implantation (a) and with established malignant disease (b).

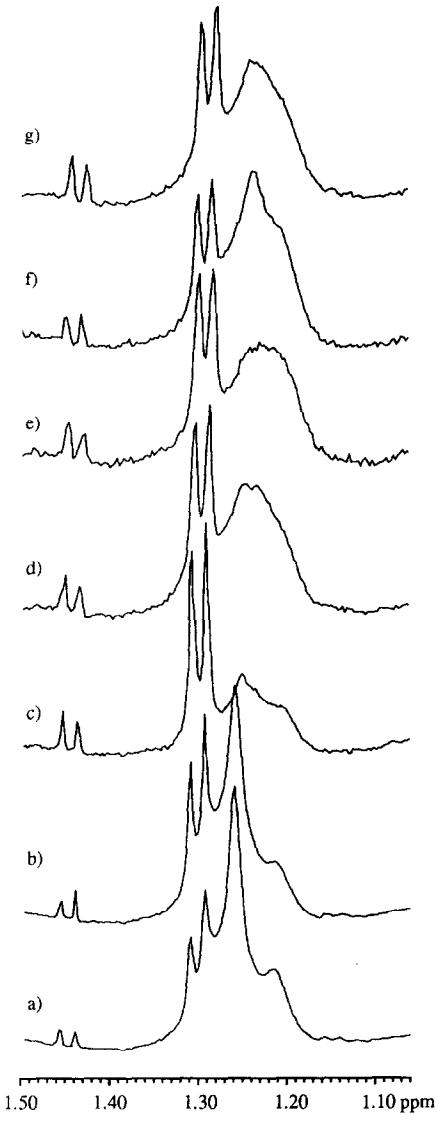

Fig. 3. Spectral changes in the methylene signals of lipoproteins due to progressive weight loss as a result of dietary restrictions. (a) 2 weeks before food restriction, (b) immediately before diet, (c) 2 weeks diet: 2% weight loss, (d) 5 weeks diet: 7% weight loss, (e) 7 weeks diet: 7% weight loss, (f) 10 weeks diet: 10% weight loss, (g) 18 weeks diet: 17% weight loss.

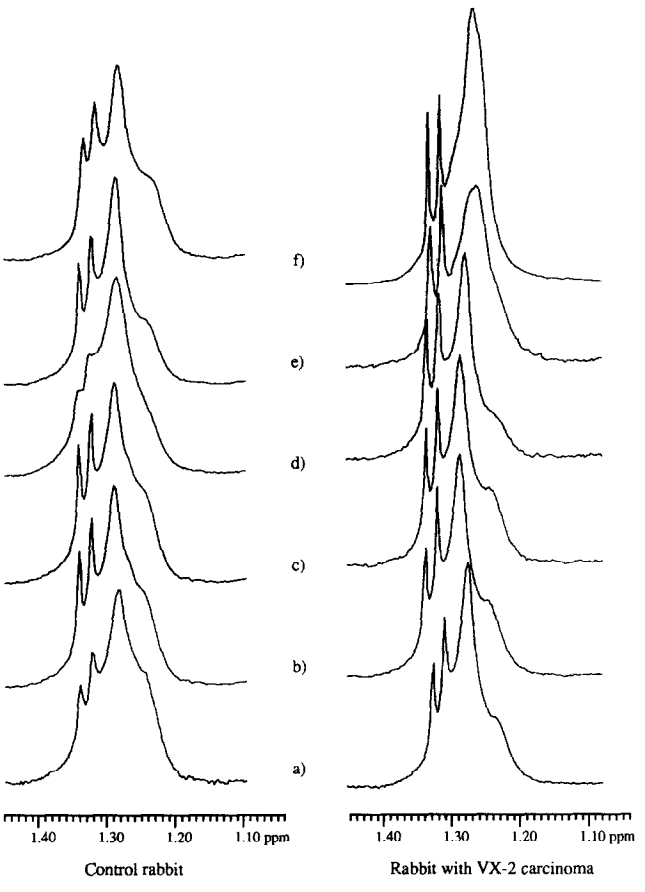

Fig. 4. Changes in spectral profiles of methylene signals in lipoprotein of serum from rabbits with VX-2 carcinoma and control group monitored over a period of 25 days. (a) 7 weeks old, (b) preoperatively, (c) immediately postoperatively, (d) 4 days postoperatively, (e) at 2/3 of observation time (median 17 days), (f) terminal illness (median 25 days).

