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Detection of Malignant Disease by 1H-NMR Spectroscopy of Blood Plasma

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

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Ottawa, Ontario, Canada
August, 1988

ISBN 0-315-46820-3

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Acknowledgements

To Dr. Carolyn Mountford, a special appreciation for the initiation of this research. Her interest and support were an invaluable source of energy, much needed at times.

Without the technical help of Therese Kroft much of this research would not have been possible. .

Many thanks to Dr. John K. Saunders whose useful discussions have greatly improved my knowledge and understanding of NMR.

From Mrs. Eva Princz, the friendship, kindness and assistance in times of desperate need will always be remembered.

A word of thanks to Beatrice Winsborrow who assumed the role as my mentor and helped to progress the thesis writing in the days of its infancy. I look forward to her lasting friendship.

I wish to acknowledge two very close friends, Jacinta Drew and Anita MacEachern with whom I have shared many unforgettable experiences and whose support has known no bounds.

My initial instructions in NMR and Biophysics I owe to Dr Laura Stewart. Her patience, helpful suggestions and many coffees were greatly appreciated.

For the suggestions, ideas and comments toward my thesis and life in general at the lab, sincere thanks to Dr Roxanne Deslauriers.

I very much appreciate the interest and suggestions of Dr. Irena Ekiel with regard to my work. Thanks to Dr. Harold Jarrell for many stimulating conversations and suggestions and to Dr. Keith Butler for sharing his wealth of knowledge with me.

My gratitude to all my friends and fellow students in the lab with whom I have shared many frustrations and much fun.

To my family, a special word for their loving support.

Many thanks to the NRC commissionnaires especially Mr Terry McConnell and Mr Ken Drew for their kindness and attentiveness.

To Dr. Jean-Marc Bonmatin, who I met later on in the course of my stay but whose friendship I value greatly, thanks for the many lessons in French.

Finally, I am indebted to my supervisor, Dr. Ian C. P. Smith, with whom I feel fortunate to have been a graduate student. He has always given me cheerful support and I admire his ability to find the positive in what appears bleak. His effervescent energy was greatly appreciated in both good and bad times. His generosity with his time and knowledge was enlightening. For all of this, Many thanks.

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LIST OF ABBREVIATIONS

AFP	- Alphafetoprotein
CA125	- Cancer Antigen 125
CEA	- Carcinoembryonic Antigen
COSY	- Correlated Spectroscopy
^{1}H-NMR	- Proton Nuclear Magnetic Resonance
HDL	- High Density Lipoprotein
HDL_1	- High Density Lipoprotein (Subclass 1)
HDL_2	- High Density Lipoprotein (Subclass 2)
LDL	- Low Density Lipoprotein
NCI	- National Cancer Institute of Health (Bethesda,Maryland)
NMR	- Nuclear Magnetic Resonance
NRC	- National Research Council of Canada
O.D.	- Outside Diameter
PBS	- Phosphate Buffered Saline
TG	- Triglyceride
VLDL	- Very low density lipoprotein

ABSTRACT

Elevation and decrease of VLDL and HDL_2 respectively, have been observed in blood plasma of cancer patients. These observations were not specific to a particular type of cancer but to cancer in general. It has been discovered that ^{1}H-NMR spectra of plasma from cancer patients differ from those of normal individuals in that the lines due to the mobile lipid components are narrower. We have done experiments which demonstrate this discovery. We have also observed (as was mentioned in the original discovery) that a great variability exists in patients who have undergone treatments of various forms. In a few cases where we had obtained blood from patients before and after therapy the linewidths increased after treatment. A patient whose plasma was measured several times over the course of this study showed a drop and then an increase in linewidths. It remains to be seen whether her cancer has been eradicated.

The linewidths, in general, varied inversely with the level of TG in the blood. Although more analyses are necessary, it appears that a positive correlation between linewidths and HDL levels exists. Our measurements agree with the literature in that elevated VLDL (the major carrier of TG in the blood in the fasting state) and relatively low HDL levels have been measured in the blood of cancer patients.

Other attempts have been made at searching for diagnostic indicators of malignant disease in ovarian cancer patients. T2

relaxation measurements have been performed on what was suspected to be a fucosylated proteolipid isolated from the plasma of cancer patients. Preliminary results have given T2 values of 100-300 ms contrary to literature values (960 ms). Further studies on such samples are necessary for definitive answers concerning the molecular species contributing the long T2 reported in the literature.

Chapter 1

A seemingly neverending battle in the ongoing war against the insidious disease known as cancer is in its detection. Researchers armed with the knowledge and machinery to fight this battle must combat not merely one but literally thousands of opponents. With cooperation between research groups success is possible.

i) Tumour Markers

Indeed, much progress has been made in recent years in the discovery of biochemical markers for human tumours (commonly known as tumour markers). However, finding a marker specific to the tumour and not to normal tissue remains a problem. The markers in use to date and those being tested for future use, appear to be in greater concentration in the presence of a tumour than in its absence but are not necessarily specific to the cancer itself.

The most widely used marker to date in the detection of recurrent colorectal cancers is CEA. The complete structure of this marker has not been fully characterised although it is known to consist of a protein dimer having similar subunits. Variability in the carbohydrate contribution has made characterization difficult (Chu, T. M., 1987)).

The primary sequence of the tumour marker AFP has been elucidated. AFP, a protein of 590 amino acids, has been said to be

perhaps the best candidate for use in the screening of primary hepatocellular carcinoma. In China, Southeast Asia and Africa the population is at high risk for the disease. Thus it follows that the prevalence (percentage of the population with the disease at any one time) should be great. A high prevalence of a disease in a population is necessary for the effective use of a marker in screening. In order to have predictive value, the marker must also be specific for the disease and detectable to a high degree of sensitivity.

In a screening study of about 1/2 million Chinese people, elevated levels of AFP were found in 57 individuals who were later diagnosed with liver cancer. These people were asymptomatic at the time of the study and thus, in this case, the use of the protein as a tumour marker looks promising. However, further testing is necessary to verify to acceptable standards that AFP is useful for this purpose.

This particular marker is specific for hepatocellular carcinoma and testicular tumours of germ-cell origin. However, measurements of the blood chemistry of cancer patients have shown trends that were consistently observed regardless of the type of cancer. Barclay and coworkers (1964, 1970) measured abnormal levels of lipoproteins (VLDL and HDL_2) in the sera of patients with a variety of malignant diseases. Specifically, HDL_2 levels were decreased in the sera of both males and females whereas VLDL levels were elevated. The utility of the measurement of such lipoprotein levels in the diagnosis of cancer would depend on several parameters. A

relatively quick test on a small sample (which would result in the measurement of a quantitative, definitive value) is, intuitively, the minimum requirement.

In a study by Barclay et al (1964) concerning individuals with a family history of cancer, it is interesting to note that the VLDL levels in men were also unusually high while HDL_2 levels in both men and women were low. Neither measurement resulted in levels as high or as low, respectively, as in cancer patients.The levels were statistically significantly different when compared with those in normal individuals. A follow-up study, five years later, of 13 women and men with a family history of cancer resulted in 4 (2 women and 2 men) with very low levels of HDL_2 and the subsequent appearance of cancer. These results suggest that it could be possible to measure circulating levels of specific lipoproteins to identify high risk individuals. It is necessary to specify that those qualifying for the kindred study were individuals with one or more immediate family members (siblings, parents) or grand-parents) or two or more aunts or uncles who had had cancer.

ii) Relevant NMR studies

Our interests in using ^{1}H-NMR in the detection of cancer stemmed from observations in Australia by the group of Mountford and co-workers. In 1982 it was observed that a high resolution spectrum was obtained from leukemically transformed lymphocytes

and a somewhat broader but similar high resolution spectrum was obtained from mitogen-stimulated peripheral blood lymphocytes (Figures 1-1A & 1-1B). The spectrum obtained from normal T-lymphocytes was broad (Figure 1-1C). Spectra of cell preparations from an Epstein Barr virus cell line (GK) were similar to those of the mitogen-stimulated lymphocytes. Isolation of the plasma membranes of cells from the Epstein Barr Virus cell line, JP, gave rise to a similar spectrum as that of whole cells (Figures 1-2A & 1-2B). Thus it appeared that the high resolution spectrum was originating from the membrane. (Mountford et. al. (1982)). A high resolution spectrum for membrane lipids is unusual due to the relatively immobile environment of the lipid acyl chains.

Further studies in 1984 led to the identification of the resonances arising from mammalian membranes (Cross et. al (1984)). Specifically, this was a study of membranes of the rat mammary adenocarcinoma (J-Clone) cell line. A two-dimensional NMR method or COSY, which emphasises the coupling between protons on adjacent carbon atoms identified the origin of the resonances to be lipid acyl chains. These were later discovered to be due, for the most part, to triglycerides. In a COSY spectrum, a unique coupling exists between geminal protons on the carbon atoms of the glycerol backbone of triglycerides. This is identified in FIGURE 1-3 as cross peak G'. (Mountford et. al. (1985). The same cross peak was evident in the COSY spectrum of the cell line. Thus the resonances arising from the triglycerides were located in membrane domains, like lipoprotein

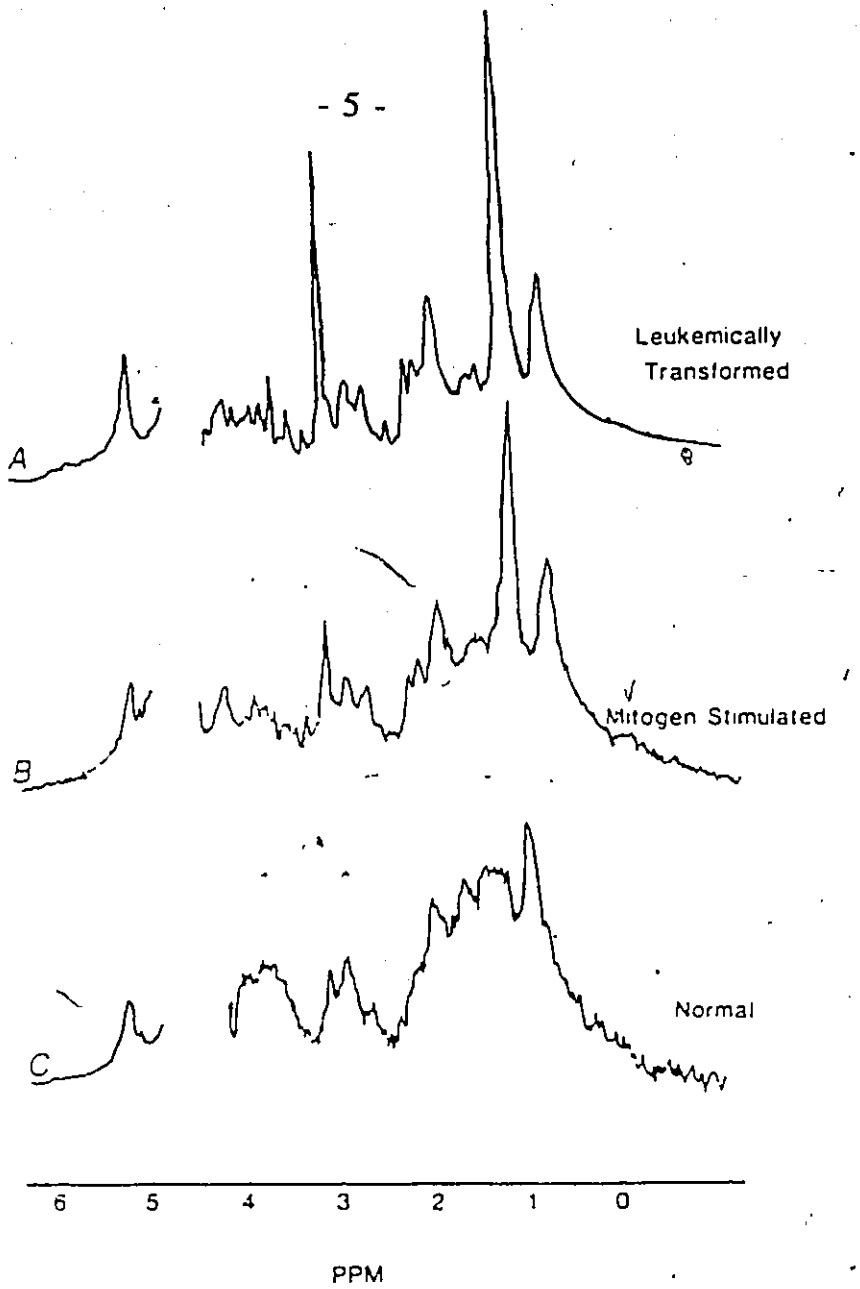


FIGURE 1-1 ^{1}H-NMR Spectra (400 M.Hz.). A, ALL T-Cell line CCRF/CEM (5 x 10^7 cells) suspended in PBS in D_2O; B, normal peripheral blood T-lymphocytes after exposure to Poke Weed Mitogen (2 µg/ml) for 5 days; C, normal peripheral blood T-lymphocytes (5 x 10^7) suspended in PBS in D_2O. Spectra were recorded in 11 min. at 37°C on a Bruker WM-400 Spectrometer with the residual HOD peak suppressed by selective irradiation.

(Mountford, C. et. al. June 1982)

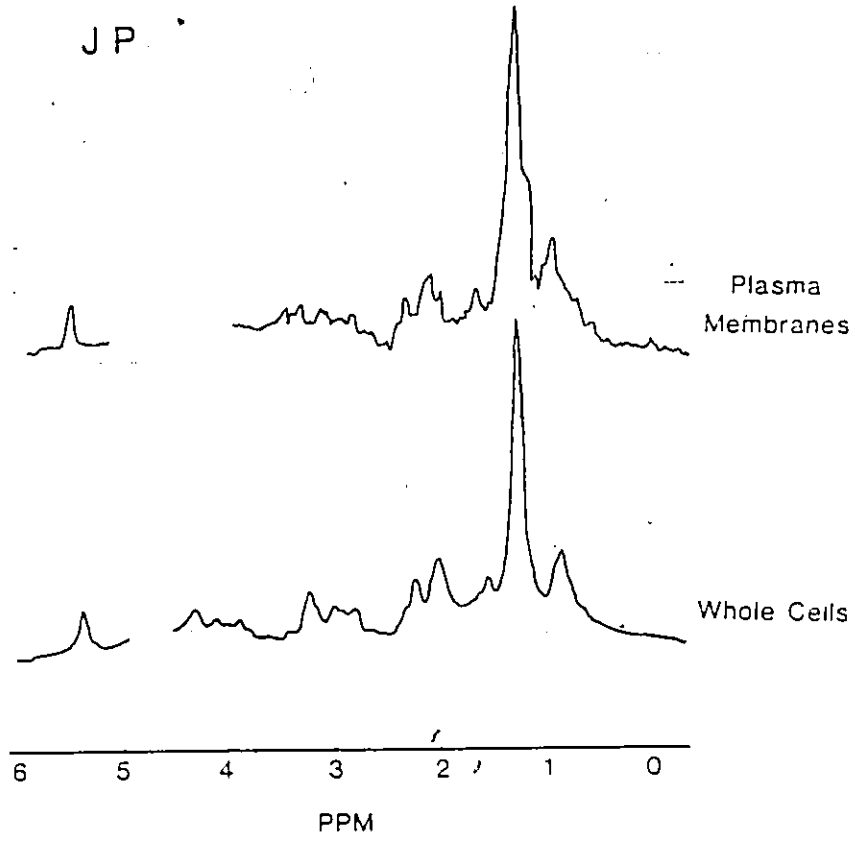


FIGURE 1-2 ^{1}H-NMR Spectra (400 M.Hz.). **A**, membrane ghosts (2 x 10^7) isolated from the EBV cell line JP resuspended in PBS in D_2O. **B**, EBV cell line JP (5 x 10^7) suspended in PBS in D_2O. Spectra were recorded in 11 min. at 37° on a BRUKER WM-400 Spectrometer with the residual HOD peak suppressed by selective irradiation.

(Mountford, C. et. al. June 1982)

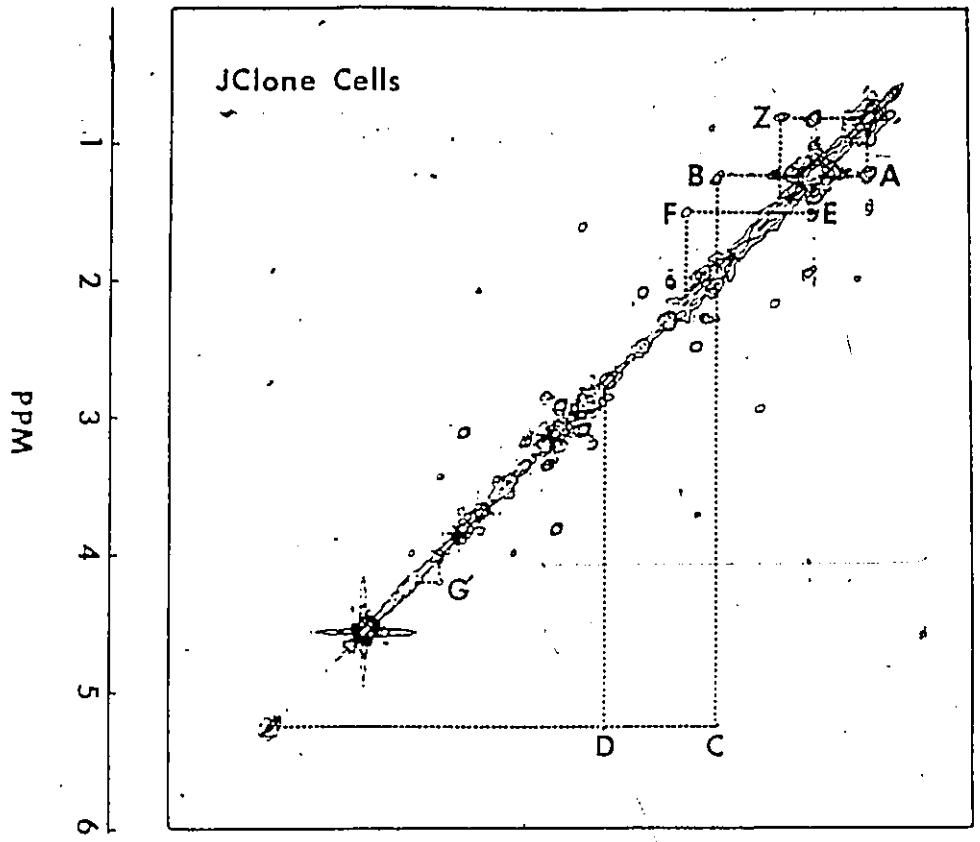


FIGURE 1-3 Symmetrised COSY spectrum of a suspension of J-Clone cells (1 x 10^8 cells) in phosphate buffered saline in D_2O. Spectra were obtained at 37°C with the sample spinning and the residual HOD peak suppressed by gated irradiation. Cross peak G' is a result of the geminal couplings between protons on the glycerol backbone of triglyceride molecules.

(Mountford, C. et. al. Nov. 1985)

domains, such that isotropic tumbling could occur. This type of motion would result in the high resolution character of the spectra.

Such results sparked an interest in the study of methylene resonances arising from tumour biopsy samples (Princz, E. M.Sc. Thesis (1988)) and components of human blood plasma samples using NMR measurements such as transverse relaxation times and, furthermore, simple spectra for the blood samples.

Proton NMR of plasma has been used by Sadler et. al (1983,1984,1987) in the past decade to study metabolic abnormalities in diseases such as diabetes. The results obtained by Fossel and co-workers (1986) generated an interest in the development of ^{1}H-NMR as a potential test for diagnosing malignant disease. The method they used consisted of measuring an average value for the linewidths of the composite methyl and methylene resonances of a ^{1}H-NMR spectrum. The Fossel test or the mean line width measurement ($\nu_{1/2}$) (it shall be called either of these in further reference) showed much promise in diagnosis in that the results obtained from patients with malignant disease were found to be significantly different from normal controls and from patients with other infections and inflammatory diseases. The conditions of pregnancy and benign prostatic hypertrophy in test subjects were sources of false positive results. It is important to note that the cancer population studied was entirely symptomatic and cancer patients who had undergone treatments showed spurious values. (i.e. results ranged over a large set of values encompassing all categories).

Many groups , including our own, have tried to verify the results obtained by Fossel and co-workers and to understand the biochemical basis of this test. Wilding et al. (1988) have discovered that the Fossel values obtained were inversely related to the plasma TG levels. Chmurny et al (1988) in an HPLC study of human blood plasma also found elevated VLDL levels. (VLDL is the major carrier of triglycerides in the fasting state). Holmes et. al. (1988) in an earlier study concluded that the biochemical basis for the decreased linewidths observed for cancer patients was an increase in their TG levels. In this case, patients with hypertriglyceridemia showed false positive results (i.e. linewidths less than 25 Hz.).

In light of the fact that Barclay and Skipski saw a decrease in the HDL levels in addition to increases in VLDL, it is possible that a distinction could be made between cancer patients and hypertriglyceridemia. In the latter case, one may expect that the VLDL level should be excessive such that the contribution of HDL to the linewidth would be negligible. In the case of cancer patients, the VLDL level is not as excessively elevated and while the HDL level is somewhat lowered, its contribution is finite. Our samples were measured at 360 M.Hz.. This distinction would be better achieved at higher magnetic fields as Chmurny and coworkers demonstrated. Another option would be a mathematical deconvolution of the spectra obtained. (Sletten, E. et al., (1988).

^{13}C-NMR studies of human blood plasma have been used to distinguish between benign and malignant primary intracranial neoplasms. (Tomchuk et. al. (1987)) The outstanding spectral features in the malignant cases, as compared to benign cases or normal subjects, were the presence of peaks due to higher concentrations of mono- and polyunsaturated fatty acids. However, some overlap between cancer and non-cancer groups was observed. A nice feature of the ^{1}H-NMR study on the same group of patients was the ability to distinguish the stage of the tumour as well as benign neoplasms.

The advantage of having a malignant condition manifested in the chemistry of the blood is that such abnormalities can be easily detected. However, it would be of greater utility to have a malignant condition expressed by a non-specific malignancy marker. An NPL was discovered in human serum HDL by Barclay and co-workers in 1971. Its biological and chemical properties were different from those of lipoproteins in that it was extractable into organic solvents, contained a large amount of monosaccharides and had a protein component in which a large proportion of the amino acids consisted of glutamine and/or glutamic acid. The NPL was present, as well, in the following tumours: Walker Carcinosarcoma 256, sarcoma 180 and the Taper liver tumour. Detection of NPL in the serum HDL was from patients with such cancers as breast, ovarian, uterine, bronchiogenic carcinoma, melanoma, neuroblastoma and chronic Myelocytic Leukemia.

Petitou and co-workers, (Petitou et. al. (1978)), using fluorescence polarization techniques, studied leucocyte cell membranes from patients with leukemia and from normal controls. It was discovered that the mobility in the membrane systems studied was significantly higher in leukemia patients than normals. In further experiments, plasma membrane vesicles with a high degree of microviscosity were isolated from pleural effusions and from the blood serum. The cholesterol/phospholipid ratio of the leukemia cell membranes were much lower than normal: 0.39 as opposed to 0.67, respectively. Using similar measurements, the isolated vesicles had a cholesterol/phospholipid ratio that was greater than 1. On the basis of these observations, the researchers hypothesised that plasma membrane vesicles containing relatively high amounts of cholesterol were actively shed from leukemia cell membranes leaving behind a more mobile environment.

A putative RNA-containing proteolipid was reported by Wieczorek and co-workers to have been isolated from the blood of cancer patients and from the media of various cancer cell lines Wieczorek et al. (1985). The discovery was significant to our work because it was claimed that the proteolipid was actively exocytosed from the cells and thus at some time would be necessarily associated with the cell membrane. It is possible that this proteolipid made a contribution to the high resolution spectrum and the long T2 relaxation value of cancerous tumours as was seen in our lab (Princz, E. M. Sc. Thesis) and that of Mountford and co-workers. The

remarkable features of the proteolipid were the presence of RNA, its association with an oligopeptide composed of 9 amino acids and the large glycosphingolipid content (24.1% - 37.6%) (Wieczorek et. al. (1985)). Nonspecificity of tumour type was also featured. The complex was found in a wide variety of cancers including myelogenous leukemia, malignant lymphoma, ovarian and uterine carcinoma to name a few. When looked for in patients with other inflammatory conditions (including pregnancy) the complex was present in rather low levels relative to those in cancer patients (Wieczorek et. al. (1985)). The isolation of the proteolipid from the blood of cancer patients followed by ^{1}H-NMR transverse relaxation experiments (T_2) could provide information on the biological origin of the long T_2 seen in the tumour samples. It appeared, that this "RNA-proteolipid" could potentially be useful in the detection of cancer.

iii) Relevant NMR theory

It is necessary at this time to introduce nuclear relaxation.

Many nuclei in nature, including the proton, have the property of magnetism. The magnetic character of the nucleus is that which allows one to perform NMR. Each such nucleus also has associated with it the property of spin. When the nuclei are placed in a magnetic field, like a regular bar magnet with north and south poles, the nuclear spin vectors will align themselves either with or against the induced field. The resultant vector is aligned with the magnetic field giving a bulk magnetization vector. The process of

alignment takes a finite period of time. The alignment itself represents the equilibrium system.

After perturbing the equilibrium magnetization using radiofrequency radiation the nuclear spin vectors acquire energy and phase. Two relaxation processes denoted T1 and T2 then occur simultaneously. A T1 or spin-lattice relaxation time differs from a T2 or spin-spin relaxation time in the method by which the processes occur. Whereas T1 is accomplished due to a transfer of energy, of the correct frequency, of a nucleus to its surroundings (or lattice), T2 is a loss of phase coherence or phase memory of the spin vectors. Interactions between spins effect this loss. This may be visualized in the T2 experiment which was used extensively for measuring this parameter known as the Carr-Purcell-Meiboom-Gill (CPMG) sequence.(Meiboom, S. et. al. (1958)). The details of this sequence will be discussed further in the last chapter.

Nuclei in environments which allow isotropic tumbling undergo rapid reorientational motions such that interactions between nuclear spins are infrequent. The molecular components of these environments are often relatively mobile, such as the neutral lipid interior of a lipoprotein-like complex or in a hydrophilic solution itself. It is the interaction between spins which effects the loss of phase coherence (detectable magnetization) in a T_2 experiment. Thus infrequent interaction would result in the slow loss of phase coherence and a long T_2 ($>$ 350 ms). On the other hand, nuclei which

are somewhat restricted in their motions lose phase coherence relatively quickly (due to frequent interactions of spin vectors) and thus give a short T2 value. Therefore, the T2 relaxation times can provide information about the relative mobilities of the nuclei in their environments. Methyl groups (like those of lactate) which undergo rapid reorientations in an isotropic environment will give T2 values greater than 1 second while the methylene protons of lipid acyl chains in the core of a lipoprotein - like particle show T2's ranging from 100-300 miliseconds. T2 relaxation measurements combined with chemical shift information can potentially elucidate the molecular species involved.

In the case of simple proton spectra of biological samples (like blood plasma) the interpretation of the motions is not straightforward due to the fact that the spectra are simple resonances composed of single lines. The significance here is that the resonance linewidths at half height are generally governed by the motions of the molecules. In simple spectra, $\Delta\nu = 1/2\Pi T2^*$. ($\Delta\nu$ is the linewidth of a peak at half height; T2* is the actual observed T2 which accounts for field inhomogeneities and other fluctuations affecting T2). In biological samples, however, the spectra are composed of overlapping resonances. These add together resulting in a resonance envelope containing unequal contributions from macromolecules and small molecules. Small molecules (like lactate) contribute narrow resonances due to the fact that the rapid tumbling in solution defines much better the frequency observed by the

spectrometer and thus gives rise to a narrow peak. Macromolecules, however, tumble slowly relative to small molecules so that a range of frequencies is observed for the species giving rise to the resonance and thus the peak is broad. Large proteins (such as serum albumin) which are of the order of 68,000 molecular weight tumble very slowly in solution in comparison to small molecules and give rise to a broad hump underlying the high resolution part of a serum spectrum.

As was indicated earlier, with mathematical manipulation, it is possible to separate components and thus derive information from such complex spectra. It is also possible, using different spectroscopic pulse sequences, to characterize better the system observed. Several researchers have gained valuable information on various aspects of the malignant condition from ^{1}H - N M R experiments. ^{1}H-NMR spectroscopy can give a better understanding of the system being studied and has great possibilities for the study and understanding of biological systems. In fact, as will be seen in the following chapters, it has the potential to assist in the diagnosis and/or prognosis of the cancerous condition.

Chapter 2

DETECTION OF MALIGNANCY IN OVARIAN CANCER PATIENTS

The circulating levels of plasma lipoproteins in patients with malignant disease have been known to be abnormal for more than two decades (Barclay et al. 1955, 1964, 1972, 1975). Plasma lipoproteins are the major carriers of triglycerides, cholesterol, esterified cholesterol and phospholipids in the systemic circulation. These are transported to organs such as the liver and adrenal glands for bile acid synthesis, steroidogenesis, and membrane synthesis, and to adipocytes for storage of excess triglycerides.

i) Lipoprotein classification

The plasma lipoproteins have different classifications based upon the techniques used to separate their various classes. The nomenclature prebeta, beta (β), and alpha (α) have been used to describe their behaviour in electrophoretic gels using various support media (Scanu et al. (1984)). These terms may correspond to, respectively, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). This latter distinction is due to the equibrilium densities of the lipoprotein classes in an isopycnic density gradient after having been subjected to an ultracentrifugation for periods of 24 hours or more (Havel et. al, 1955). VLDL particles have been measured at densities between 1.006 g/ml and 1.019 g/ml, LDL have been located between 1.019

g/ml and 1.063 g/ml and HDL between 1.063 g/ml and 1.210 g/ml. (FIGURE 2-1)

Normal circulating levels of lipoproteins have been extablished and differ according to several factors including sex and age (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES (1980)). Notably, males tend to have higher circulating levels of VLDL than do premenopausal females, while the latter have higher levels of HDL.

ii) Lipoprotein levels and Cancer

Barclay et al. (1964) measured serum lipoprotein levels of premenopausal women. The subjects consisted of two groups from a convent, which suggests somewhat similar diets, two groups from the institutional staff and one group of patients with advanced carcinoma of the breast. Significantly higher levels of VLDL (30 mg/100 ml) were found in the patient group as opposed to the normal groups (3, 3, 2 and 8 mg/100 ml). Significantly lower levels of HDL's (48 and 59 mg/100 ml) were found in the patients as opposed to the normal women (111 and 141 mg/100 ml). These levels were specifically for the high density lipoprotein subclass HDL2 which floats at solution density 1.125 g/ml.

In a subsequent and more extensive study, Barclay et al. (1970) studied lipoprotein levels in men, women and children with and without cancer of various types (ovarian carcinomas in women, lung cancer and melanoma to name a few), VLDL levels were increased significantly in cancer patients as compared with levels

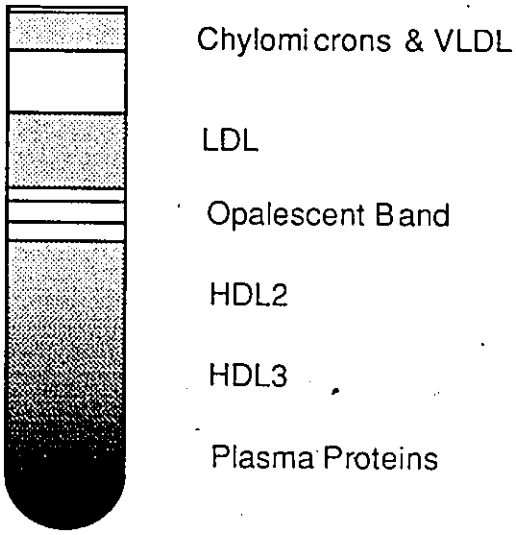


FIGURE 2-1 Gradient with demarcation of layers, **A**, Chylomicron-VLDL, **B**, LDL, **C**, Opalescent band (at a density of approximately 1.085 g/ml), **D**, HDL_2->HDL_3, **E**, Plasma proteins

obtained from normal subjects. HDL_2 was decreased significantly in all three categories of cancer patients as compared with normals. Another high density lipoprotein HDL_1, whose density coincides with LDL, was elevated in women with advanced inoperable breast cancer and other cancer types as opposed to primary operable breast cancer. At a time when a bilateral oophorectomy was performed as a palliative procedure for women with advanced cancer of the breast, Barclay et al. (1955) found that a favorable response resulted in an increase in HDL but an unfavorable response showed increased LDL.

Spiegel et al. (1982) studied lipoprotein levels in patients who had conditions of leukemia or lymphoma. Elevated TG and VLDL levels were discovered with decreased overall HDL levels although only the latter measurements showed significantly different results. It appears that a general trend of an increase in plasma VLDL and a decrease in HDL (especially HDL_2) levels occurs in malignant disease.

iii) ^{1}H-NMR of Lipoproteins

Proton Nuclear Magnetic Resonance (^{1}H-NMR) work on the various classes of lipoproteins has shown assignments for the resonance contributions from the various lipoproteins. (Segrest, J.P., Albers, J.J. Methods in Enzymology, 1986)).

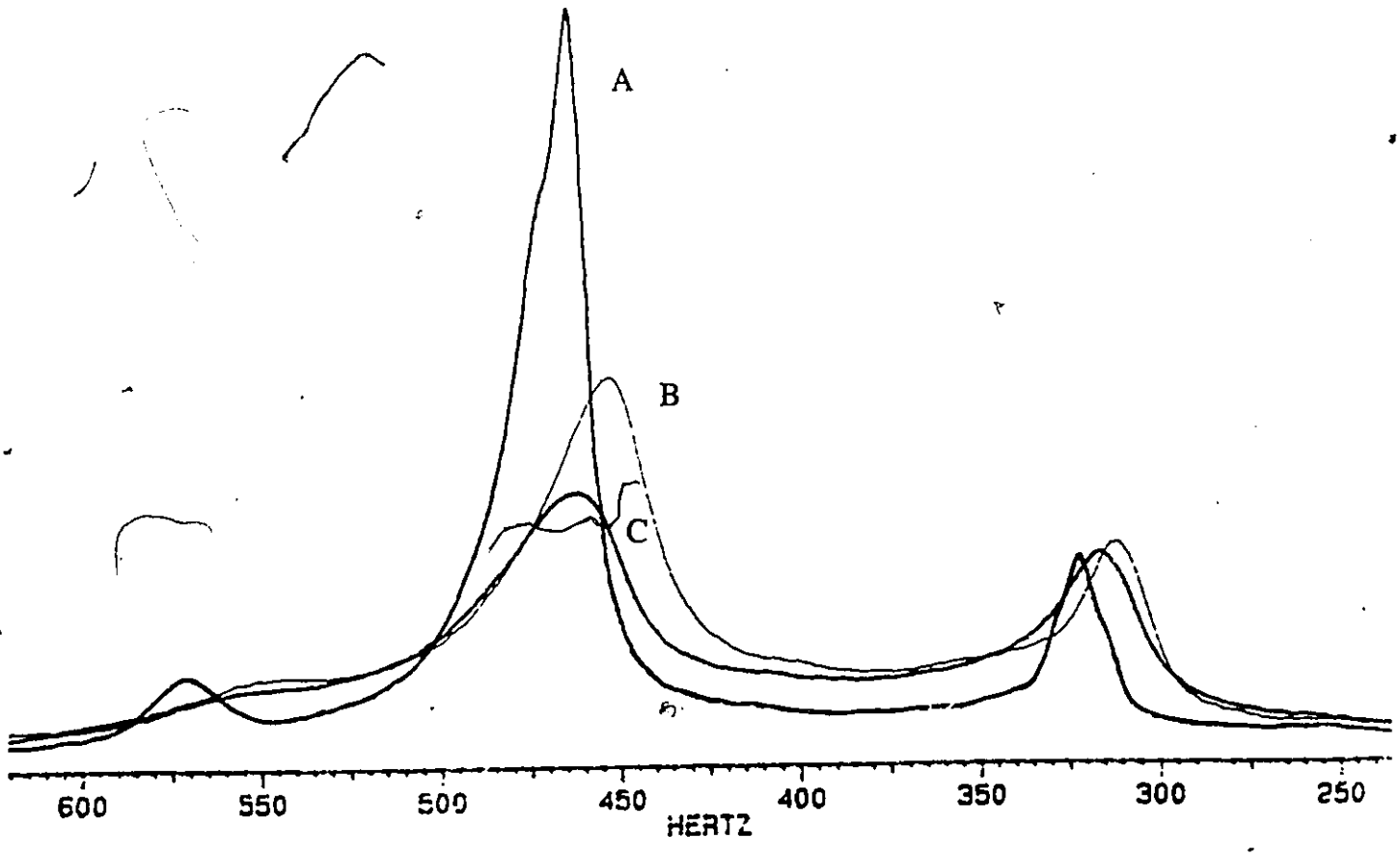
Here it was proposed that this technique is not useful in the quantitation of lipoprotein components or for obtaining structural and/or motional information of same. This is because

mobile hydrogen in biological and chemical systems does not experience a wide range of magnetic environments and means that the frequency range of ^1H signals is not broad (10 ppm). In comparison the magnetically active nucleus ^{13}C can experience a range of frequencies as broad as 200 ppm. The ^1H-NMR signals centered around 1.3 and 0.9 ppm are respectively, contributions from the methylene and methyl ^1H's of primarily triglyceride lipid chains in a membrane bound environment. (Mountford et. al.,(1986)) However, the protons from the CH_3 groups of amino acid side chains of threonine, valine, isoleucine, from metabolites such as lactate as well as fatty acid $(CH_2)_n$ groups, contribute to ^1H-NMR spectra within the same narrow frequency range. Effectively then, peaks composed of such ^1H's will overlap.

In simple systems studied by NMR a characteristic parameter is width of peaks at half height ($\nu_{1/2}$). From $\nu_{1/2}$, information can be extracted about the motions of the nuclei in their environments. This becomes much less straightforward if peaks are overlapping. The predominant contributions to these lipid peaks in blood plasma are from the fatty acid CH_3 and CH_2 protons with some influence by lactate. Because the plasma lipoproteins are the major carriers of fatty acids in the blood the percentage contributions from each of HDL, LDL and VLDL will greatly determine the line widths of the CH_3 and CH_2 peaks. Chmurny et al. (1988) found for VLDL, $\nu_{1/2}$'s of 18.5 and 19 for CH_2 and CH_3 respectively. FIGURE 2-2 shows the superposition of VLDL, LDL and HDL spectra. A difference in

FIGURE 2-2 Superposition of ^{1}H-NMR spectra of HDL, LDL, and VLDL isolated from a patient with Ovarian cancer. The methyl-methylene region or upfield region between 0.85 ppm and 1.32 ppm is shown. Spectra were referenced to TSP (Sodium-3-trimethylsilylpropionate-2,2,3,3-d_4-$(CH_3)_3SiCD_2CD_2CO_2Na$) at 0.0 ppm. Linewidths for peaks, which were measured as stated in text, are as follows:

	A) VLDL	B) HDL	C) LDL
CH_2	24	38	42
CH_3	18	26	31



chemical shift (δ) of 0.02 PPM occurs between the HDL and VLDL CH_2 resonance. Here, the CH_2 and CH_3 $\nu_{1/2}$'s for VLDL, LDL and HDL are as indicated in the figure. One would expect that greater amounts of VLDL and lesser amounts of LDL and HDL should result in a much narrower composite $\nu_{1/2}$. Holmes et al. (1988) found results to this effect.

Fossel et al. (1986) discovered that if one measured the $\nu_{1/2}$ of the methyl and methylene resonances from a ^{1}H-NMR plasma lipoprotein spectrum and calculated the average of these values (FIGURE 2-3) , the number (in Hz) that one would obtain would be lower in patients with various cancers as opposed to normal subjects. Some overlap with both of the above-mentioned groups was obtained for people with non-cancerous disorders but differences were significant. For this group of cancer patients no treatment of any form had been administered and blood was drawn prior to surgery. The test was therefore considered to have potential for the diagnosis of cancer as well as management of malignant disease. A $\nu_{1/2} < 33$ Hz indicating malignant disease was considered to be a positive result while $\nu_{1/2} >= 33$ Hz indicated a negative result.

iv) <u>NRC Study of Ovarian Cancer</u>

Patients with ovarian cancer, since it is such an elusive type of malignancy, would benefit greatly from the detection of disease by such a method. At present the only reliable method of detection is by surgery. A radioimmunoassay using the cancer antigen-125 (CA-

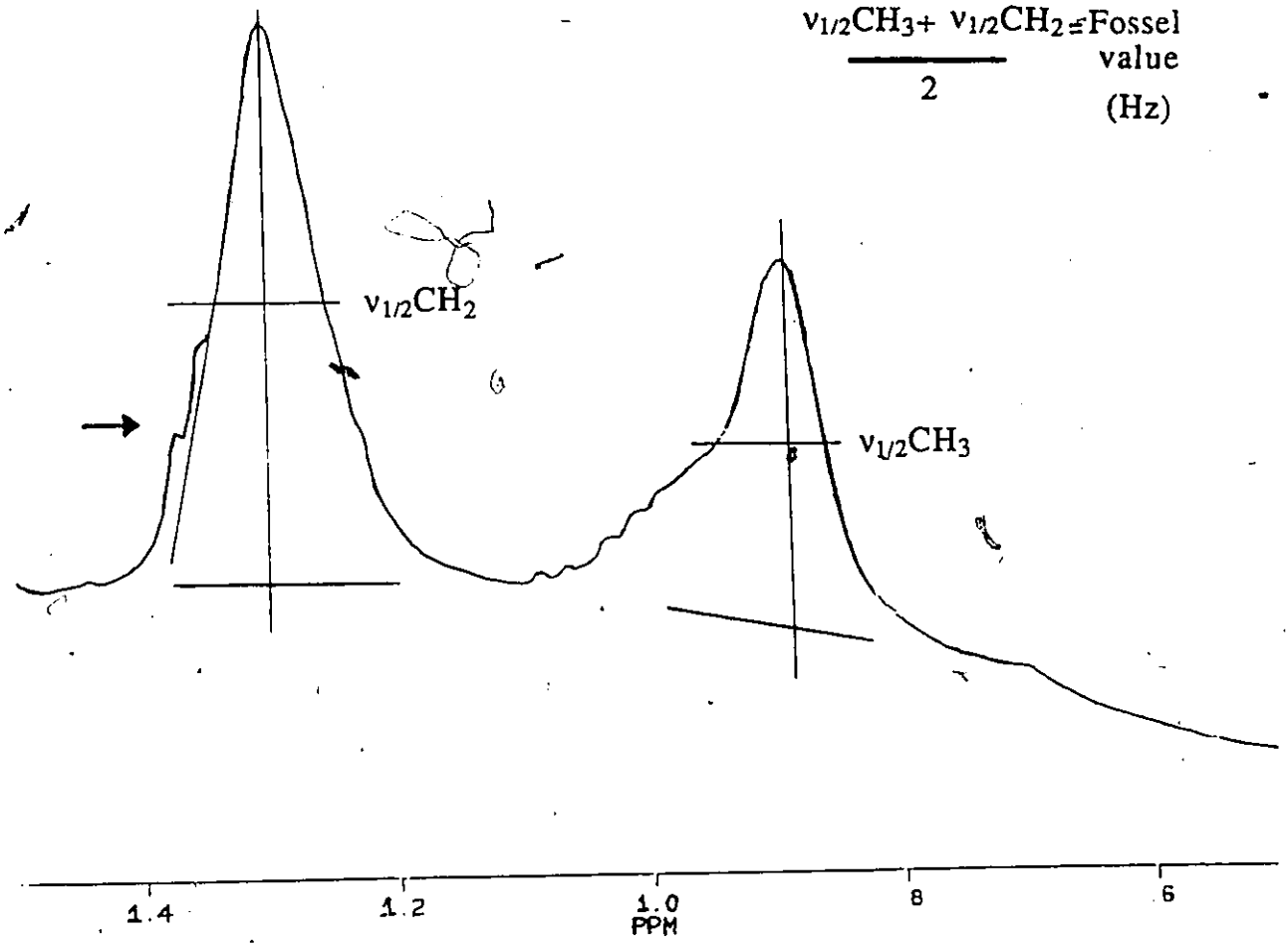


FIGURE 2-3 ^1H spectrum of human blood plasma from an Ovarian cancer patient. Measurement of the linewidth at 1/2 height ($\nu_{1/2}$ in Hz) is as indicated in the figure. Any lactate interference (not apparent here) was manually removed from the measurement. Although lactate interference is not apparent the method of removing its contribution to the linewidth is shown at the arrow.