The large water signal was suppressed by application of an 8 s decoupler presaturation pulse, followed by a single pulse. Most spectra were acquired at a spectral width of 9000 Hz, 16 K data points, 32 scans and transformed with no line broadening. The $N(CH_3)_3^+$ phospholipid resonance at 3.25 parts per million (ppm) served as reference. TMA resonance at 3.15 ppm proved to be a suitable internal intensity standard for freshly tapped serum samples.

RESULTS

The 12 rabbits with VX-2 carcinoma were killed due to progressive illness at median 25 days after tumor implantation, range 21–69 days. The average weight loss was 17% of the peak weight. At autopsy all had a large renal tumor with extensive local infiltration, disseminated disease with metastases to regional lymph nodes, lungs, mediastinum, and frequently to the contralateral kidney, liver and spleen. There were no pathological findings in the six control rabbits that were autopsied 70 days after sham operation.

Figure 1 shows that the high resolution proton spectra of serum from healthy human volunteers and normal rabbits are quite similar.

The resonances at 1.2 and 0.8 ppm arise from the methyl and methylene groups of the lipoprotein lipids. The contributing lipids are present in a variety of molecular aggregates: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and free fatty acids [2, 10, 11]. In addition, methyl resonances from varying amounts of lactate are seen as two distinct shoulders on the low-field side of the broad methylene peak.

Figure 2a shows two main types of methylene resonances of serum lipoproteins from normal healthy rabbits. The occurrence of spectra types I and II is randomly distributed throughout the data. The broad profile type I differs distinctly from profile type II. The lipoprotein spectra of rabbits put on a restricted dietary scheme eventually exhibited a methylene resonance profile similar to the type I pattern (Fig. 3).

For implanted rabbits with type I profile the same final resonance pattern emerged as for rabbits of type II (Fig. 2b). The progressive spectral changes in type II methylene resonances of serum from VX-2 implanted rabbits and controls are shown in Fig. 4. In all rabbits with advanced malignant disease marked

changes in the signal profiles were observed. No significant changes were seen in 6–8-week-old rabbits, in control rabbits after sham operation, or in 5–6-year-old rabbits (unpublished results).

Figure 3 shows typical lipoprotein methylene resonance profiles from one of the control rabbits during weight loss. With gradual weight reduction to approximately 17% of peak adult weight all control rabbits exhibited similar changes in lipoprotein resonance profiles and intensity at 1.2 ppm.

DISCUSSION

Rabbits were chosen for this study because they have a lipoprotein pattern quite similar to humans, and they are large enough to allow frequent sampling of an adequate volume of blood for NMR analysis of lipoproteins. The VX-2 carcinoma in rabbit is well known in our laboratory. When implanted in a kidney, a specified number and volume of VX-2 carcinoma cells reliably produce tumors of approximately the same size and lung metastases after the same time interval [12]. In this study 11 of 12 rabbits had terminal disease at autopsy 21–35 days after tumor implantation. One rabbit survived for 69 days which may be explained by chance, by individual immunity or by a poorer quality implant.

The proton NMR spectra of lipoproteins from serum represent several molecular species, including cholesterol, triacylglycerols, phospholipids and free fatty acids. Bell *et al.* [10], using the Hahn spin-echo method, have assigned the proton resonances in the lipoprotein fractions. The major parts of the methyl and methylene signals were shown to represent LDL, HDL, VLDL and chylomicrons.

A careful examination of the spectral profiles of serum from rabbits in the control group and in the tumor group reveals some characteristic features (Figs 2, 4): all methylene peaks in the spectra from the control rabbits exhibit, invariably, a distinct shoulder or a double peak on the high-field side. This particular resonance, however, is missing in the spectra of the rabbits with established VX-2 carcinoma. The shoulder gradually decreases shortly after implantation until complete disappearance at approximately 2/3 of observation time after implantation. The same variation was observed in all rabbits with VX-2 carcinoma implanted with only small differences in relation to time scale for the disappearance of the shoulder. In the control group a distinct shoulder or double peak persisted during the whole time period.

We have also observed the same characteristic high-field shoulder in the methylene profile of serum from healthy human volunteers, while the shoulder in most cases is lacking in spectra from cancer patients (unpublished results). Berger *et al.* [4] have made similar observations, and conclude that the shoulder stems from HDL and/or the LDL fractions. Herring *et al.* [11] suggest that the line shape is controlled by the relative concentration of VLDL, LDL and HDL. In computer simulation they have demonstrated how elevated VLDL and decreased HDL give narrow line widths.