125) in the early development stages was used with great success for detecting all types of ovarian carcinoma except for the type termed mucinous. It was subsequently found to test positive for many more conditions including pericarditis and pregnancy. Still in the testing stage it has shown great promise for the management of ovarian cancer. Management refers to the ability of a test to detect relapse of disease after treatment.(Alberts, D. S. and Surweit, E. A., (1985))

A study was undertaken to determine if the Fossel test would be useful in the detection of ovarian cancer.. Blood plasma or serum from ovarian cancer patients, from healthy volunteers and from volunteers with nonmalignant disorders was studied using modified methods of the Fossel test. Subjects tested were classified according to treatment, residual disease, and a classification called "No Evidence of Disease" (NED). NED refers to the situation in which the examining surgeon has not found diseased tissue after an operative procedure called "Second Look Surgery" but suspects disease on the basis of the overall condition of the patient. In this last classification all patients have had treatment. Treatments range from surgery to a combination of surgery and chemotherapy. The patient histories in terms of treatments, medications, family histories, etc. were unknown at the time of sampling. For the majority, 105 to be exact, patients in the study were fasted. Three of the subjects studied were pregnant. One of these was sampled in each trimester of pregnancy. Pregnancy in women was a source of false positive results in the study by Fossel et. al. (1986).

Some of the subjects in this study have contributed blood samples two or more times. In the case of controls and cancer patients who have not undergone treatment, an average of values was used in the results. For cancer patients whose treatment regime has changed, only the first result was reported and any further results were commented upon. A data sheet as in FIGURE 2-4 was prepared, thanks to sources other than the author, with the information required for each subject.

Several experiments were performed by various research groups to determine whether conditions such as test temperature (i.e. ^{o}C at which NMR was run), storage temperature, use of serum vs plasma, or lipoprotein abnormalities, had any effect on the linewidths. In 17 cases an average of 2 Hz variability in the linewidth due to running temperature was observed. (Author's results). Differences in the linewidths of serum vs plasma samples from the same subject were insignificant. Neither storage at $4^{o}C$. and NMR the same day nor storage at $-20^{o}C$. and NMR for as long as two years after blood letting was found to affect the results. However, an inverse correlation was found between the plasma triglyceride concentration and the linewidth. People with varying hyperlipidemias were specifically included to test this observation. On average the value obtained was significantly lower than that for normal controls. Results from subjects with hypertriglyceridemia gave a lower mean linewidth measurement than those from patients with various malignant disorders. However, the linewidths were not significantly lower. Subjects with triglyceride levels exceeding 200

NRC *	88
Hospital *	
Name of patient	[redacted]
Doctor	
Hospital	CHRO
Sex	F
Age	26 Weight 60Kg
Smoker	N
medication	Anovulant
Other infections	None
Anticoagulant	EDTA
Fasted before sampling	N
Cancer in family	None
Previous cancer	None
Date of sample	06/04/87
Time of sampling	
Date & type of surgery	none
Date of last treatment	
Type of treatment	
Type of cancer	
Histological type	
Stage of cancer	
Grade of cancer	
Residual disease & Size	
Sample preparation	
Banding	No band x
Fossel Number	33
CPMG *	31
CPMG T1 *	32
T2	
class	control
Blood Type	
CA-125 (<35U/ml)	
CEA (<2.5ug/l)	

FIGURE 2-4 Data required of each patient. NRC #, Name of Patient and Hospital were necessary for filing purposes. At the time of sampling, all information was not available. Other data necessary for classification consisted of Histological type, Stage of cancer, Grade of cancer, whether Residual Disease was present or not, Date and type of surgery (if any), date of last treatment (if any).

mg/dL were placed in the category of hypertriglyceridemia.(Wilding et. al. (1988))

Due to the various conditions that give false positive results, pregnancy, diabetes induced hyperlipidemia and other lipidemias to name a few, one would not expect that such a test would be useful for diagnosis of disease. However, with further study the Fossel test may prove to be a prognostic indicator in the role of cancer management rather than as a screen for cancer patients.

EXPERIMENTAL

i) Sampling Procedures

The majority of blood samples from patients with ovarian malignancies was obtained from the Ottawa General and Civic Hospitals. Three samples were obtained from the National Defence Medical Centre. Control donors were employees from the National Research Council of Canada (NRC) (Ottawa Branch), several healthy female housewives, hospital workers, employees at the Canadian Red Cross and acquaintances of NRC employees. Average ages of patients and controls were 55 $\pm$ 15 and 48 $\pm$ 17, respectively.

Blood was drawn into vacutainer tubes containing EDTA(10.5 mg K_3EDTA), Lithium Heparin or no anticoagulant, placed immediately on ice and kept for further processing. As quickly as possible (usually within one hour) blood was centrifuged at 4^{o}C.to remove erythrocytes. 0.7 ml. of plasma was pipetted off into a 1 or

2-dram vial and kept at 4°C. Analysis by NMR was performed within one week of collection except where otherwise noted.

40 ml. (3ml for each centrifuge tube) of the same plasma/serum was taken for separation of lipoprotein components by density gradient ultracentrifugation (Beckman L8-70 Ultracentrifuge). Plasma/serum was increased in density to 1.225 g/ml using potassium bromide (KBr) and layered at the bottom of a discontinous density gradient. All gradient solutions consisted of sodium chloride (NaCl) and (KBr) in increasing amounts (Havel et. al. (1955)) to provide density layers (g/ml) of 1.006, 1.019, 1.063, and 1.221. Gradients were layered by first introducing the lowest density layer into a 14mm x 95mm centrifuge tube (Beckman Ultra Clear Plastic for use with Beckman swinging bucket SW-40 rotors). Increasingly higher density layers were introduced to the bottom of the gradient with a pasteur pipette that was drawn out over flame to a narrow opening at the tip as in FIGURE 2-5. Plasma/serum was the final layer.

Centrifugation was for 16 to 24 hours at 105,000 x gravity (g) and 4°C. to give a good separation of the major density classes of lipoproteins. Plasma lipoproteins were removed from a gradient by piercing the centrifuge tube with a 25 gauge-5/8 in. canula at the meniscus of the appropriate layer and drawing the layer into a syringe. The fraction was stored at -20°C. for further processing.

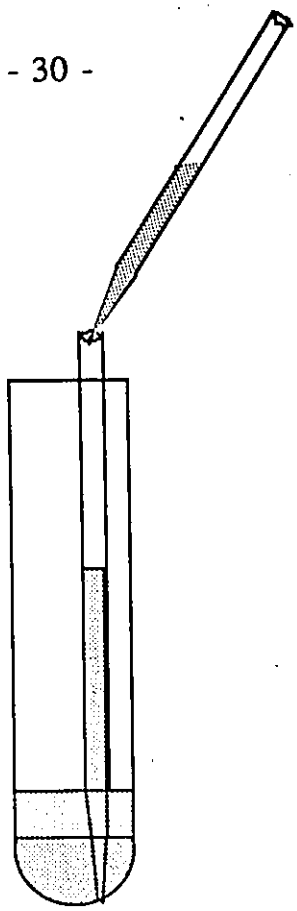


FIGURE 2-5 Apparatus for layering discontinous density gradients. Density layer 1.0063 g/ml was placed in the bottom of the centrifuge tube. Layers introduced by pasteur pipette in order of increasing density were as follows: 1.019 g/ml, 1.063 g/ml and 1.21 g/ml. The plasma which was density increased to 1.225 g/ml was layered last.

ii) ^{1}H-NMR studies

A Bruker AM-360 spectrometer was used to obtain ^{1}H-NMR spectra of all samples. Deuterium oxide (D_2O), 0.1 ml, was added to 0.7ml plasma/serum samples to provide a field-frequency lock which is used to minimize drifts in the magnetic field during spectral accumulation. Samples were put in NMR tubes of o.d. 5 mm and mixed well before being placed in the magnet. The probe with sample was tuned to obtain minimum reflectance of radiofrequency radiation. All samples were run at 27°C..

Biological samples such as blood plasma contain an abundance of water whose protons contribute a very strong signal. To saturate and thus dampen this signal selective irradiation at the resonant frequency of the water ^{1}H resonance was used. This weakened the signal and allowed the measurement of signals from lipoprotein lipid ^{1}H's. Irradiation at the water frequency was followed by a delay of 3 seconds, a 90° pulse of 7.0 microseconds on average and an acquisition time of 0.5 or 1.7 seconds. The number of spectral accumulations depended strongly on the amount of spectrometer time available, 8, 16 or 24 in our case.

iii) Temperature dependence of the Fossel Test

The effect of temperature on the $\nu_{1/2}$ was investigated. Spectra were accumulated initially at 21°C. as in Fossel et. al.(1986). The temperature was adjusted to 27°C. at which samples were

equilibrated for 15 minutes prior to a second accumulation. Maintenance of 21°C. is very difficult in a spectrometer due to the fact that it is nominally room temperature. Heating and cooling of the sample is used to achieve optimum temperature and therefore on the average the temperature would be 21°C. but would fluctuate between approx. 20°C.and 22°C.

iv) Measurement of Linewidths at Half Height

The line widths at half height of the composite methylene and methyl resonances were measured and averaged as in FIGURE 2-3. $\nu_{1/2}=(\nu_{CH3} + \nu_{CH2})/2$. A polynomial baseline correction routine, available on a Bruker AM data station equipped with an Aspect 1000 computer, was performed prior to calculation of $\nu_{1/2}$. This provided a well defined baseline from which the height of the peak could be measured more accurately.

v) CPMG Measurements of Linewidths at Half Height

Large, relatively immobile protons of large proteins such as serum albumin do not have well defined frequencies in a magnetic field and therefore appear as broad humps underlying the more aesthetically pleasing resonances contributed by the lipoprotein methyl and methylene protons. This leads to baseline distortions of the latter resonances in a ^{1}H-NMR spectrum. (FIGURE 2-6)

In order to define better the baseline of these spectra, advantage was taken of the fact that ^{1}H's in a relatively immobile

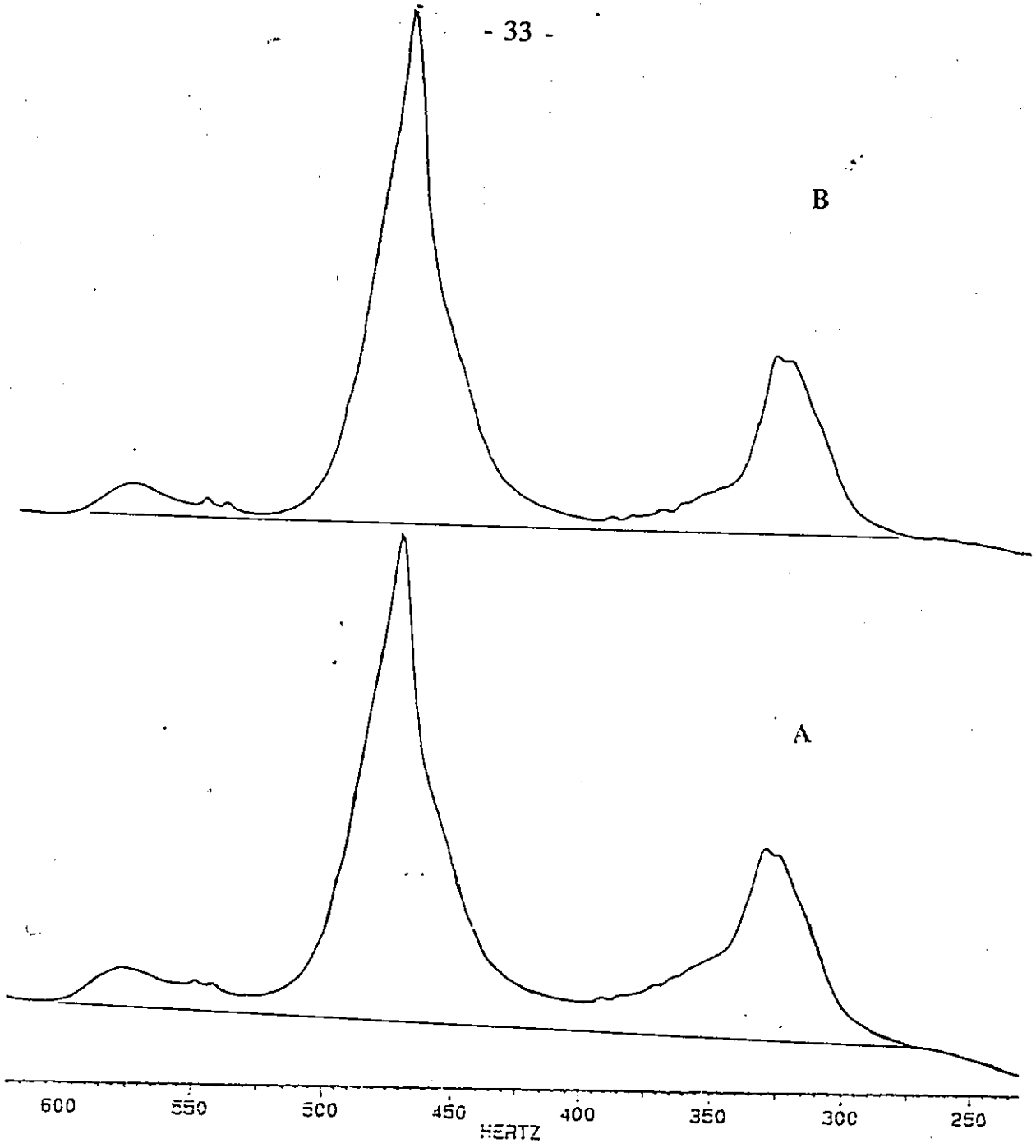


FIGURE 2-6 Comparison of **A**, Methyl-methylene region of a ^{1}H-NMR spectrum taken with a 90°-Acquire (one) pulse sequence **B**, Methyl-methylene region of a ^{1}H-NMR spectrum taken with a 90_x-$(\tau$-180_y-$\tau)_4$-Acquire (CPMG) pulse sequence. More of the broad underlying component is evident in **A** than in **B**.

environment lose their magnetization rapidly as compared to mobile ^1H's. The Carr-Purcell modification of the Meiboom-Gill pulse sequence (CPMG) was used with the idea of removing the contributions to the broad hump in the spectrum.

The pulse sequence that was used is summarised by 90_x-(τ-180_y-τ-ECHO)$_N$ where 'N' is the number of repetitions of the 180 pulse-echo train. 'τ' was a delay of 1 millisecond (ms), the N used was 4 , thus a total of 8ms passed before spectra were acquired. This was observed to decrease the baseline distortion in most cases. All other conditions of spectral acquisition remained the same as in the single pulse experiments.

vi) Statistical tests

Predictive value calculations were performed as in (Galen, R. S. (1975)). Explanations are as in Table 3 for the Fossel Test and in Table 4 for the CPMG modification to the Fossel test.

RESULTS AND DISCUSSION

i) NMR results

Results on the temperature dependence of the Fossel test are summarized in FIGURE 2-7. At the lower temperature the linewidth measurements were on average 2 Hz higher than at the higher temperature. This can be explained by the fact that at higher temperatures molecular motions are relatively faster. Since peak

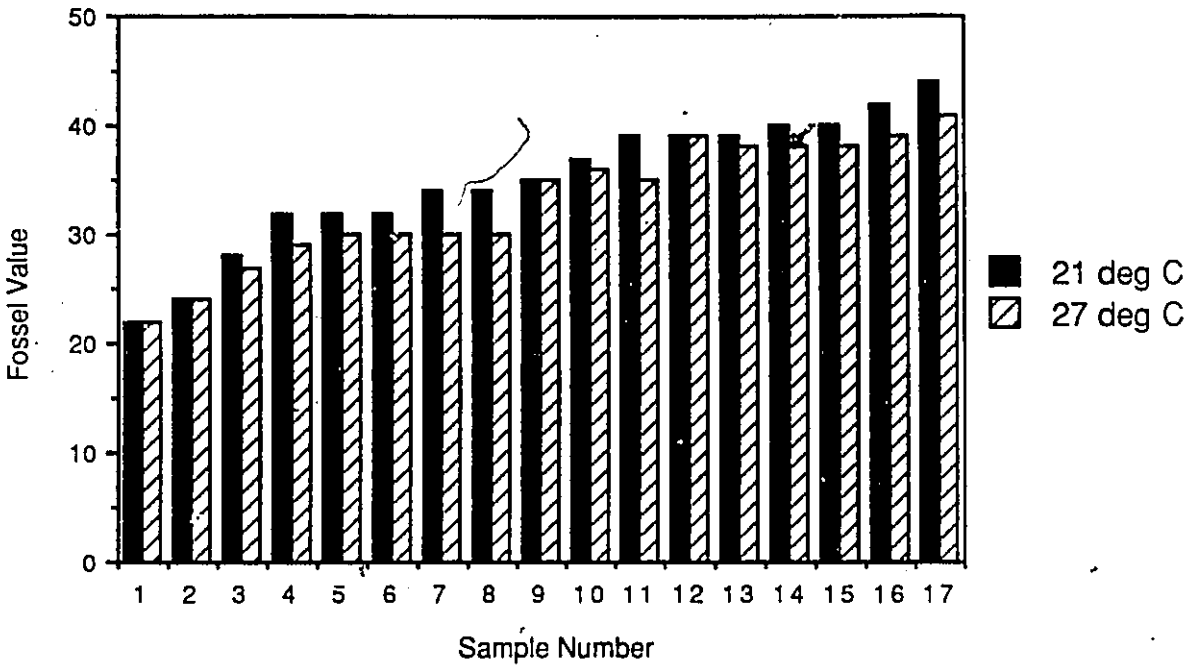


FIGURE 2-7 Line widths (in Hz) of samples measured at 21°C and at 27°C. Measurements were performed as stated in text.

linewidths are related to rates of motion, narrower linewidths corresponding to faster motion at higher temperatures as dictated by theory were consistently found. Although this difference is not large, samples whose line widths fall at the demarcation value (disease vs no disease) could be classified wrongly if one temperature is not consistently observed for all samples measured.

The measured mean $\nu_{1/2}$ for the blood samples studied are summarised in Table #1. Only results from fasted subjects are included. Each circle represents a mean $\nu_{1/2}$ measurement for a single plasma sample; adjacent numbers being the corresponding fossel values. The categories are as follows: **CNT** (Cancer No Treatment) = untreated cancer patients; **CTR**, (Cancer, Treatment and Residual Disease) = patients who were treated by surgery, or chemo- or radiation therapy and in whom cancer remains; **NED** (No Evidence of Disease) = patients after second look surgery in whom no cancer remains but the surgeon suspects disease on the basis of the overall condition of the patients; **CTNR** (Cancer, Treatment and No Residual Disease) = similar to **CTR** except that no detectable cancer remains; **SC** (Sick Control) = Included controls with colds, flus, pneumonia, benign tumours and other non-malignant disorders; **C** (Control individuals); **P** (Pregnancy).

The average values of the linewidth measurements for cancer patients and normal subjects and their percentage contribution to the total number of subjects was used as the demarcation line between

$\nu_{1/2}$(Hz)	CNT	CTR	NED	CTNR	SC	C	P
40		o					
39	o	o			o	o	
38		o				o	
37							
36	o	o		o	o	oooooo	
35	o			o	o	oo	
34		o		oo	oo	ooooooo	
33		oo		o	o	oo	
32	o	ooo		ooo	o	ooo	o
31		ooo		o	o		o
30		o	o	o	oo	o	
29	oo	oooo	o		ooo	o	o
28	o	o			ooo	o	o
27		ooooo			o		
26		o			o		
25				o			
24		oo	o				
23		oo	o				
22	o	oo	o				
21			o				
20		o					
AVG	31.2	29.3	24.8	32.0	31.0	34.0	30.0
N	8	32	6	12	18	25	4
RANGE	22-39	20-40	21-30	25-36	26-39	28-39	28-32

Total N = 105

Avg. of Cancer (In Hz) = 28.4 Avg of No-Cancer (In Hz) = 31.8

Overall Avg (In Hz) = 30

TABLE 2-I Results of the Fossel test for a one-pulse sequence. The line widths at half height (left vertical column) of all cancer patients and normal controls were included for all categories CNT...P as were described in the text. AVG - the average linewidth for each vertical column. N refers to the number of entries in each vertical column. Range - the range of linewidths for each category. Avg of Cancer - the mean of line width measurements for all cancer patient categories (as described in text). Avg of No-Cancer - the mean of line width measurements in all categories for which cancer is not apparent.

$\nu_{1/2}$(Hz)	CNT	CTR	NED	CTNR	SC	C	P
40							
39					o		
38							
37							
36		o				oo	
35	o	o					
34	o	o		o	o	o	
33	o					oo	
32		oo		o	oo	oooooo	
31					o	oo	
30		ooooo		o		o	
29	o	oo		oo	oo		o
28	o	o		o	oo		o
27	o				oo	oo	
26	o	ooooo		o		o	o
25		o			o		
24		o			o		
23		o					
22		oo					
21	o		o				
20							
AVG	29.1	28.3	21.0	29.7	29.6	31.5	27.6
N	8	23	1	7	13	17	3
RANGE	21-35	22-36	21-21	26-34	24-39	26-36	28-32

Total N = 72

Avg. of Cancer (In Hz) = 26.1 Avg of No-Cancer (In Hz) = 29.2

Overall Avg (In Hz) = 28

TABLE 2-II Results of the Fossel test for a CPMG pulse sequence. Categories are as explained in the text. All other information is as explained in the caption to table 2-I.

high and low measurements. The number of values, their ranges and averages of linewidth values were included at the bottom of the corresponding category.

CNT, CTR and NED were classified as persons with malignant disorders. CTNR, SC, C, and P were considered to be subjects without malignancies. 27 cancer patients were found to give true positive results whereas 19 showed false negative values. 46 controls showed true negative as opposed to 13 with false positive results. The majority of false positive results in SC were from patients who had been subsequently treated for Ovarian cysts. These are in some cases considered to be pre-malignant conditions (Sloane, E., (1985)) although personal communication with the operating surgeon in this study has indicated that this has not been confirmed. Pregnancy was given a distinct category because false positive results have been obtained (Fossel et. al., 1986). Two positive results were obtained from the plasma of pregnant women. The results of plasma tested in each trimester for one of these women showed a decrease in the mean linewidth from 32 Hz to 29 Hz corresponding to the 10^{th}, and 34^{th} weeks respectively. Amounts of triglycerides have been known to increase considerably as gestation is reached Desoye et. al. (1987). In view of the results by Wilding and co-workers (and results as will be described in the following chapter), an increase in triglycerides could be responsible for the decrease in the mean $\nu_{1/2}$ in the 34^{th} week.

Of the two false positives in the control category, one was a cigarette smoker. Preliminary results on HPLC studies of plasma samples (performed by visitors interested in these studies) have implied a correlation between high VLDL levels and smoking. These involved only two samples and therefore require more intensive study to be conclusive.

It is interesting that of the four cancer patients with a high mean $v_{1/2}$ in the CNT category three had ovarian malignancies with a surgical classification called borderline. These tumours are unusual in that they very rarely metastasize. A considerable number (14/32) of the CTR values show a negative result. Fossel et. al. (1986), as indicated previously, also found a wide range of mean $v_{1/2}$'s for a population of treated cancer patients. This could be a manifestation of an increased serum LDL or HDL_1 level as was noted by Barclay et. al. (1975). Quantitative measurements on these samples would be necessary to verify this. A distinct possibility is that the measurements on plasma have been altered by the various treatments administered. Time of sampling after treatment was not a controlled variable. Treatment in all patients ranged from surgery to a combination of surgery and chemo and/or radiation therapy. Samples were not available from these patients before treatment.

The classification NED, although n=6, is notable in that all but one give positive results. All of these patients had undergone anywhere from 2 to 12 cycles of chemotherapy. Four of these

patients have been improving in successive examinations and have not shown signs of relapse.(Information thanks to Dr. Michel Prefontaine, Ottawa General Hospital). Follow-up tests on these patients over at least a six month period would provide interesting and possibly informative results in the determination of the validity of the Fossel test. Conclusive evidence cannot be based on four results. Thus follow-up tests and subsequent diagnoses should be made on patients in the other categories defined as positive for cancer and negative for residual disease.

ii) Statistical Analyses

Predictive value analysis indicated that from the results of the Fossel test for the population studied the test would correctly predict a disease condition 68% of the time. 70% of the time the test would correctly predict no disease. Sensitivity refers to the ability of the test to respond to the condition being tested. 60% sensitivity was determined for this population. A specificity of 78% was obtained indicating that false positive results would interfere with the test 22% of the time. The values are reduced to close to 0% when corrected for prevalence in the population (i.e. less than 5%) (FIGURE 2-8).

The CPMG modification of the test was performed on only 72 subjects due to the fact that the idea of this experiment was conceived after the investigation of the Fossel test had begun. Predictive value analysis showed somewhat similar results giving

Predictive Value Analysis of Fossel Test

	# Fossel Positive	# Fossel Negative	Totals
# Cancer Positive	28	19	47
# Cancer Negative	13	45	58
Totals	41	64	105

$$\text{Sensitivity} = \frac{TP}{TP + FN} = 0.60$$

$$\text{Specificity} = \frac{TN}{TN + FP} = 0.78$$

$$\text{Probability of a Positive Predictive Value} = \frac{TP}{TP + FP} = 0.68$$

$$\text{Probability of a Negative Predictive Value} = \frac{TN}{TN + FN} = 0.70$$

TABLE 2-III Statistical analysis of Fossel test results. # Fossel Positive = #Fossel values < 30 Hz., # Fossel Negative >= 30 Hz. Sensitivity refers to the responsiveness of the test to the condition being tested. Specificity indicates the percentage of negative results obtained in healthy subjects. Probability of a Positive/ Negative predictive value refers to the validity of the test as a predictive value indicator.

Predictive Value Analysis of Fossel Test with CPMG Modification

	# Fossel Positive	# Fossel Negative	Totals
# Cancer Positive	14	9	23
# Cancer Negative	18	31	49
Totals	32	40	72

$$\text{Sensitivity} = \frac{TP}{TP + FN} = 0.44$$

$$\text{Specificity} = \frac{TN}{TN + FP} = 0.78$$

$$\text{Probability of a Positive Predictive Value} = \frac{TP}{TP + FP} = 0.61$$

$$\text{Probability of a Negative Predictive Value} = \frac{TN}{TN + FN} = 0.63$$

TABLE 2-IV Statistical analysis of CPMG test results. # Fossel Positive = #Fossel values < 28 Hz., # Fossel Negative >= 28 Hz. Sensitivity, Specificity and Positive/Negative predictive value are as stated in the caption to **TABLE** 2-III and in the text.

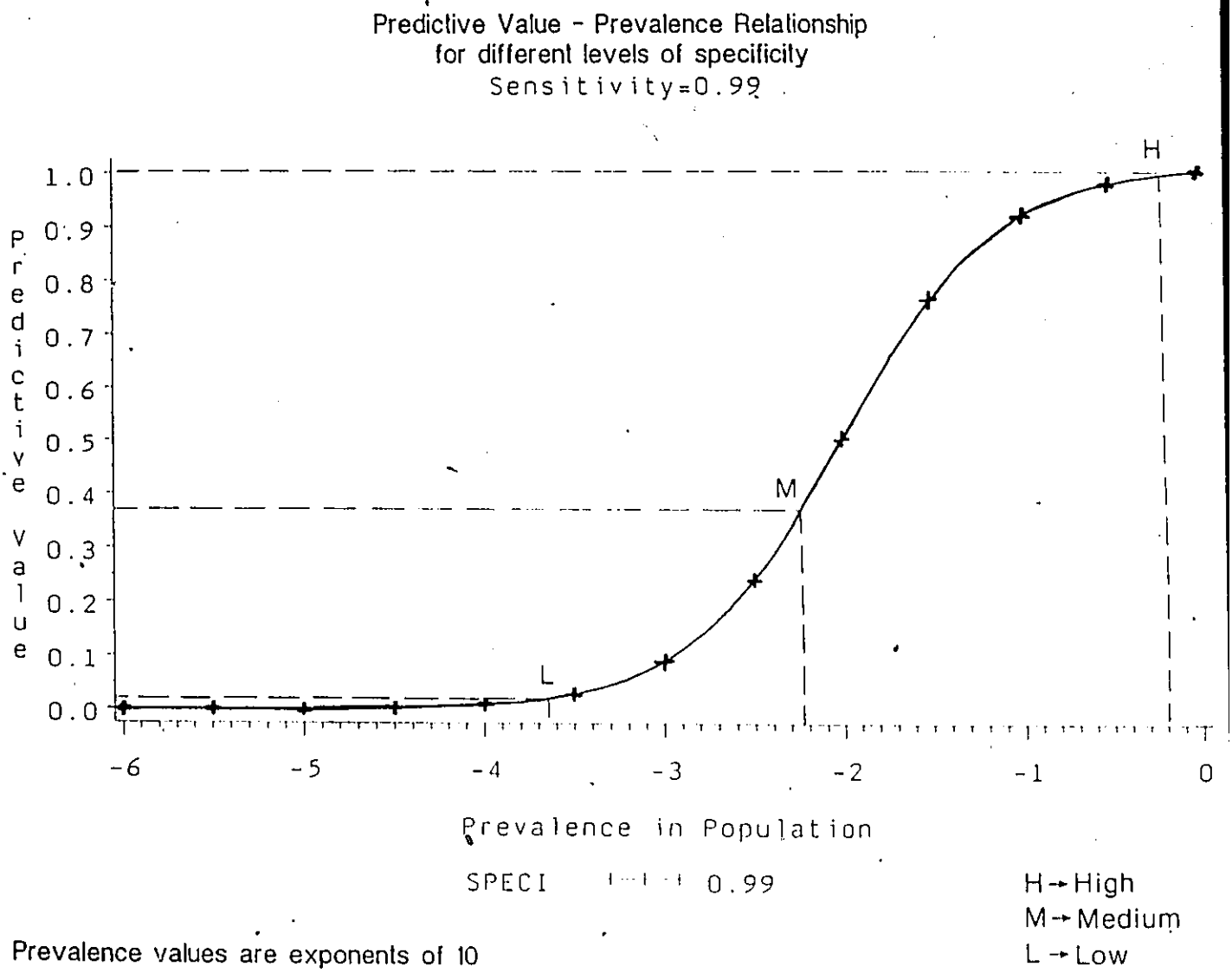


FIGURE 2-8 Graph indicating predictive value of a test for disease given 99% test sensitivity and specificity. The predictive value is dependent upon the prevalence of the disease in the population.

values of 44%, 78%, 61% and 63% for sensitivity, specificity, positive and negative predictive values respectively. The one very different result from the conventional Fossel analysis was 44% for sensitivity.

iii) Summary of findings for multiple sampling

One very cooperative patient in this work has donated blood for study nine times. The patient has an inoperable adrenocortical carcinoma and had had blood taken over the course of approx. 1.5 years, and both before and after chemotherapy/radiation therapy. (FIGURE 2-9) Values for the linewidths were 25, 23, 26, 25, 25, 27, 29, 30 and 30. The first six values were obtained prior to any form of treatment. The values obtained were consistently low and all positive for the Fossel test.

Two samples were obtained from an ovarian cancer patient who has malignant disease in stage IV. Samples were obtained one year after surgery at which time residual disease was found and again in 11 days when metastases to the liver were discovered. Linewidths were 37 and 33 corresponding to earlier and later dates at which samples were received. These are both negative values relative to the determined cut off value but may reflect the findings of Barclay et al. (1975) in which high levels of HDL1 were found in ovarian cancer patients in advanced stages of disease relative to patients whose cancers were not so advanced.

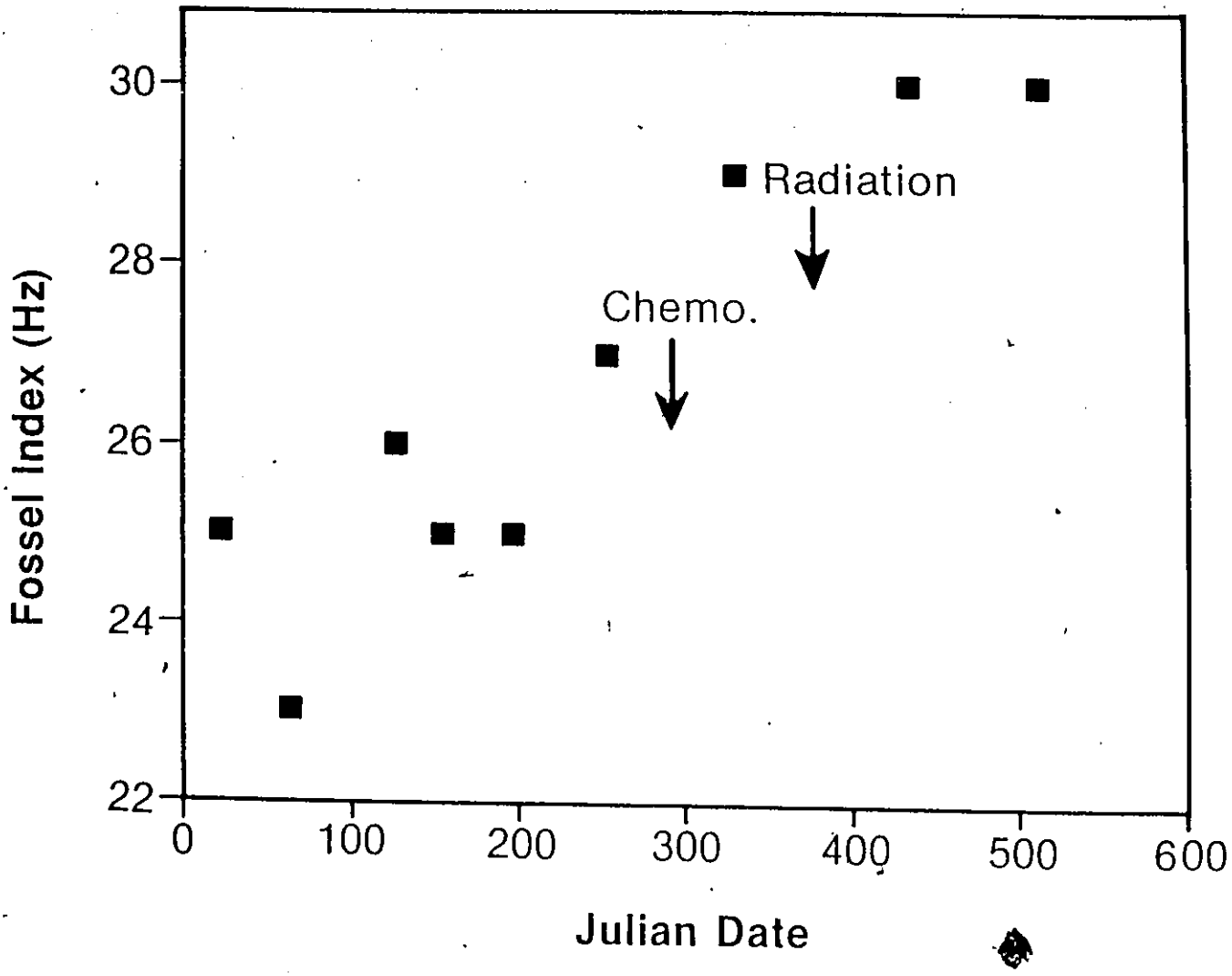


FIGURE 2-9 Fossel Values for a woman with an inoperable adrenocortical carcinoma. Blood samples were measured both before and after chemo and/or radiation therapy. Dates of sampling were as indicated on the figure

In another ovarian patient who donated two blood samples the linewidths were 29 Hz and 25 Hz, respectively 14 days and one month post operatively. This woman was undergoing chemotherapy at the time of sampling. The prognosis just after the last sampling was carcinoma of the ovary with bowel obstruction. The values obtained are positive for the Fossel test but may possibly have been influenced by the treatment. Further samples post-therapy are necessary in this case to state conclusively whether the low values are due to disease, chemotherapy, surgery or a combination of the three.

A patient who tested positive with a mean $\nu_{1/2}$ = 21 Hz was entered in the sick control category due to the fact that the diagnosis was mucocele of the appendix (i.e. an appendix filled with clear mucous). In addition the patient is an insulin-dependent diabetic. This latter condition in which triglycerides are mobilized for energy usage may have influenced the result.

A patient who had undergone a sub-total abdominal hysterectomy for a stage III ovarian carcinoma 7 months prior to sampling and 6 cycles of therapy on cyclo/carboplatinum up to sampling time, showed a linewidth of 33 at time of sampling. At that time the patient by a surgical 2nd look operation was seen to have 5mm nodules of residual disease in the abdominal cavity. Eight months later the patient was free of disease. In this instance it is

possible that the Fossel test indicated improvement in the patient prior to disappearance of the disease.

Finally, a linewidth of 35 Hz was obtained for a patient with ovarian cancer who had undergone a total abdominal hysterectomy, had been diagnosed previously as having 2-10 cm residual disease eight months prior to sampling, and was diagnosed at the time of sampling to have no residual disease. Cyclo/cisplatin was the administered treatment post-surgery but the date of last treatment was not known. Again the treatment may have influenced the value obtained depending upon date of last dosage.

CONCLUSIONS

Although the present study does not strongly support the Fossel test as an unequivocal indicator of cancer in an individual, a more extended study is necessary to support or reject the test conclusively. It is imperative to have stricter requirements on the time the blood samples are taken. Necessarily, patients must be fasted, samples must be taken prior to surgery and prior to administration of anaesthesia. Patients should not be undergoing hormone replacement therapy (Barclay et. al. (1964) and (1975)), due to the influence of hormones on lipoprotein lipids. Testing should be done prior to the introduction of chemotherapy or radiation therapy due to the possible influence of these on the lipoprotein composition of the blood of the patients studied

Several disorders which are known to increase plasma VLDL significantly such as insulin-dependent diabetes, hypertriglyceridemias and smoking should be investigated thoroughly as probable false positives.

Several of the results obtained have supported the possibility of using the Fossel test as a cancer management tool. This can be studied by long term sampling of many individuals commencing prior to surgery and continuing for at least several months during and post-treatment. Biochemical quantitation of plasma lipoproteins should be performed simultaneously to observe whether changes in the levels of these occur with changes in the linewidth. The changes in plasma lipoproteins have not been rigorously followed through and post treatment of patients. The results from all of these proposed studies would be interesting regardless of the interpretation and may invoke ideas for new areas of study within the vast field of cancer research.

Chapter 3

A DOUBLE BLIND STUDY OF THE FOSSEL TEST

Several research groups have attempted to reproduce and/or explain the results observed by Fossel et. al. in 1986. Experimental conditions were varied and the effects of the different conditions on the linewidths obtained were examined. Variations included the use of Plasma vs. Serum, sample storage at 4° C and -20°C, length of time prior to separation of red blood cells from plasma/serum (both at room temperature and at 4°C), the use of samples from subjects with hypertriglyceridemia (those whose triglyceride levels exceeded 220 mg/dl plasma, (Wilding et. al.(1988)), and the temperature at which NMR experiments were run. The majority of these researchers had few samples and therefore could not obtain statistically significant results. Some had enough samples but not enough from any one category (such as untreated cancer patients) to support or reject the test (Wilding et. al. (1988), Holmes et. al. (1988), Chmurny et. al. (1988), Peeling et. al. (1988)).

In view of these uncertainties a double blinded study was undertaken as an attempt to determine the usefulness of this test. Dr. Smith's group at the National Research Council of Canada and Dr Fossel's group at Beth Israel Hospital in Boston, Massachusetts, were chosen to study blood samples from 950 individuals using the method of Fossel et. al. (1986). Samples were also measured for

cholesterol, triglyceride, HDL and LDL levels at an independent laboratory. Blood samples were taken from individual donors and cancer patients at the Latter Day Saints Hospital in Salt Lake City, Utah. At the time of the study the researchers conducting the experiments in the NRCC Laboratory, including the author, had no knowledge of the histories of the blood samples except for the fact that they originated in Salt Lake City.

After completion of all sampling followed by decoding and collation of data (subject histories as well as measured data), records on each of the subjects were made available to researchers participating in the study. Results from 25 control individuals were rejected on the basis of histories such as serious illnesses, pregnancy or a previous history of cancer. The summary indicated that the post-prandial mean time after which blood was taken was 3 hours. It was also found that the mean $v_{1/2}$ varied inversely with the plasma triglyceride concentration. A peak in plasma triglyceride concentrations has been found to occur in individuals approx. 5-6 hours after ingestion of 1.5g. oral fat/Kg body weight (Tall A.R. (1986)). This is an average value and varies according to the individual.

The response to a meal high in triglycerides (TG), as observed by the levels of these lipids in the plasma, may vary considerably depending upon the level of triglycerides already in existence in the circulation. This was found to be true for both normo- and

hyperlipidemic individuals (Fasting Levels of TG<200mg/dl and TG>=200mg/dl respectively) (Nestel, P.J. (1964), Grundy S. M. (1976)). These researchers have suggested that in studies involved in such measurements, a necessary requirement is that subjects fast for at least 14 hours prior to commencement of study.

Patsch et. al (1983) found an inverse relationship between the fasting TG levels after a fatty meal and the fasting HDL-2 and HDL cholesterol levels. Interestingly, Barclay et. al. (1975) observed the same trends in individuals with malignant disease. Three independent research groups found that HDL cholesteryl esters increased (Tall et. al., 1982), decreased (Kay, R.M. et al. (1980) or remained the same (Havel et al. (1973)) in response to a fatty meal. It is feasible that such trends in the Triglyceride and HDL-Cholesterol levels could interfere tremendously with measurements taken of samples in this study.

EXPERIMENTAL

All plasma samples were packed on ice for shipment and were sent by courier from Salt Lake City in batches of 100 or 150. No other information was given. Data sheets were provided for entry of Eossel values as measured by NMR. These were to be returned to the location of origin for collation of results from the three

laboratories (i.e. Abbott Laboratories, the Fossel Laboratory and the Smith Laboratory).

A 0.6 ml aliquot of plasma was pipetted into an NMR tube of o.d. 5mm . As in chapter 2 spectra of all samples were obtained at 360 MHz. In order to eliminate the possibility of differences in the Fossel measurement due to differences in the NMR technique, the spectrometers in the two laboratories were set up identically prior to sampling. An initial 20 plasma samples were provided for this purpose in addition to the 950 samples provided.

i) Linewidth Measurement Specifications

The ^{1}H-NMR probe was detuned meaning that the frequency of observation was purposely set to approximately 3 MHz from the resonant frequency of ^{1}H's at 360 MHz. This avoids a problem phenomenon known as "Radiation Damping" in which the H_2O signal is very strong, saturates the receiver and therefore artificially broadens the intrinsic linewidth of H_2O in an NMR spectrum. By detuning, the H_2O signal is weakened and allows one to vary the local magnetic fields in the vicinity of the sample within the large magnetic field, a process called "Shimming". One shims to optimise the homogeneity of the magnetic field around the sample and this was monitored by the linewidth of the H_2O peak. A linewidth <= 4.5 Hz for this peak was the requirement for each sample prior to measurement.

```
     PRESAT.AUR
D1=   4.0000000   S3 =   12L          RD=    0.0
PW=       23.00   DE=      158.75     NS =       8
DS =          0
```

Figure 3-1 Spectral Acquisition parameters. **PW** = 90° pulse length. **D1** = Relaxation delay time during which solvent suppression was performed. **S3** = Decoupler power. **RD** = Relaxation delay (unused). **DE** = Delay (automatically set) between the end of the 90° pulse and the beginning of spectral acquisition.