The connection between cancer and lipid metabolism has been established through several independent reports [13, 14]. The progressive depletion of fat depots in cancer patients is well known. In order to compare the effects of weight loss due to dietary restrictions and malignant disease, the lipoprotein spectra of a control group were monitored during a period of reduced food intake. The spectral changes observed (Fig. 3) are shown to be dramatic, but significantly different from those observed during progressive growth of the VX-2 carcinoma. Evidently, the effects of malignancy on the lipoprotein fractions are different from the effects resulting from prolonged dieting.

So far we have shown that by visual inspection of spectra it is possible, in most cases, to discriminate between healthy controls and rabbits with tumors. We have also started a series of experiments with VX-2 carcinoma implanted in the rabbit hindlimb, as a slower progress with later development of metastases is expected compared with renal implantation. However, a major problem still remains: how to devise a quantitative, diagnostic criterion and determine at which stage of malignancy this criterion is reliable. Fossel's use of line widths to distinguish between malignant and non-malignant samples will in our opinion be of limited value as a discriminatory test. A non-Lorentzian, composite signal can never be properly defined by a single value of the line width at half-height. This is clearly demonstrated in the spectra shown in Fig. 4, where typical methylene resonances have a distinct shoulder. The line widths at half-height in such a case will often be subjected to large errors since these measurements are critically dependent on a reliable estimate of the true base line. The line width of peaks with no apparent shoulder, is of course, less influenced by ill-defined base lines.

In order to circumvent this problem we suggest that the digitally defined profiles of the methylene peaks should be used rather than a single line width measurement. We have undertaken a multivariate data analysis, called principal component analysis [15, 16] using the major part of the spectral profile as input variables. Preliminary results of this type of analysis on a limited data set from humans are encouraging.

The specific effect on VLDL status in serum caused by the presence of malignant disease may give an important clue to the mechanism involved. By delipidation of isolated lipoprotein fractions from serum from cancer patients and controls, purified apolipoproteins may be produced by HPLC. These proteins have relatively low molecular weight and are thus well suited for characterization by NMR spectroscopy. By comparing spectra of both native and apoprotein one may be able to reveal whether changes induced by malignancy are connected to the lipid or the peptide part.

We conclude that rabbit with implanted VX-2 carcinoma is a suitable model for a controlled study of spectral changes in lipoprotein pattern in neoplastic disease. The same changes in spectral pattern are seen as in humans with progressive malignant disease. Surgery did not produce significant changes in the lipoprotein pattern. The changes in the lipoprotein pattern induced by slimming were different from those due to progressive malignant disease.

1. Fossel ET, Carr JM, McDonagh J. Detection of malignant tumors: water suppressed proton nuclear magnetic resonance spectroscopy of plasma. *N Engl J Med* 1986, **315**, 1369–1376.
2. Holmes KT, Mackinnon WB, May GL *et al.* Hyperlipidemia as a biochemical basis of magnetic resonance plasma test for cancer. *NMR Biomed* 1988, **1**, 44–49.
3. Dowd TL, Kaplan BA, Gupta RK, Aisen P. Detection of malignant tumors: water suppressed proton nuclear magnetic resonance spectroscopy of plasma. *Magn Resonance Med* 1987, **5**, 395–397.
4. Berger S, Pflüger K-H, Etzel WA, Fischer J. Detection of tumors with nuclear magnetic resonance spectroscopy of plasma. *Eur J Cancer Clin Oncol* 1989, **25**, 535–543.
5. Fossel ET. Commentary on 'Detection of tumors with nuclear magnetic resonance spectroscopy of plasma' by S. Berger, K-H. Pflüger, W.A. Etzel, J. Fischer. *Eur J Cancer Clin Oncol* 1989, **25**, 925–927.