Plasma was measured between 20-22°C. Aquisition parameters were set as in FIGURE 3-1. Specifically, pulse width (pw) refers to the time necessary to tip the magnetization 90 degrees into a plane where it can be observed, recorded and subsequently analysed as a spectrum, D1 is a delay time on the order of seconds required for adequate saturation (cancellation) of the H_2O signal, spectral width (SW) is the width of frequencies (Hz) recorded, S1 is the amount of power used to saturate the H_2O signal, Number of scans (NS) refers to the number of times the spectrum was sampled for time-averaging and Acquisition time (ACQ) is the time taken to record a spectrum. All of these parameters were identical for the two laboratories. Total time for each sample measurement averaged 3 min.. Samples were allowed to equilibrate at room temperature for at least 1/2 hour prior to measurement

ii) Measurement of Mean Line Widths

Spectra were processed similarly by the two laboratories. A baseline correction was performed using phase correction software provided on the Bruker AM data station and works in the following manner. If the transmitter and the receiver are not exactly 90 degrees out of phase during an experiment the difference is manifested in the resulting spectrum and it is the entire spectrum that is affected (FIGURE 3-2A). The phase of the spectrum can be easily corrected using a zero order phase correction which affects the spectrum equally (FIGURE 3-2B). Since the baseline of a plasma

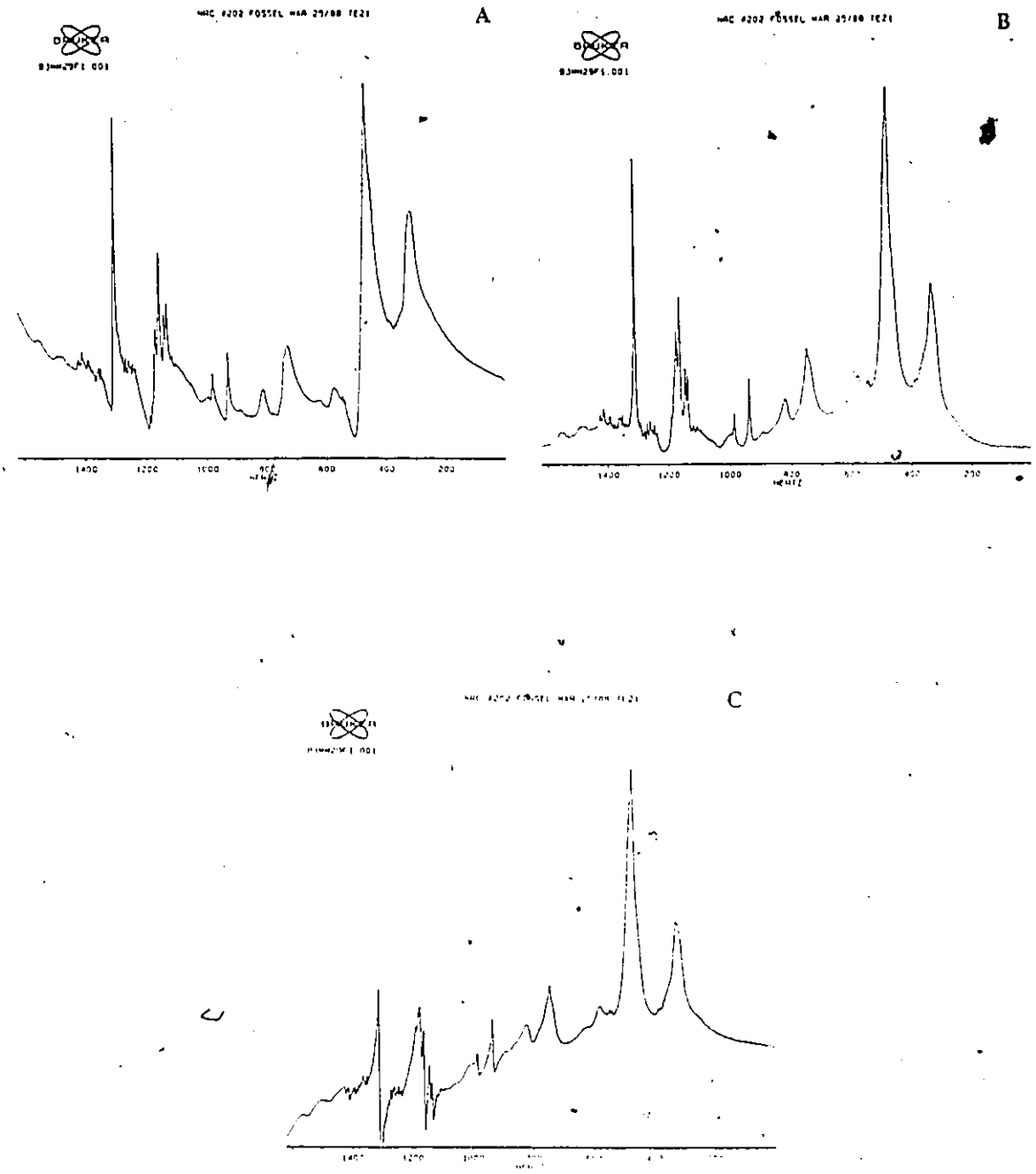


Figure 3-2 1H-NMR spectrum of human plasma A, Fourier transformation, no phase correction B, Fourier transformation with zero order phase correction C, Fourier transformation with both zero order and first order phase correction.

spectrum is often distorted by broad underlying components, it is possible to use a first order correction method to provide a flat baseline in the region of interest (FIGURE 3-2C). A first order correction is applied linearly with frequency. It was used for correction in the methyl and methylene region of the above spectra. This procedure would not normally be used since it leads to lineshape aberrations in other spectral regions. It was used here to duplicate Fossel's methods.

iii) Mean Line Width as a Function of Storage

In order to determine the stability of the Fossel test over a period of time, 25 of the samples which had been measured previously were remeasured by NMR after 1.5-2.5 months of storage at 4°C. The experimental procedure was carried out identically for both measurements.

iv) Linear Regression Analysis

A simple linear regression analysis was used to compare the mean line width results with each of triglyceride, total cholesterol, LDL and HDL cholesterol levels. The R^2 value, used to evaluate correlations between variables, was examined for each comparison. R^2 values close to 1.0 are considered to show a correlation:

R^2 = Sum of squares residuals due to regression/
Sum of Squares about the mean

v) Comparison of Means

A two-tailed t-test was used to compare the mean values for similarity (Freund, J. (1962)). The test used was

$$Z = \frac{X_1 - X_2 - \delta_0}{\sqrt{(s_1^2/N_1 + s_2^2/N_2)}} \quad \text{where } \delta_0 = 0$$

X_1 = mean of sample 1

X_2 = mean of sample 2

s_1^2 = variance of sample 1

s_2^2 = variance of sample 2

N_1 = number in 1st sample

N_2 = number in 2nd sample

RESULTS AND DISCUSSION

i) Fossel Values

The Fossel measurements for the following cases were not used in this analysis. Thirty-one cases of a group consisting of families with known cases of familial polyposis were excluded in order that the distinction between cancer and control groups is clear. Cancer patients were classified into three groups: 1) Untreated cancer 2) Treatment given and residual disease 3) Treatment given and no residual disease. This third group was also excluded from the

analysis of results. Information on 2 individuals was not obtained and, as mentioned previously, 25 individuals were excluded on the basis of their health.

The mean line widths of the control samples (N = 826) and cancer patients (N=41) were 36 and 32, respectively. The Standard deviation on each of these measurements was found to be 6. In the two groups of cancer patients, the means (standard deviations) of the Fossel values corresponded to 33.4(6.1) and 29.5(4.1) respectively for N = 19 and 22 samples. From simple inspection of these results one may find that prior to treatment, the physical manifestation of the diseased state is not pronounced enough to be detected by the Fossel test. Statistically the means for both cancer vs. control and cancer treated vs. cancer untreated are significantly different. However, the overlapping distributions of Fossel values indicate that the comparison of means is not sufficient to determine the value of the test in screening the population.

Predictive value analysis was performed on the mean fossel values of true positive, false positive, true negative and false negative categories (FIGURE 3-3). When uncorrected for prevalence in the population, the analysis gave a sensitivity = 48.78%, specificity = 71.79%, predictive value = 7.87% and 92.09% false positives. When corrected for prevalence in the population the predictive value was

Predictive Value Analysis of NCI Fossel Data

	# Fossel Positive	# Fossel Negative	Totals
# Cancer Positive	20	21	41
# Cancer Negative	233	593	826
Totals	253	614	867

$$\text{Sensitivity} = \frac{TP}{TP + FN} \times 100\% = 48.78\%$$

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100\% = 71.79\%$$

$$\text{Positive Predictive Value} = \frac{TP}{TP + FP} \times 100\% = 7.87\%$$

$$\text{False Positives} = \frac{TN}{TN + FN} \times 100\% = 92.09\%$$

Figure 3-3 Predictive value analysis for NCI Fossel Data. Fossel Positive = Line width >= 32, Fossel Negative = Line width < 32. Other parameters were as described in Chapter 2.

reduced to 0%. These results suggest that a Fossel test result would be highly unreliable in predicting malignant disease. It would be the decision of the medical community, however to determine whether the use of such a test would be of value in any other facet of medical diagnosis.

ii) Fossel Value as a Function of Storage

The results of this experiment are shown in FIGURE 3-4. The Fossel values for the first measurement and for the second measurement (1.5-2.5 months post-storage) indicate that line width increases with prolonged storage of samples. FIGURES 3-5 and 3-6 show representative spectra corresponding to the 1st and 2nd sample measurements. It can be seen in the methylene & methyl regions (FIGURE 3-6) that the lines have become broadened more than in the initial measurement. The sample has probably undergone degradation over time which is manifested in the spectrum as an increase in line widths due to a greater number of component species each contributing its own line width and chemical shift. It is imperative, then, that samples are run as soon as possible.

iii) Triglyceride Levels vs Fossel

The mean line width is inversely related to the level of triglycerides (FIGURE 3-7) as can be seen visually and by linear regression analysis. $R^2 = 0.6$ was obtained when triglyceride levels <= 320 mg/dl were considered. $R^2 = 0.5$ and thus little change was

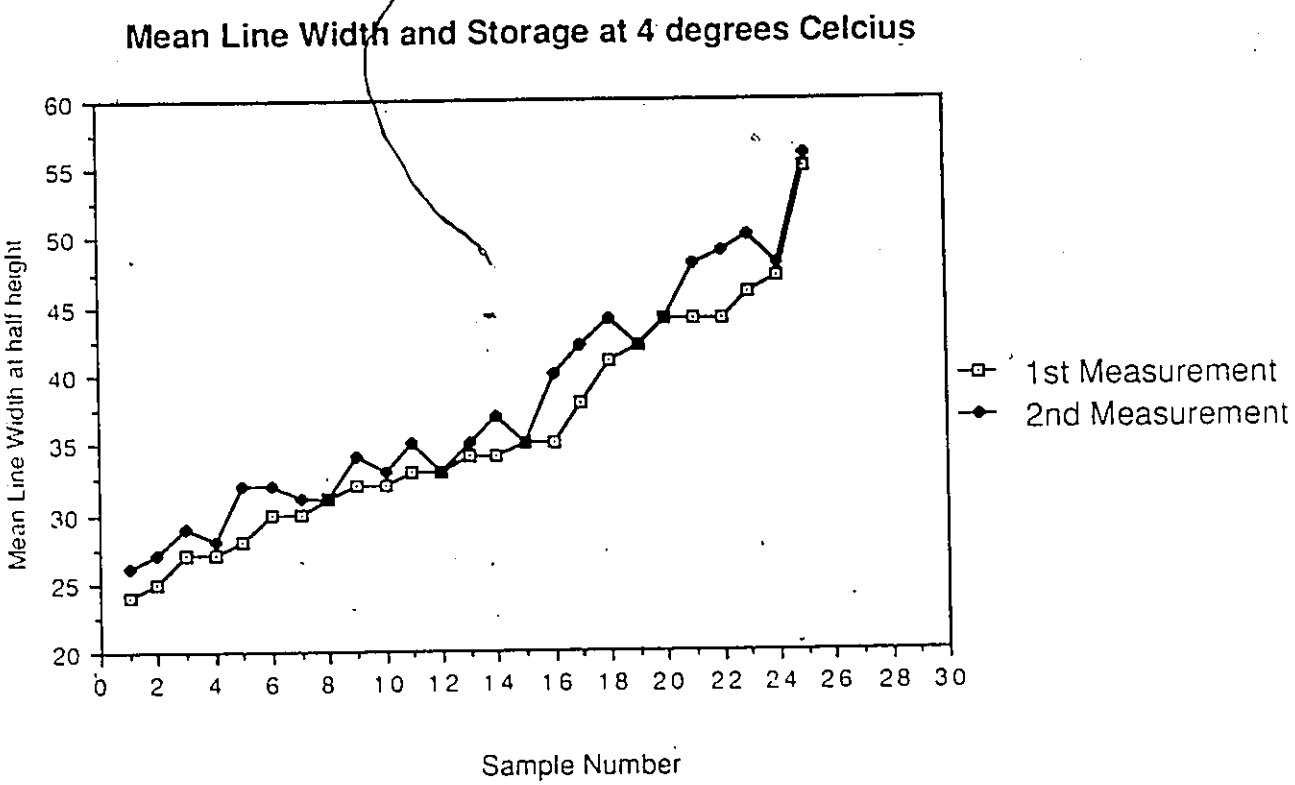


Figure 3-4 Sample measurements taken initially (⊡) and after storage at 4°C for 1.5-2.5 months (◆). For each sample number the mean line widths correspond to the initial and storage measurements.

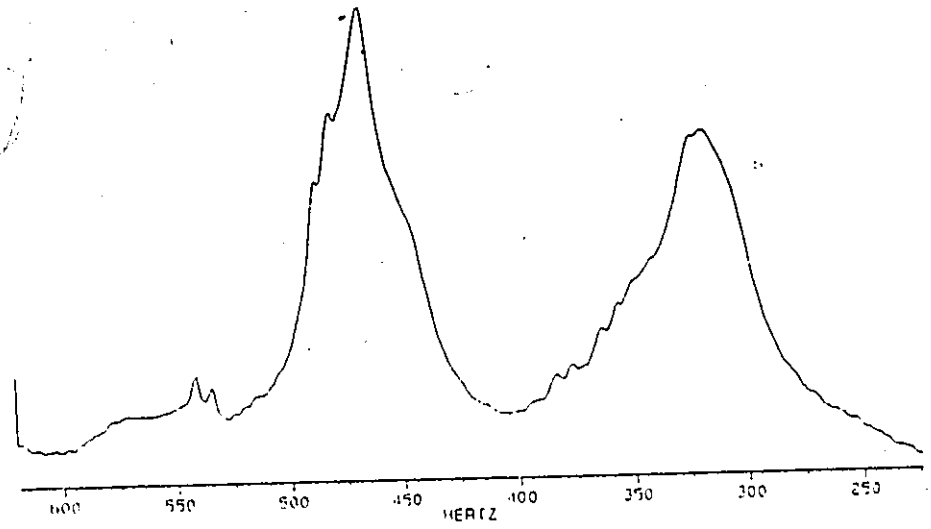


FIGURE 3-5 Sample ^{1}H-NMR spectrum of human plasma measured initially as opposed to after storage. Methods were as described in the text (experimental section).

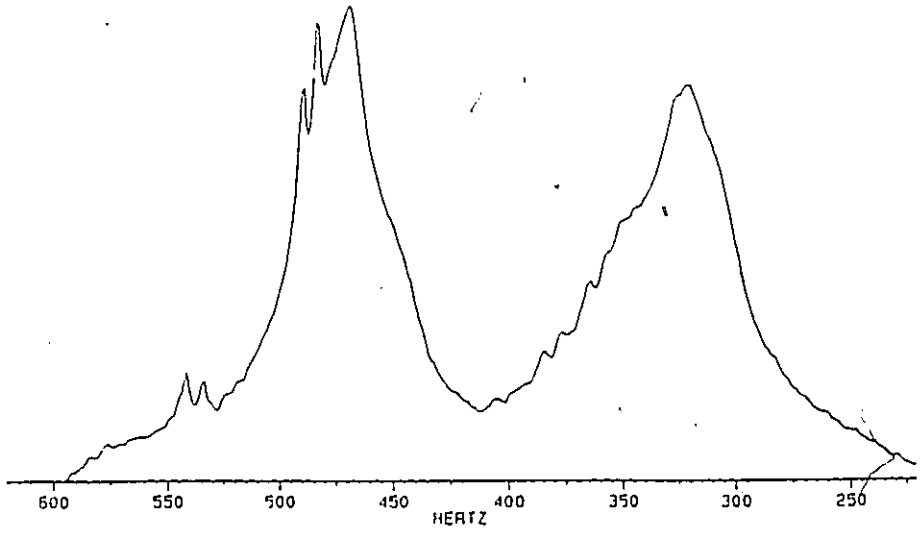


Figure 3-6 Sample ^{1}H-NMR spectum of human plasma measured after storage of sample for 1.5 months at 4°C. Methods were as described in text (experimental section).

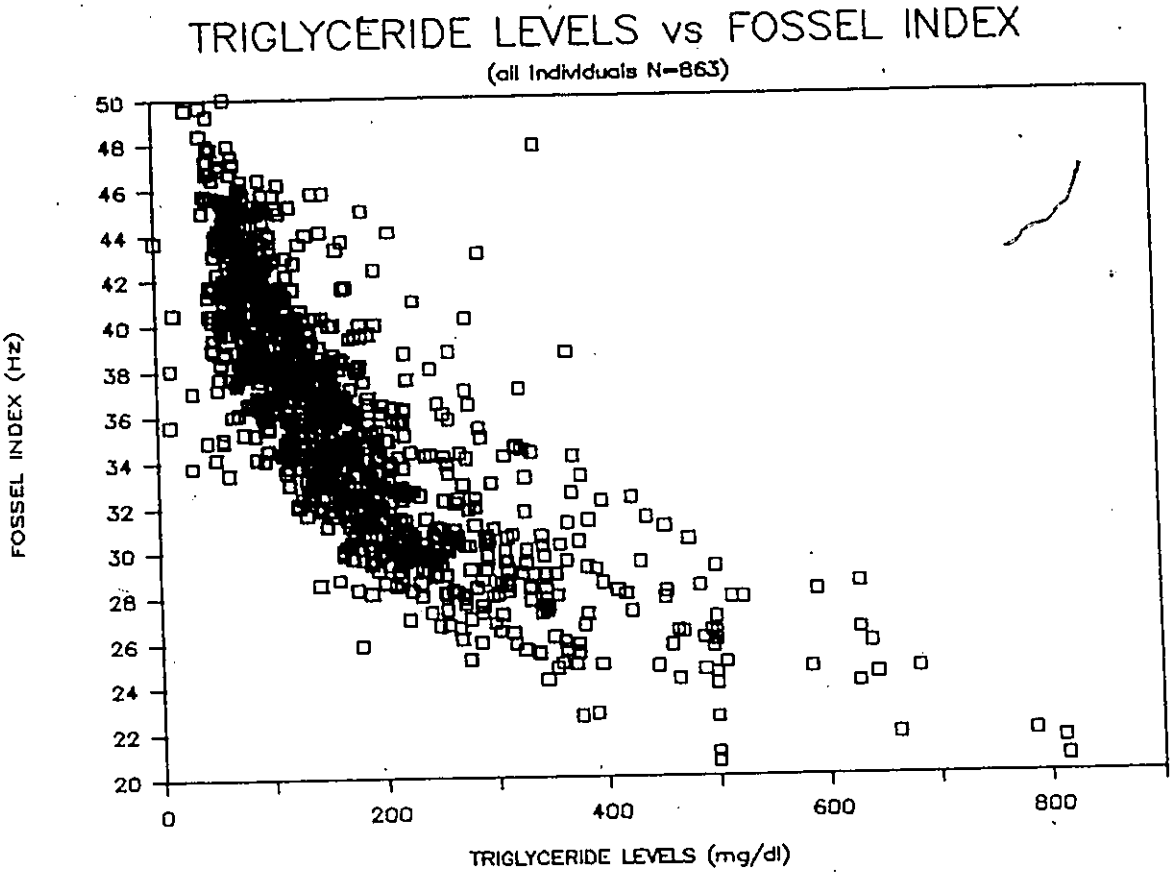


Figure 3-7 Triglyceride levels vs Fossel index (mean line width). Parallel measurements were performed on duplicate aliquots of the same human blood plasma. Conditions of fossel index meaurement were as described in text. Fossel indices used were an average of the line width measurements from the Fossel laboratory and the Smith laboratory

observed when all samples were included. Above levels of 320 mg/dl, as indicated by R^2 = 0.2, there is little dependence of the fossel value on triglyceride levels.