6. Wilding P, Senior MB, Inubushi T, Ludwick ML. Assessment of proton nuclear magnetic resonance spectroscopy for detection of malignancy. *Clin Chem* 1988, **34**, 505–511.
7. Sträuli P, Haemmerli G. The V2 carcinoma of the rabbit as an integrated model of tumor invasion. *Bull Cancer (Paris)* 1984, **71**, 447–452.
8. Vargha G, Miltényi L, Endes J, Endes P, Kelemen J, Szücs L. Über eine neue Transplantationsmethode des VX2-Nierencarcinoms von Kaninchen. *Res Exp Med* 1974, **164**, 77–82.
9. Göthlin JH, Barbaric ZL. Tissue adhesive in surgical and crush renal trauma. An experimental angiographic investigation in the rabbit. *Scand J Urol Nephrol* 1978, **12**, 145–149.
10. Bell JD, Sadler PJ, Macleod AF, Turner PR, La Ville A. ^{1}H NMR studies of human blood plasma. Assignments of resonances for lipoproteins. *FEBS Lett* 1987, **219**, 239–243.
11. Herring FG, Phillips PS, Pritchard PH. Proton magnetic resonance spectroscopy of plasma from patients with dyslipoproteinemia: identification of factors governing methyl and methylene proton line widths. *J Lipid Res* 1989, **30**, 521–528.
12. Gadeholt G. Effects of intra-arterial and systemic infusion of doxorubicin treatment in rabbits with implanted renal VX-2 carcinoma. *J Interv Radio* 1989, **4**, 67–71.
13. Nydegger UE, Butler RE. Serum lipoprotein levels in patients with cancer. *Cancer Res* 1972, **32**, 1756–1760.
14. Barclay M, Skipski VP, Terebus-Kekish O, Greene EM, Kaufman RJ, Stock CC. Effect of cancer upon high-density and other lipoproteins. *Cancer Res* 1970, **30**, 2420–2430.
15. Wold S, Espensen K, Geladi P. Principal component analysis. *Chemometrics Intell Lab Syst* 1987, **2**, 37–52.
16. Kvalheim OM, Karstang TV. A general-purpose program for multivariate data analysis. *Chemometrics Intell Lab Syst* 1987, **2**, 235–237.

Acknowledgement—This work was supported with grants from The Norwegian Cancer Society.

Eur J Cancer, Vol. 26, No. 5, pp. 615–618, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

Detection of Malignant Tumours by Multivariate Analysis of Proton Magnetic Resonance Spectra of Serum

Einar Sletten, Olav M. Kvalheim, Svetlana Kruse, Mikael Farstad and Odd Søreide

Proton magnetic resonance spectra of blood serum have been subjected to multivariate data analysis to discriminate between samples from cancer patients and from controls. The main feature was the use of digitally defined resonance profiles. The methyl and methylene lipoprotein signals centred at 1.3 and 0.9 parts per million are non-lorentzian composite peaks that cannot be described properly by the line width at half-height. Instead 71 and 76 data points were used to describe the methylene and methyl peak profiles, respectively. These data points were used as input to a principal component analysis to distinguish between malignant (n = 29) and control samples (n =55). At a probability level of 0.01 (F-test) modelling classified all patients except 2 correctly, while 1 control was slightly above the predictive level for malignancy.
Eur J Cancer, Vol. 26, No. 5, pp. 615—618, 1990.

INTRODUCTION

CANCER may influence lipid metabolism in man [1, 2]. Interest in proton magnetic resonance (NMR) as a diagnostic tool for cancer was revived by Fossel *et al.* [3]. The mean line widths of the methyl and methylene resonances of lipoprotein lipids in plasma correlated with the presence of disease, values being lower in patients with malignant tumours. Several research groups have failed to produce the same excellent descrimination [4–10]. Fossel [11] pointed out several methodological factors to account for this discrepancy: field strength, recording temperature, field homogeneity and storage conditions.

In our opinion the major problem with the Fossel test is found in the univariate description of the resonance profiles. The lipoprotein proton NMR spectra from serum represent several molecular species, including cholesterol, triacylglycerols, phospholipids and free fatty acids. The major parts of the methyl and methylene signals [5] represent low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) and chylomicrons. A non-lorentzian composite signal cannot be properly defined by a single value of the line width at half-height. In addition, variable amounts of lactate, manifested as two satellite peaks on the low-field side of methylene, may affect line width measurements.

To circumvent this problem we suggest that digitally defined profiles of the methyl and methylene peaks should be used in the analysis. We have applied multivariate principal component analysis [12, 13] with the major part of the spectral profile as input variables. We have successfully used a similar method to analyse diffuse reflectance infrared spectral profiles of coals [14].

Correspondence to: E. Sletten, Department of Chemistry, University of Bergen, Allegt. 41, N-5007 Bergen, Norway.
E. Sletten, Olav Kvalheim and S. Kruse are at the Department of Chemistry and M. Farstad and O. Søreide are at the Laboratory for Clinical Biochemistry, University of Bergen, Bergen, Norway.