The demarcation Fossel value between cancer patients and control individuals was found to occur at 32 Hz. To verify further the influence of triglycerides on the Fossel value obtained, an average triglyceride level of 129.6($\pm$63.2) mg/dl was calculated for Fossel >32 and 306.7($\pm$125.1) mg/dl for Fossel <= 32. The difference in the means was not statistically significant due to the great variability in the sample measurements. However, one may account for the variability using the knowledge that the individuals studied had not fasted prior to blood-letting. Given this factor, it is highly possible that the difference in the triglyceride levels are real. As in other reports, mentioned in chapter 2, hypertriglyceridemics would be expected to give a false positive result.

iv) Anomalous cases

Some anomalies arise in the inverse relationship between triglycerides and the fossel value. 41 individuals with a plasma triglyceride level >= 220 mg/dl have given a fossel value > 32 Hz. The average triglyceride level for this group was 281 mg/dl. In many of these cases the HDL component of the composite methylene resonance has been large enough to contribute to the line width. Another probable component, since the majority of the subjects had not fasted, is the contribution from chylomicron triglycerides. The

chemical shift of the latter is downfield from that of VLDL triglycerides (Sadler et. al (1987)) in both the methyl and methylene peaks. The addition of this component would cause a broadening of these peaks and thus a wider linewidth. A representative spectrum which demonstrates this broadening is shown in FIGURE 3-8.

At the other extreme, 72 individuals show a Fossel value <=32 with a plasma triglyceride level <= 220 mg/dl. In most of these cases the methylene peak in the spectrum demonstrated a low HDL level (FIGURE 3-9) which did not contribute to the line width. The average triglyceride level for this group at 184.82mg/dl remained high in comparison with the general population (U.S. DEPT. OF HEALTH AND HUMAN SERVICES, July (1980)). Except for a single Fossel value the range of these values was between 28 and 32 Hz. The one individual who gave a Fossel = 28 Hz gave corresponding triglyceride level of 177 mg/dl.

An examination of the mean triglyceride levels determined for the study population as compared to the general North American population are given in FIGURE 10(A&B). Levels were calculated on the basis of sex and age-in-decades. Contrary to levels found in the North American study, 151 mg/dl for males and 105 mg/dl for females, average levels of 180.78 mg/dl were determined for the NCI study population. This number is highly variable given a standard deviation of 117.32 and may be a result of the variability in the times postprandially after which blood was taken from the individuals. Mean HDL cholesterol levels of 37.64 mg/dl, when

examined in a similar way, were lower for the study population (FIGURE 11(A&B)). Again, the variability is great as viewed in a standard deviation of 13.57.

v) Cholesterol Levels vs Fossel

No correlation was found between total cholesterol or LDL cholesterol levels and the line width measurement. Data were analysed in the same manner as the triglycerides and the following information was extracted. The average total cholesterol was 207.0($\pm$42.6) mg/dl for linewidth <= 32 Hz and 187.9 ($\pm$91.6) for line width >32 Hz. LDL cholesterol average values were 96.2($\pm$58.3) and 115.1($\pm$37.7) for line widths <= 32 Hz and >32 Hz respectively. Considerable overlap can be seen within each of these value pairs. It appears reasonable to assume for this data that neither total cholesterol nor LDL cholesterol greatly influences the Fossel number.

Alexopoulos et al. (1987) found higher cholesterol levels in healthy controls as opposed to cancer patients at ages > 70 and 30-39 but lower levels in individuals less than 30 years of age. For all other age groups the cholesterol levels were similar between cancer patients and controls. In the study population the number of cancer patients of any one age was few. Therefore a comparison between cancer patients and controls on the bases of age and cholesterol levels would not present representative differences, if any, between cancer patients and control individuals. The average age of the cancer patients in the study population was 61 for both males and

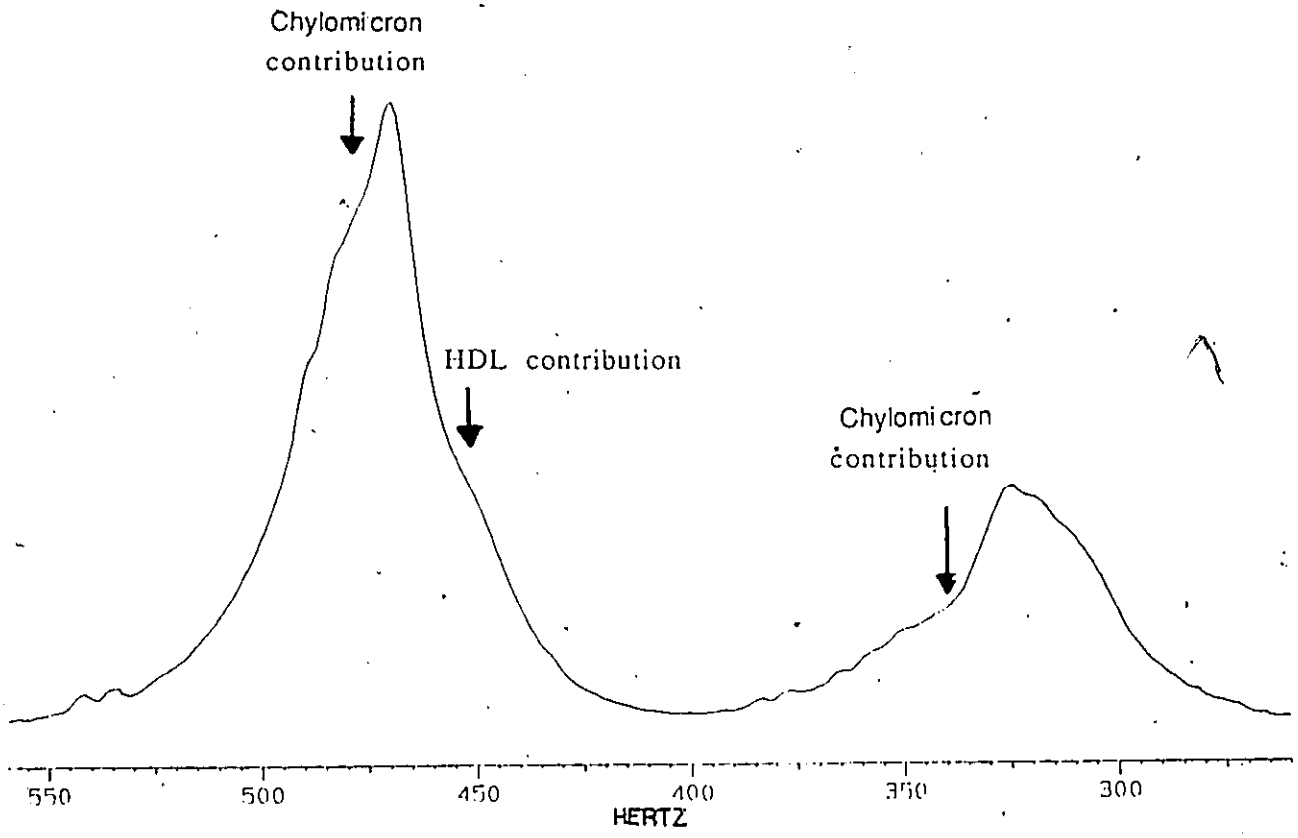


Figure 3-8 Proton NMR spectrum of human plasma samples. Broadening to the methylene peak has been effected by chylomicron and HDL contribution and to the methyl peak by chylomicron contributions (as indicated in the figure).

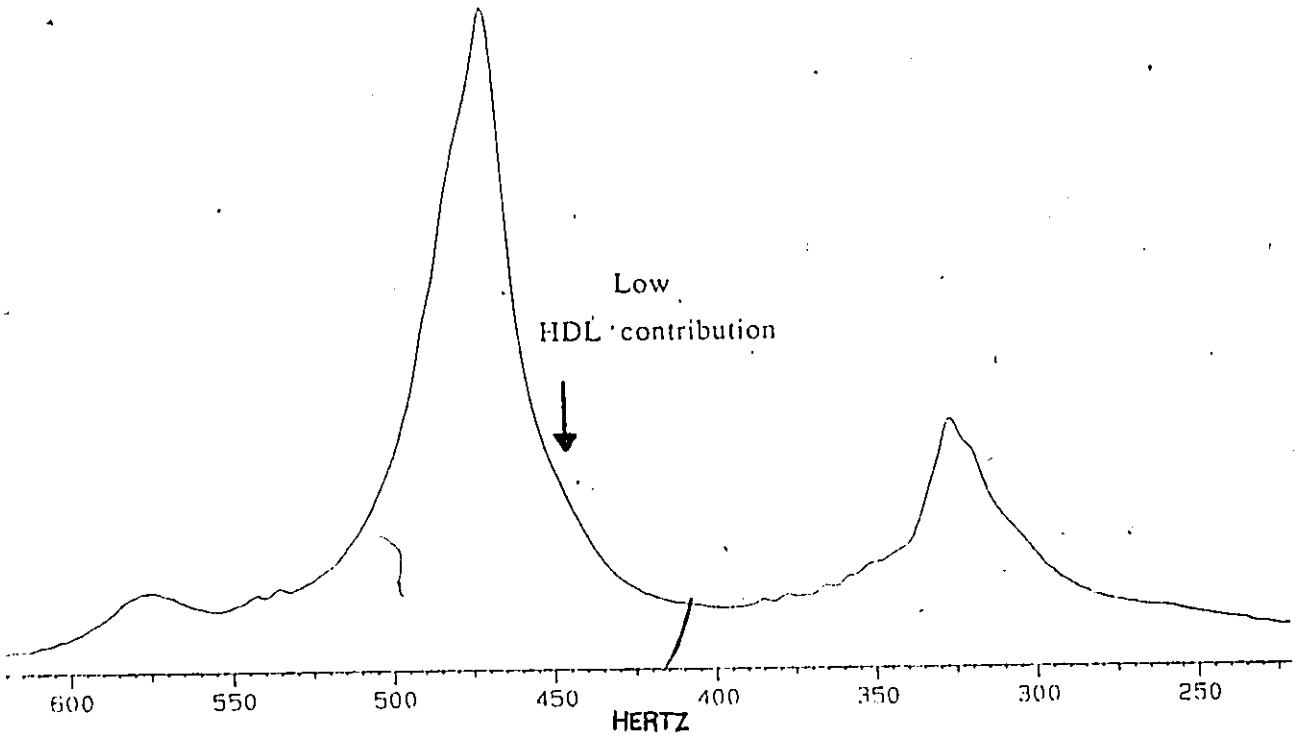


Figure 3-9 Sample proton NMR spectrum in which the HDL contribution does not increase peak width at half height as indicated by the arrows.

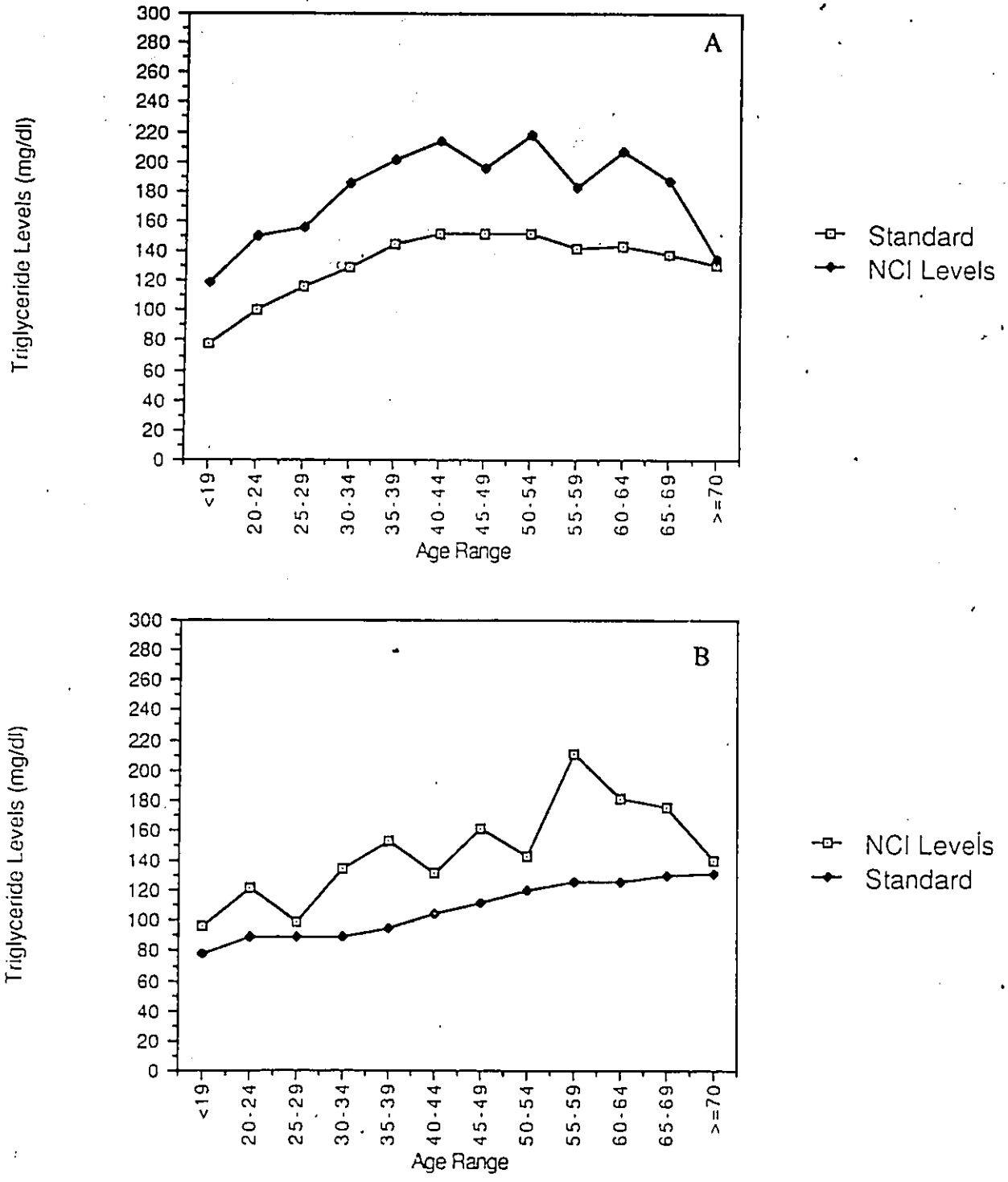


Figure 3-10 Triglyceride levels measured from samples in the study for the age groups indicated and average triglyceride levels in the appropriate age range for the general North American Population **A**, Levels for men **B**, Levels for women.

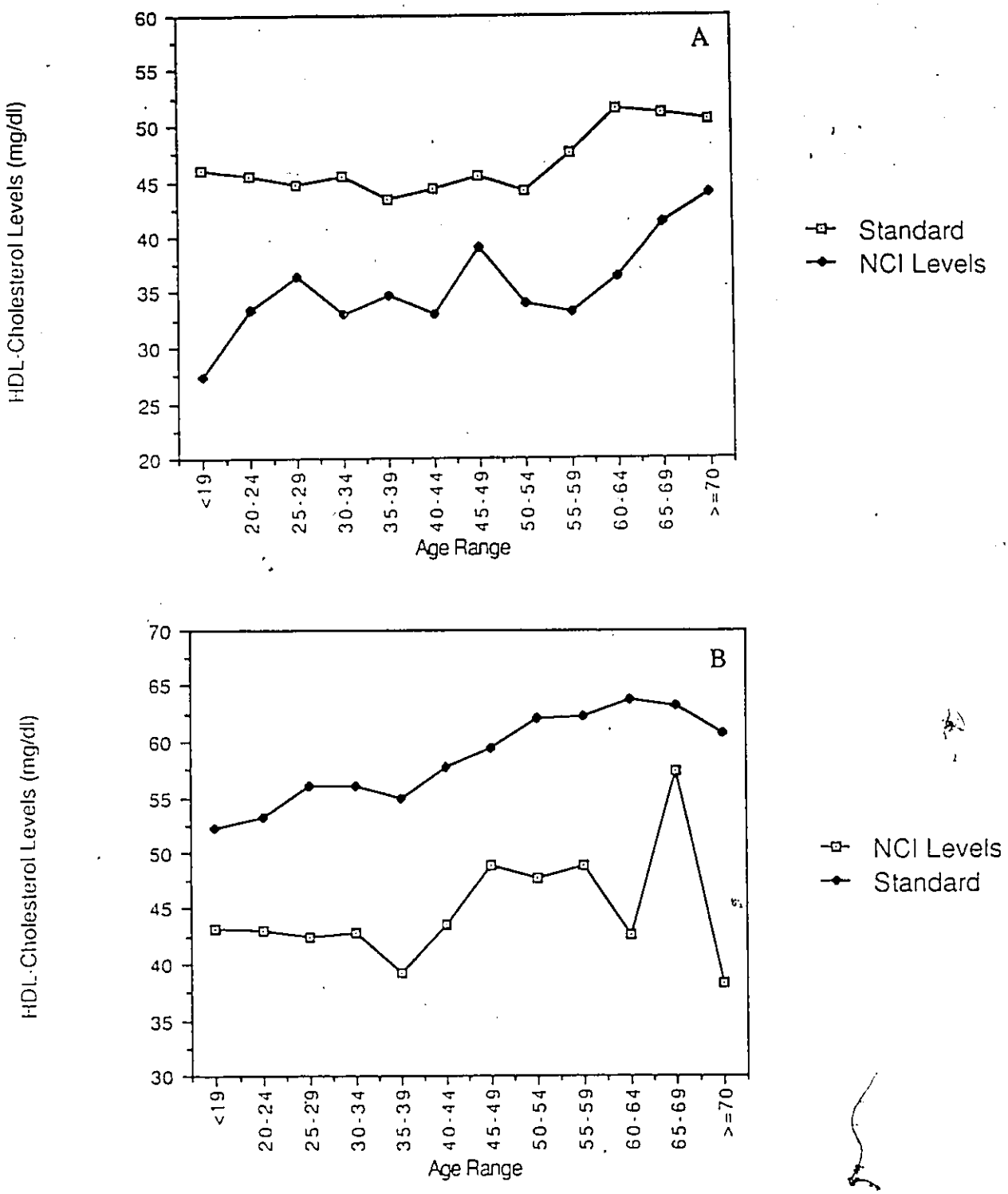


Figure 3-11 Average HDL levels measured from samples in the study for the age groups indicated and average HDL levels in the appropriate age range for the general North American Population. A, Levels for men. B, Levels for women.

females as opposed to 39 and 41 years for male and female controls respectively. Perhaps the information obtained would have been more informative if the cancer and control subjects were age matched.

HDL cholesterol (with a good imagination) showed some correlation when plotted against line width (FIGURE 3-12). However, mean line widths of 30.6 ($\pm$14.1) for Fossel <= 32 and 40.4 ($\pm$12.2) for fossel >32 indicated that there is considerable overlap between the groups and therefore little difference in the populations. Regression analysis gave $R^2 = 0.1$ for control individuals and $R^2 = 0.3$ for cancer patients. Perhaps if HDL_2 cholesterol levels were measured separately, one would see a greater correlation between the levels of this lipoprotein subclass in the cancer patients and Fossel values as did Barclay et al. (1964) for HDL_2 levels and cancer.

SUMMARY AND CONCLUSIONS

In summary, the preceding study observed a population in which many variables remained uncontrolled. Subjects had not fasted prior to blood-letting so that the circulating levels of triglycerides were examined in various stages of metabolism. This is likely one reason for the great variability in triglyceride levels. Mormons generally have a high intake of triglycerides given that their diet includes a large amount of dairy products. They have the

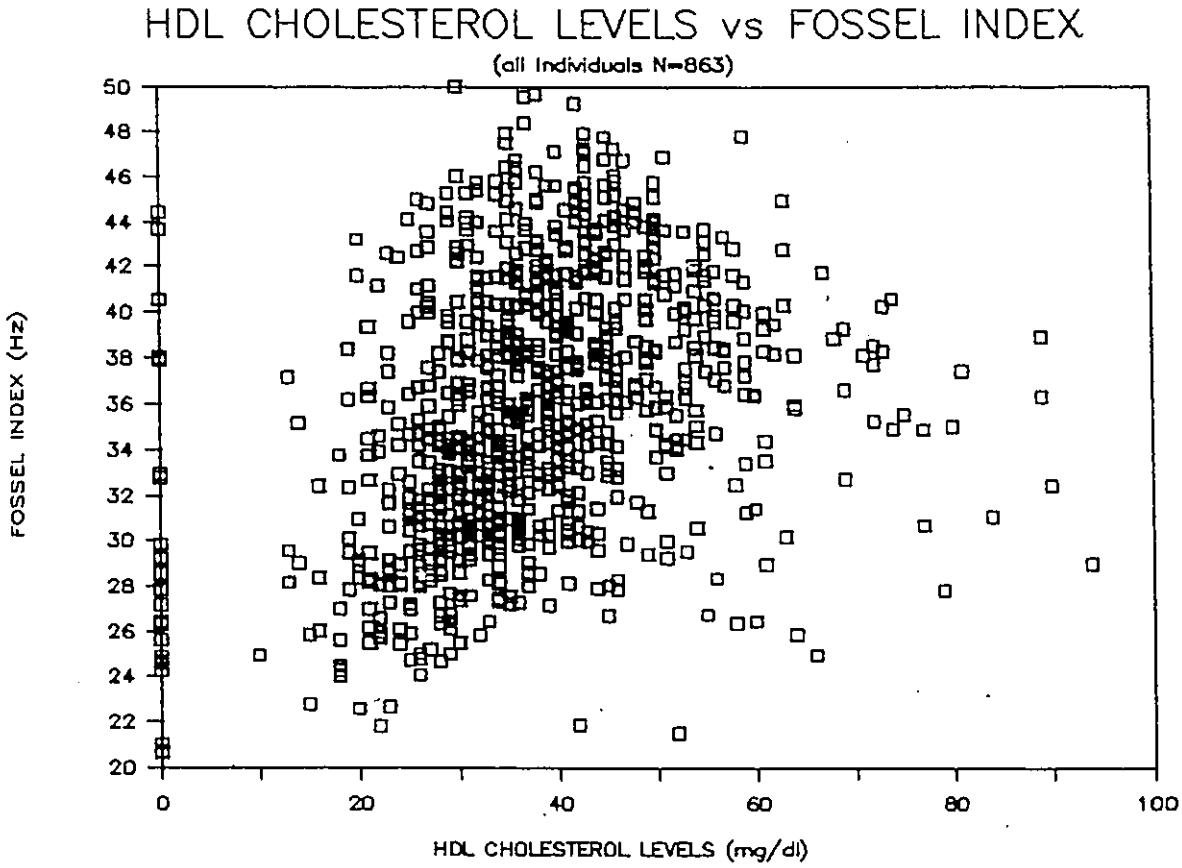


Figure 3-12 HDL-Cholesterol levels vs Fossel index (mean line width). Individual measurements were performed on aliquots of the same human blood plasma. Conditions of fossel index measurement were as described in text. Fossel indices used were an average of the line width measurements from the Fossel laboratory and the Smith laboratory

lowest national alcohol consumption level per capita and the lowest cigarette smoking level. (Kay et al. (1980)) observed that circulating levels of triglycerides are elevated with prolonged consumption of triglyceride containing substances.

An inverse relationship between triglyceride levels and HDL cholesterol levels was observed in 1964 by Barclay et. al.. Low HDL cholesterol levels were also observed for this study population. The cancer patients were generally older than the control individuals so that age matching in the population was an uncontrolled variable. This is a potential problem due to the fact that people in the general population of significantly different age groups tend to exhibit different triglyceride and cholesterol levels.(U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES)

Predictive value analysis did not show the Fossel test to be analytical for the screening of disease. However, a lower average Fossel value was observed for patients who did not respond well to therapy.

Considering the above variability in the sample population, the actual test itself has not been satisfactorily studied. Further line width measurements are necessary on fasted subjects taken from the general population in order to obtain a true analysis of its potential predictive value. This information will allow a reliable judgement to be made on the usefulness of this measurement as an indicator or for the monitoring of malignant disease.

Chapter 4

FOSSEL TEST FOR THE ANIMAL MODEL

Although many Fossel type experiments have been done for human populations, it appears that similar studies have not been reported with animal models. Such experiments would be useful to explore trends in the blood plasma with tumour growth and development. Furthermore, one could examine anomalies such as high Fossel (i.e. >32 Hz) values which are associated with increased triglyceride levels as was seen in Chapter 3. A major advantage of using an animal model is having greater control of variables which ultimately simplifies the analysis of results.

Many researchers have studied the effects of carcinogens and implanted tumours on plasma lipoproteins in rats. Specifically, Barclay et al. (1967) looked at the changes in the lipoprotein levels with time in two systems: 1) Female Sprague-Dawley rats injected with the chemical carcinogen DMBA (9,10-Dimethyl-benz-1,2-α-Anthracene) and 2) CFN Wistar male rats implanted with Walker Carcinosarcoma 256 cells. Several useful observations were made.

An increase in the levels of VLDL with time resulted for both of the above animal models. However, this observation was inconsistent from animal to animal. LDL behaved differently in the two systems, as was observed in the analytical ultracentrifuge. In all

control rats only one S_f class (of flotation rates) could be observed. In the DMBA-fed animals, the S_f class of LDL particles appeared as two classes. This was found only in blood samples taken after tumours had appeared. A similar observation was not made in the rats with implanted tumours.

The HDL_2 levels showed most interesting results in both of the above cases. As an initial observation, HDL_2 levels were lower than those in control animals for each pair of determinations. In DMBA-fed animals, the HDL_2 levels were low regardless of whether a tumour developed. In the CFN Wistar rats the HDL_2 levels dropped only if a tumour developed. However, if the tumour was rejected, the HDL_2 rose to levels higher than those in the control rats.

Given these results of the effects of chemical carcinogenesis on lipoproteins in model systems, it was thought that tumour development could be monitored using the line width measurements made by Fossel and coworkers. The model chosen was DMBA induced carcinogenesis in the female Sprague-Dawley rat.

In addition, due to the fact that one source of false positive results observed by Fossel et al. was pregnancy, a second study was undertaken. This involved monitoring the effect of gestation in rats on linewidths. The model, in this case, is useful due to the relatively short gestation period of 20-22 days.

EXPERIMENTAL

i) DMBA Study

All animal handling was carried out at Health and Welfare Canada. Twenty-four female Sprague-Dawley rats from 35 days of age and fed a normal diet were used in the experiments. Three groups of 8 were fed 1) 2.0 ml. of corn oil, 2) 10 mg. DMBA dissolved in 2.0 ml. corn oil or 3) 20 mg. DMBA dissolved in 2.0 ml. corn oil.

Once a week blood was drawn for analysis by NMR. To draw, blood the rats were anaesthetised with halothane. Approx. 1.5 ml. of blood was taken from the jugular vein into an EDTA containing Vacutainer. Blood was immediately centrifuged (at 2000 rpm for 10 min.) to obtain a plasma supernatant. Plasma was removed and stored at -18°C until such time as NMR could be performed. Plasma was thawed and 0.6 ml. pipetted into an NMR tube o.d. 5 mm for analysis the same day. NMR measurements were performed and spectra were processed as described in the previous chapter.

At the Health and Welfare laboratories other examinations of the rats were carried out. These included palpation of the breast area twice a week in order to look for tumour development and to record the time, location of tumour and the identification number of the animal. The expected time of tumour appearance depended upon the dosage of carcinogen but was believed to be 1-2 months.

ii) Gestation of Rats as Measured by NMR

Ten female Sprague-Dawley rats participated in the experiments of Fossel value as a function of gestation. The animals included in this study were as follows: one non-pregnant rat, one rat at 6 days, three rats at 9 days, one rat at 12 days, one rat at 15 days and three rats at 20 days of gestation.

Animals were anaesthetized with halothane and then sacrificed on the appropriate day (as indicated above). Blood (5-7 ml.) was drawn from the abdominal aorta. The anticoagulant used was 0.1M Sodium citrate (0.5 ml. per 5 ml. blood). Centrifugation, separation, storage and NMR were as for the DMBA study.

RESULTS AND DISCUSSION

i) DMBA Study

One of the 24 rats was not used in this study due to death caused by anaesthesia. Fossel values as a function of time are shown in FIGURE 4-1 for the other twenty three rats. These graphs are representative of experimental results. Generally the Fossel values do not appear to decrease with time.

It was interesting that the animals generally took 2-3 times longer than expected to develop tumours (i.e. Anywhere up to 6 months as opposed to the 1-2 months expected. This was thought to be due to frequent blood letting which would decrease the DMBA

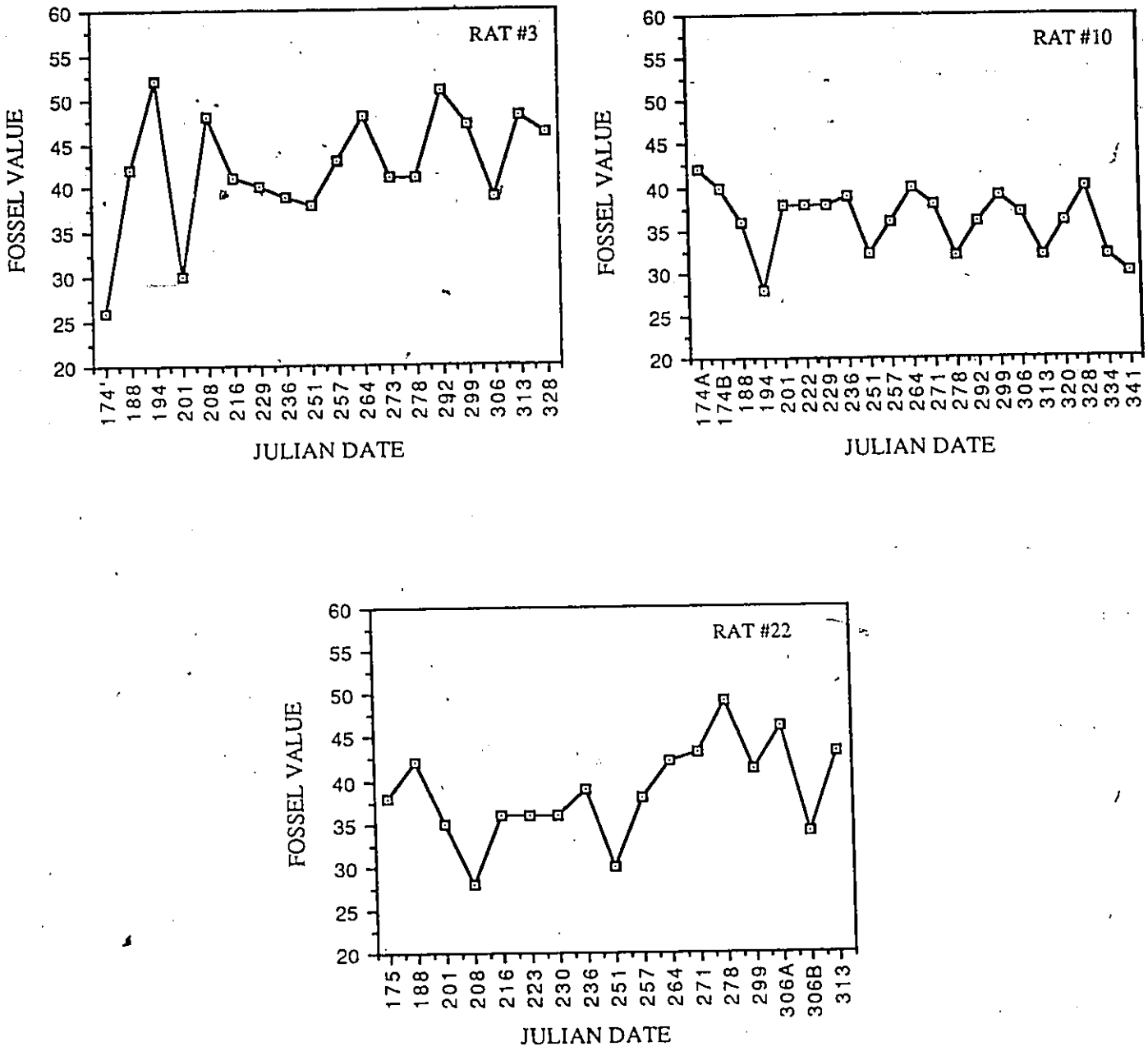


FIGURE 4-1 Fossel value vs time after DMBA administration RAT #3 - No treatment (CONTROL). RAT #10 - One dosage of DMBA (10 mg) administered. RAT #22 - Two dosages of DMBA (20 mg) administered.

concentration in the blood and presumably lowering the intensity of the carcinogenic effect.

In rats numbered 13, 18 and 21 a maximum Fossel value was obtained 3-4 weeks prior to the appearance of the tumour followed by a decline in value of 5Hz - 10Hz. This cannot be used as a general observation because, for example, rat #16 showed similar trends in values but was not reported to bear a tumour (FIGURE 4-2).

The range of Fossel values did not differ among the three groups of rats that were studied. Thus in this case the dosage of DMBA administered did not apparently affect the value. Generally the range of values was from 24Hz - 53Hz.

Barclay and Skipski, as mentioned previously, measured lipoprotein levels in rats fed the carcinogen DMBA over a period of 6 - 7 months. They found that HDL_2 levels were consistently lower in rats fed DMBA when compared with levels in control rats. No other lipoprotein class showed a consistent increase or decrease in level.

Tumours had appeared in all animals after 100 days. Low HDL_2 levels were observed 60 days after carcinogen feeding. Blood was taken from the rats at Health and Welfare prior to carcinogen feeding, immediately after and each week thereafter. If it is necessary that a tumour is present in order to observe a positive Fossel value then one would not expect to have observed a difference between DMBA-fed and controls early in the study.

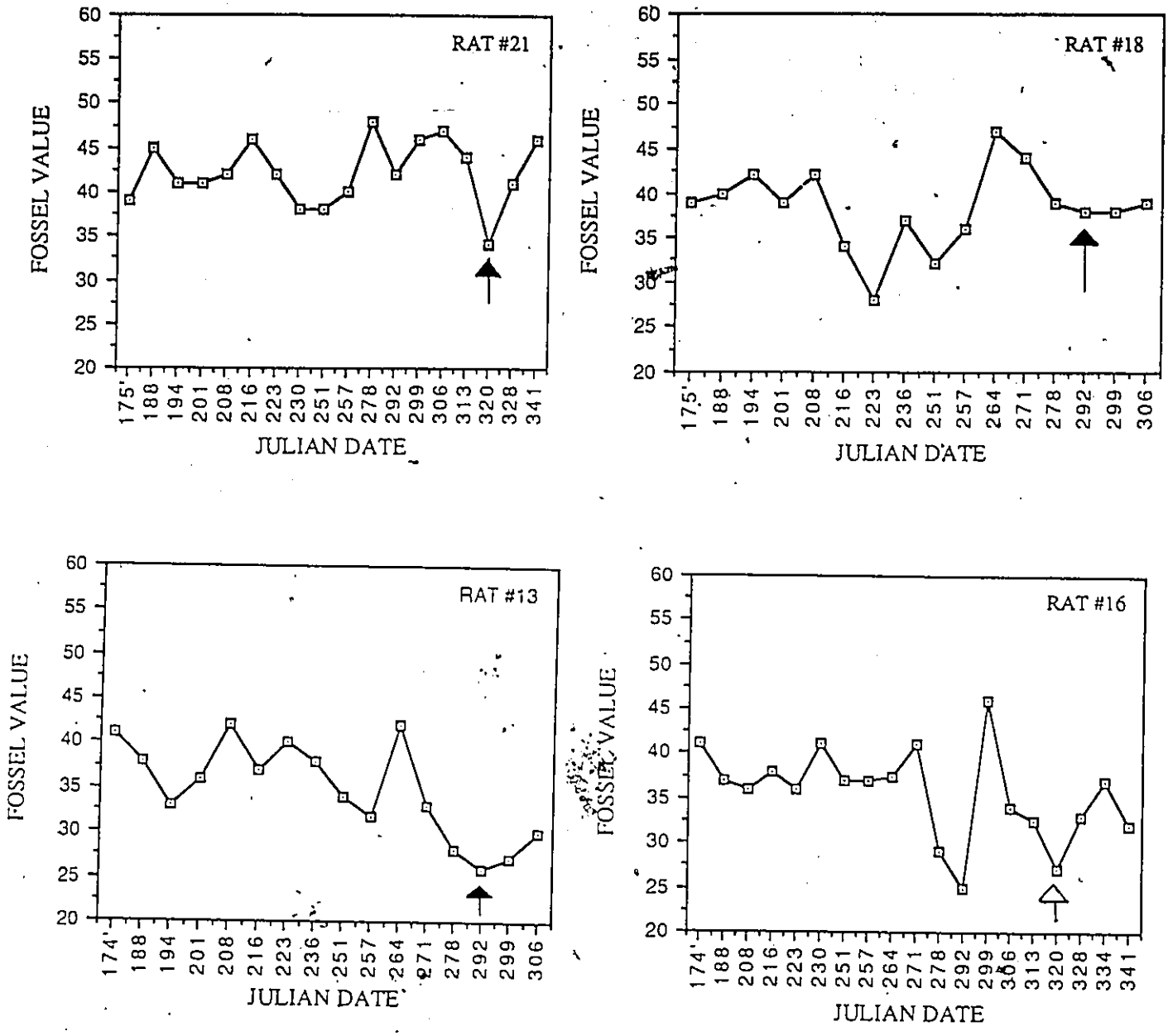


FIGURE 4-2 Fossel value vs time after DMBA administration. The closed arrows in graphs labelled Rat #21, Rat #18 and Rat #13 indicate where a tumour has appeared (i.e. 3 - 5 points after the maximum fossel value). The open arrow in Rat #16 indicates no appearance of tumour.

Other possibilities for lack of detectable differences between carcinogen-fed and control animals must be considered. 1) A decreased Fossel value may be dependent on a significant increase in the triglyceride level as well as a lowered HDL_2 level. Since an increase in VLDL levels in carcinogen-fed rats was not consistently observed (Barclay et. al. (1967)), it is possible that the rat animal model is not suitable for measurement by the Fossel method. Perhaps another experimental animal model would be necessary to observe biochemical changes by this method. 2) Detectable changes in the linewidth may not occur until the appearance of the tumour in which case further experiments covering a longer time frame and with less frequent blood-letting may be necessary.

ii) Study of Fossel Value vs Gestation in Rats

The results of the study using pregnant rats can be seen in FIGURES 4-3(A-F). Multiple samples from the same rat gave identical Fossel values (within experimental error ($\pm$2 Hz)). The Fossel value for each sample is plotted in the figure to demonstrate this reproducibility, thus the label "Sample Number" on the x-axis. Fossel values remained high (36 Hz-45 Hz) for all rats from which the blood was taken in the pre-gestational time. The rats at 20 days of pregnancy prior to sacrificing showed Fossel values ranging from 24Hz-27Hz.

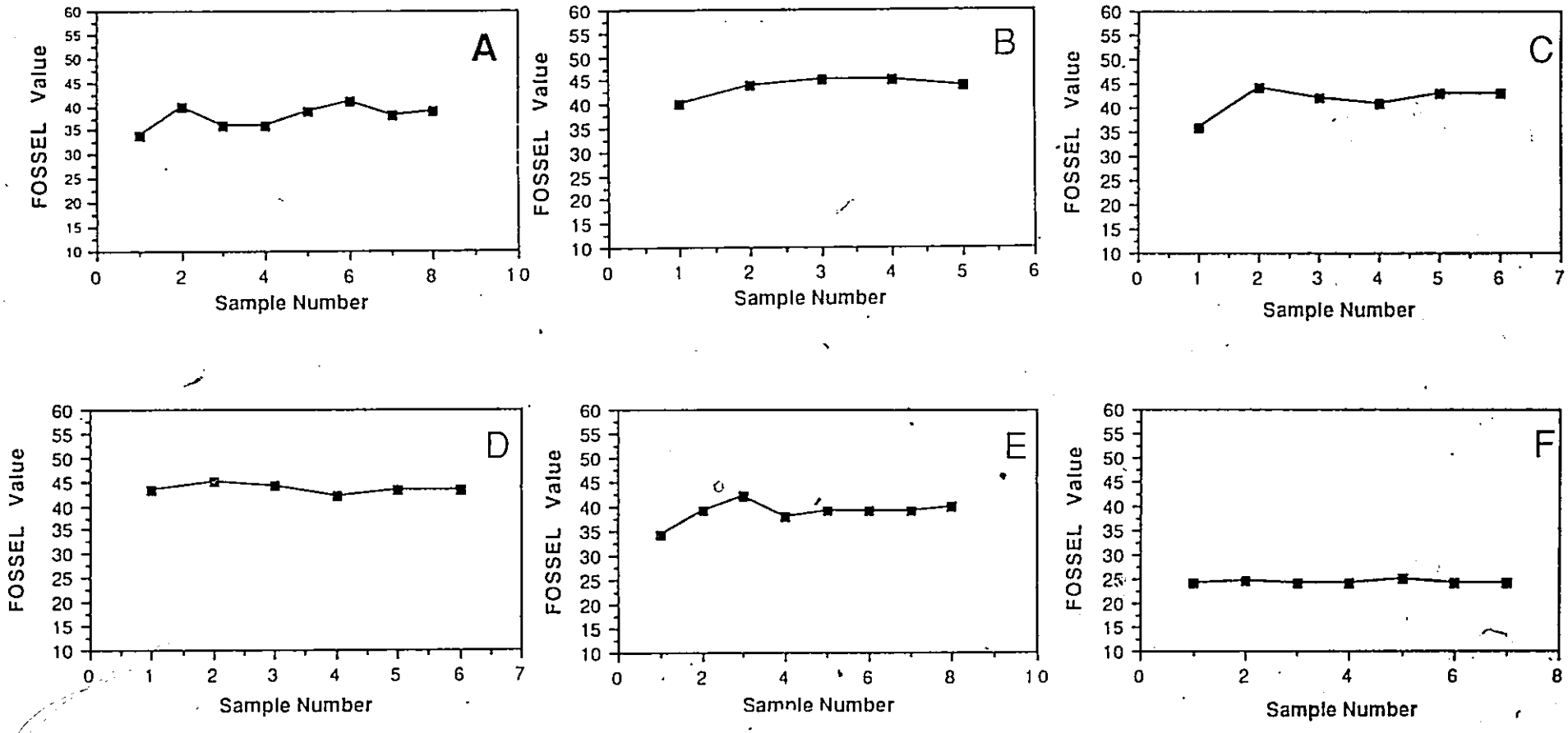


FIGURE 4-3 Fossel values for each sample from female SPRAGUE-DAWLEY rats in the following stages of gestation. **A**, Nonpregnant **B**, 6 Days **C**, 9 Days **D**, 12 Days **E**, 15 Days **F**, 20 Days.

Such low values are consistent with the results of Fossel et al. (1986) in humans in which several pregnant women showed false positive results. As previously mentioned in Chapter 2, Desoye et al. (1986) studied lipid and lipoprotein levels during pregnancy and postpartum in women. Triglyceride levels of non-pregnant control women and women at 8 weeks of pregnancy were very similar, 87 $\pm$ 16 mg/dl and 77 $\pm$ 28 mg/dl respectively. However, at 38 weeks of gestation triglyceride levels rose to 247 $\pm$ 84 mg/dl. Total cholesterol was found to increase from 167 $\pm$ 26 mg/dl at 8 weeks to 286 $\pm$ 46 mg/dl at 38 weeks. The other two increases found were for free cholesterol (42.2 $\pm$ 12.5 mg/dl at 8 weeks to 120.5 $\pm$ 30.4 at 38 weeks) and phospholipids (199 $\pm$ 34 mg/dl at 8 weeks to 323 $\pm$ 42 mg/dl) at 38 weeks). The greatest increase, however, was found for the triglyceride levels.

These results not only suggest reasons for the false positive findings in pregnant women but also verify again the dependence of the Fossel value on triglyceride levels.

SUMMARY AND CONCLUSIONS

The information from the Fossel values of DMBA treated animals over the course of time suggest that further studies are necessary using either a rat model with less frequent blood-letting or using a different animal model which may reflect more closely the biochemical changes that occur in humans with tumour development.

In the case of the rat model it would be appropriate to use saline-fed controls as well as corn oil-fed controls due to the fact that the Fossel measurements are probably influenced by the level of triglycerides. This experiment would provide a baseline set of values and the possibility of monitoring differences with the corn oil-fed group.

The decrease of the Fossel value in late gestation parallels the results in humans (Fossel et al.(1986)) This observation and the results by Desoye and co-workers of an increase in triglyceride levels in late gestation (Desoye et. al. (1986)) provides further evidence for a dependence of the test on triglyceride levels. Lipid levels in the blood of the rat should be measured in conjunction with the Fossel measurement. In order to verify this hypothesis samples taken at days 16-20 days of gestation would indicate if the time of increase in triglycerides is similar to that in humans.

Finally, the animal model has the potential of improving our understanding of this test from a biochemical point of view. For example, animals with conditions such as diabetes could be tested with the benefit of having all other variables controlled.

Chapter 5

MEASUREMENT OF A POTENTIAL MALIGNANCY MARKER, PRELIMINARY RESULTS

i) Identification of Tumour Markers

Several attempts to isolate and characterize various tumour markers have been mentioned in the introductory chapter. One of the rather successful attempts in recent years has been carried out by Hakomori and co-workers. This group has identified tumour markers of human colonic adenocarcinomas which are identical to, or slight modifications of the blood group antigens (Nudelman et al. 1986). In a review article, Hakomori pointed out that these antigens have also been demonstrated to be oncofoetal thus appearing at different stages in the development of the unborn foetus. The antigens identified (Nudelman et. al.,1986) all contained one, two or three terminal fucose residues as part of the carbohydrate moiety. Indeed, it has been noted that enhanced fucosyl transferase activity is associated with malignancies of various types (Chatterjee et. al. 1978, 1979).

These findings are significant in that Mountford et. al. (1986), using two dimensional NMR (COSY) experiments on a cell suspension of the 13762 metastatic rat mammary adenocarcinoma cell line, observed a unique cross peak between resonances located at 1.3 ppm and 4.2 ppm in the spectrum obtained. As stated in Chapter 1, a

COSY provides information on spin-spin coupling between nuclei. Thus the location of this cross peak in the spectrum could be attributed to coupling between the protons at C-6 and C-5 of a terminal fucose molecule. It is possible that fucose is the origin of the long T2 found in the tumour samples.(Princz, E., M.Sc. Thesis (1988)).

ii) RNA Proteolipid

The putative RNA-proteolipid observed by Wieczorek and co-workers, in relation to the above, had a high content of ceramide hexasaccharide raising the possibility of a fucose-containing moiety and the potential of observation by ^{1}H-NMR. If such a proteolipid could be isolated from the blood of cancer patients in a relatively pure form it should be easier to identify the resonances giving rise to a long T2 than it is from a tumour biopsy sample. This is because in solution, such as in blood plasma, the motional averaging of equivalent species better defines their resonant frequency such that the inhomogeneously broadened peak obtained from a tumour biopsy sample (broadened due to the fact it is composed of many resonances) is more easily separated into the individual resonances in a blood plasma sample. Furthermore, with regard to the concern for humanity, if identification of a tumour marker due to malignancy can be achieved in a blood sample, the possibility exists that extensive surgery can be avoided.

In the isolation of the proteolipid from human plasma samples, it was emphasised that the conditions of ultracentrifugation were critical. Electrolyte balance as well as temperature maintenance were necessary for the appearance of the opalescent band. Wright et al. (1986) were not successful in identifying the mRNA that Wieczorek reported to be associated with the band. In Wright et al. (1986), however, a resonance was reported which gave a long T2 in the region of interest. The value of 960 ms was close to that of lactic acid and could be due to this particularly ubiquitous compound. The compounds with protons in the correct chemical and magnetic environment which could resonate at the frequency of the long T2 are fucose, lactic acid and threonine. To identify the correct species it is first necessary to determine whether a long T2 is apparent and then eliminate alternatives. Identification has proven to be difficult due to the fact that lactic acid is of high concentration in the blood.

Lactate anion, (CH_3-CHOH-COO^-), whose methyl protons give a long T2 (>= 1 second), can be detected by NMR in micromolar concentrations. Due to active proliferation of cancer cells lactic acid production is increased in the individual with the disease. It will extract with an opalescent band in an ultracentrifugation and is very difficult to dialyze away. We have observed it in all blood samples received from cancer patients as well as in normal individuals.

EXPERIMENTAL

i) Processing of Samples

All blood samples were from the same groups as those described in Chapter 2. Preparation, ultracentrifugation and processing of the blood (including the method of extracting the opaléscent band) were also as before. Dialysis of the samples was performed using one of two methods. Dialysis bags had a pore size that allowed a 10,000 M.Wt. exclusion limit. Samples were either dialysed 3 times 20 minutes with 1 litre H_2O or 3 times 20 minutes with 100 ml H_2O. In both cases a final dialysis of 3 times 20 minutes with 10 ml D_2O was performed. This final dialysis was necessary to effect an isotopic exchange of ^1H for ^2H. The ^2H_2O, as stated in Chapter 2, provided a field-frequency lock necessary for the stability of the magnetic field in a high resolution experiment. Of this sample 0.6 ml was then placed in a 5 mm outside diameter NMR tube for T2 analysis.

ii) The T2 method

The T2 experiment can be briefly explained as follows. A 90 degree pulse will tip the bulk magnetization vector into a plane in which magnetization can be detected. The maximum intensity of the signal may be observed just after the 90 degree pulse. (FIG 5-1A). As was described in Chapter 1, due to interactions between magnetic

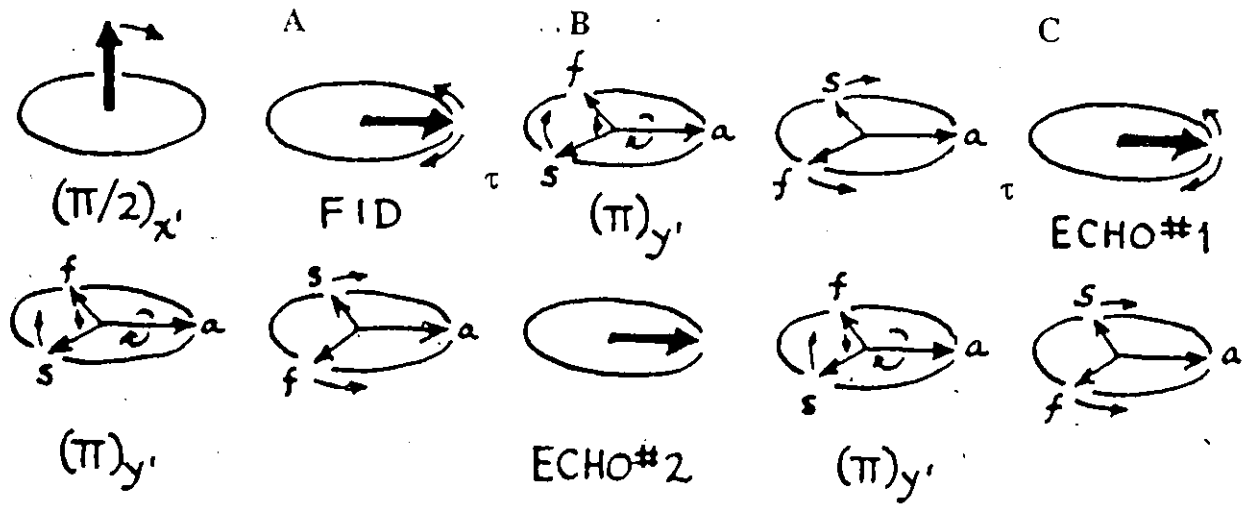


Figure 5-1 The fate of the bulk magnetization vector in a CPMG experiment for measuring T2. A, Bulk magnetization in the x-y plane just after a 90° pulse. Individual magnetic moment vectors fan out in a time τ. B, A 180° pulse rotates individual vectors by 180° (refocussing of chemical shift). C, In a time τ the individual magnetic moments will join the bulk magnetization to produce an ECHO. The 180° pulse is repeated N times. (Fukushima et. al. (1981)).

moments, phase coherence can be lost (the T2 mechanism) and the observable magnetization will decrease. A decrease in magnetization may also be effected by inhomogeneities in the magnetic field in the vicinity of the sample. This latter condition may be somewhat corrected for by a process called refocussing the chemical shift. A 180 degree pulse is applied along the vector of bulk magnetization to effect this process. After a time τ, the magnetic moment vectors which have been influenced to defocus (due to the inhomogeneities in the magnetic field) will be rotated by 180 degrees around the axis on which the pulse was applied. (FIG 5-1B) In a time τ, the precession of the individual magnetic moments will be identical to that of the bulk magnetization vector creating an echo. (FIG 5-1C). The entire experiment can be summarized by the pulse sequence:

$$90_x - (\tau - 180_y - \tau)_N$$

where 'x' and 'y' refer to the cartesian coordinate axes along which the respective pulses are applied. The τ - 180 - τ sequence is repeated N times and a spectrum may be sampled after 4N repetitions.

Experimental parameters used were as in Figure 5-2. The P1 and P2 refer to the 90^o and 180^o pulses respectively. These were found to be somewhat variable according to the sample. However, usual values were 7 μs and 14 μs respectively D1 (as described in Chapter 2) was a relaxation delay time of the order of 3 x T2 during which the magnetization of the system is allowed to achieve its

(SAMPLE I.D.) NRC #	LONGEST T2 (ms)	# EXPONENTIAL TERMS
147	82	1
153	123	2
154	266	2
163	188	2
164	205	2
165	134	2
166	153	2
168	132	2
169	148	2
170	323	3
172	223	2
173	172	2
174	125	2

TABLE 5-1 Longest T2 values (in milliseconds) for samples of opalescent band isolated from discontinous density gradient. Measurement of T2 was as described in text.

equibrium state. Water saturation was also performed during D1. D2 was the delay, τ, used in the CPMG sequence. A variable counter (vc) list contained the list of N values or the number of times a 180^{o} pulse was applied.

iii) Analysis of T2

Measurement of T2 was performed by a non-linear least sum of squares regression analysis program (Marquardt (1963)). Input data necessary for the program were the number of exponentials (1, 2, or 3) to which the data should be fitted, delay times used in the measurement (2N ms) and the corresponding peak intensities from the spectra obtained. (In many cases, at longer delay times no reliably measurable peaks were present and the data were thus omitted from the fit). After several iterations, an exponential decay curve which would best fit the data was calculated. The curve can be described by the equation:

$$\text{Intensity} = \alpha_1 e^{-t/T_{21}} + \alpha_2 e^{-t/T_{22}} + \alpha_3 e^{-t/T_{23}}$$

in which the number of exponential terms depended on the number of exponentials to which the data had been fit. The number of exponentials which would best fit the data was determined from a statistical F-test which compared the sums of squares of residuals between consecutive analyses (i.e. 1 exponential vs 2 exponentials).

The F-test equation can be summarised as:

$$\frac{(\text{SSE LF} - \text{SSE HF})/2}{\text{SSE HF}/\text{DFHF}}$$

SSE = Sum of Squares of Exponentials
LF = Lower number of Exponentials Fit
HF = Higher number of Exponentials Fit
DFHF = Degrees of Freedom for Higher Fit

For the fit which was accepted the longest T2 value was recorded as the T2 for that particular sample.

RESULTS AND DISCUSSION

Observation of the extensively dialysed samples was very difficult due to the large dilution of the sample. CPMG experiments were performed with many accumulations in order to be able to observe the signals at longer delay times. Resonances which were observable at longer delays in the appropriate chemical shift region could be attributed to lactic acid.

The samples which were dialysed for a shorter period of time gave a much better resonance signal, as was expected due to less dilution of sample. The T2's which were measured for all peaks in the region of interest gave values ranging from 100-350 ms (TABLE 5-1). This places the results in a short T2 category. An example of the high field region of the spectra obtained for a T2 experiment at the delay times indicated can be seen in figure 5-3. One can clearly see the resonances which could be attributed to lactate. In light of

```
      CPMGBAND.AU
D1 =    1.0000000   S1 =   12L          P1=          7.0C
D2=      .0010000   P2=       14.00     LO VCLIST.001
    1 =          4
    2 =         40
    3 =        128
    4 =        256
    5 =        400
    6 =        512
    7 =  END
RD=     0.0            PW=         0.0     DE=       106.30
NS =         28         DS =          0     NE =           6
```

Figure 5-2 Experimental parameters used in the CPMG experiment. **D1** = As described in figure 3-1. **S1** = Decoupler power for water saturation. **P1** = 90° pulse length in μs. **P2** = 180° pulse length in μs. **D2** = Delay time τ as described in the text. **VCLIST.001** = List of values of N (also # of times 180° pulse was applied) **RD** = Delay time (unused). **PW** = Pulse width (unused). **DE** = Dwell time or time between the end of the pulse train and spectral acquisition. **NS** = Number of spectral accumulations **DS** = Dummy scans (to ensure equilibrium of system prior next experiment). **NE** = Number of Experiments (corresponds to N).

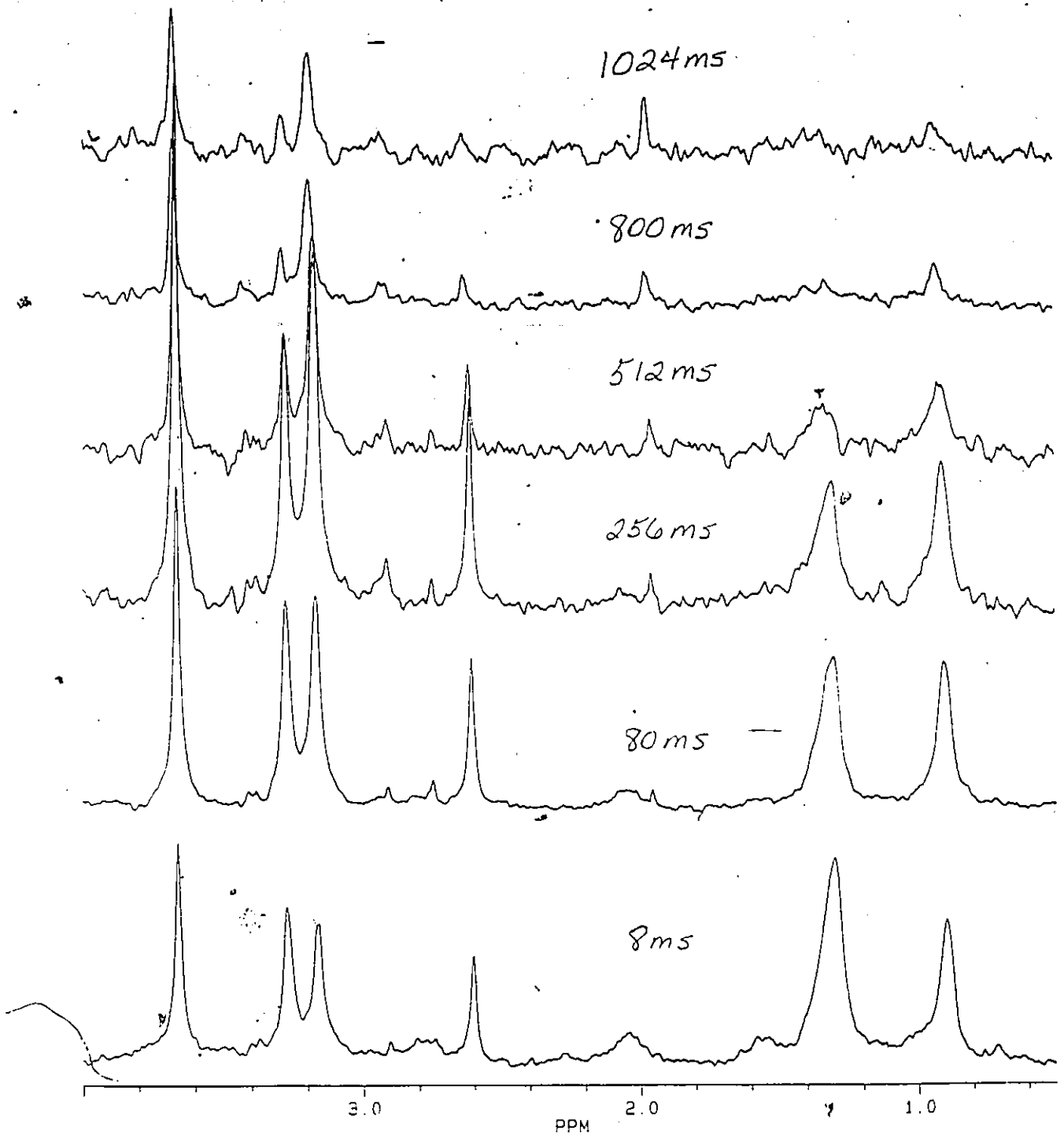


Figure 5-3 T2 experiment of an opalescent band isolated and prepared as described in the methods section. Spectra were acquired after delay times as indicated in the figure. The arrow indicates the location of the lactate ion resonance in the spectrum. It appears in this sample. The two prominent peaks centred at 1.3 ppm and 0.9 ppm are the composite methylene and methyl resonances respectively.

the present results, the lack of detection could be due to one or more possibilities.

i) Possible reasons for lack of detection

A) Treated patients would initially tend to have a lower concentration of a tumour marker in the event that the treatment was successful in eradicating the disease or at least slowing its course. Thus, if the patient has had recent treatments of chemotherapy or radiation therapy, or has undergone surgery, the marker may not be present at a level detectable by NMR. In the case of the ovarian cancer patients from whom blood was examined for the presence of a fucosylated marker, only six individuals were untreated.

B) The present method of centrifugation may have been too harsh for the survival of a detectable concentration of the marker.

C) ^{1}H-NMR may not be sensitive enough to detect the concentration of the marker that is present.

D) A fucosylated marker does not exist in the blood of patients with ovarian cancer. Alberts and co-workers in 1985 reported that fucosyl transferase activity was not enhanced in the blood of patients with certain ovarian cancers. Thus, tumour markers to which fucosyl transferase activity is directed may not be exported by ovarian cancer cells. One may suggest that a proteolipid

could exist for cancer in general but that the composition of at least the carbohydrate moiety is altered for each type of malignancy.

It is quite possible that the proteolipid observed by Wieczorek and co-workers was a modified lipoprotein whose function in the blood is as yet, unknown. Mountford and co-workers have speculated (personal communication) that the proteolipid is actually a subclass of the elusive lipoprotein(a) (Lp(a)). The structure and physical properties of the lipoprotein have been fairly well elucidated (Scanu et. al. (1984)). It has been thought that elevated levels of the complex are associated with coronary artery disease (Scanu et. al. (1984)) due to the fact it has been isolated from the intima of atherosclerotic lesions. In fact, no direct relationship has been found. Lp(a) has a relatively high carbohydrate component and thus it could be a good candidate for a fucosylated tumour marker.

The possibility of a general cancer-marker is exciting. Further experiments in ^{1}H-NMR coupled with biochemical assays should provide some information on, 1) the existence and, depending on #1, 2) the composition of such a complex. Optimization of sample preparation which would result in a higher concentration of the species being observed should enable a greater amount of information to be extracted from the NMR experiment.

FUTURE CONSIDERATIONS

The experiments described in the preceding chapters have brought to light, among other things, the importance of the experimental protocol in achieving useful, if not the intended, results. The rat animal model, as was described in Chapter 4, may not have been appropriate to study the Fossel test. Perhaps the use of the rabbit in experiments, as was performed by Goldstein and Brown, would be a better model for several reasons. A) The effects that blood-letting would have on the depletion of a carcinogen in the circulatory system would not be as pronounced. B) The lipoprotein metabolism in the rabbit much more closely mimics that of the human condition. C) It is much easier to induce certain hyperlipidemias in the rabbit model than in the rat.

The experiments using human blood plasma required more careful attention. The sample population that one chooses to study is very important if one wishes to describe a larger population. The Mormon community used to examine the Fossel test was not representative of a North American population. However, in terms of biochemical and physical information, and patient histories, it was useful. Because of experimental considerations, however, a strict regime in terms of fasting prior to blood-letting and the exclusion of people on hormone replacement therapy were not carefully followed. The number of samples taken from each sex was highly unequal in that men contributed approximately 70% of the samples. The

number of cancer patients tested was only approximately 2% of the study population. Thus, further experiments are necessary on a more carefully selected population which includes a higher proportion of cancer patients, equal numbers of men and women and a stricter requirement of subjects fasted and without hormone supplementation to gain information that would apply to the greater North American population.

It would be interesting to follow-up the linewidth experiments on patients with ovarian cancer throughout the course of their treatments to determine if the linewidth measurement will indeed indicate the success or failure of the treatment and will be of subsequent use to confirm remission or cure. Frequency of sampling for a follow-up study remains to be determined but should require that the patient experience the minimum discomfort and simultaneously allow the progressive monitoring of the disease. Weekly sampling may be the optimum.

Finally, experiments involving the identification of fucosylated tumour marker in the blood will initially require the use of blood from untreated cancer patients. These experiments have exciting possibilities in that the identification and subsequent characterization of malignancy markers in the blood can lead to development of antibodies against these markers.

CONCLUSIONS

Evidently, any work that contributes to a clearer picture of the biochemical events underlying malignant changes can aid medical science in its fight toward the eradication of the disease. From the work presented here, the use of ^{1}H-NMR to identify the diseased condition does not stand alone as a diagnostic tool. It has provided some valuable information which indicates that it can easily identify abnormalities in the blood that are associated with the cancerous condition and thus it may be useful as a diagnostic indicator for the disease.

Furthermore, with the development of the ^{1}H-NMR technique to look at tumour markers, ^{1}H-NMR can play a role as a stepping stone toward the final development of diagnostic monoclonal antibodies. Due to the ability of NMR to characterize the motions of molecules in their environments, as well as to provide structural information, experimental design in terms of characterization, isolation and purification of these markers may be facilitated.

The research in this thesis has investigated the cancerous condition from a novel, chemical perspective. The consequent insight at the molecular level has suggested some possibilities for future experimentation. It has furthermore led to treatment of the information received with novel mathematical methods. It is to be

hoped that it has made some contribution towards bridging the gap between the molecular and the clinical sciences.

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