Assessment of human sarcoma tissues by proton NMR spectroscopy: Correlation with histopathology

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Two-dimensional total correlated spectroscopy (TOCSY) was carried out on soft tissue tumour samples. A total of 10 samples of histopathologically classified sarcoma tissues and three benign tissues were analysed. Levels of phospholipid metabolites, triglyceride and the resonance patterns from fucose were found to differ between benign and tumour tissue and tumour tissue of different grade. The results demonstrate the use of the TOCSY technique *ex-vivo*, to identify and semi-quantitate various metabolites, which could then be used in the gradation of the tissue.

CANCER cells differ from non-tumorigenic cells in their abnormal biochemistry. An impaired carbohydrate metabolism with a high rate of glycolysis[1], lactic acid production, a capacity to store glycogen[2], modulated phospholipid pathways (of particular significance being the phosphocholine and phosphoethanolamine pathways[3]), and an altered cell surface chemistry, especially increased synthesis and organizational differences in cell surface glycolipids and glycoproteins[4], are characteristic of tumour cells. There is sufficient data to suggest that these modulations correlate with tumour development and progression. That these differences are also discernable by NMR is of interest because a non-invasive means of monitoring altered cell surface chemistry and metabolic differences could provide a practical method in the diagnosis and study of treatment response in cancers. Proton NMR in cells and tissues have provided information about the plasma membrane structure and function. It has been possible to distinguish highly tumorigenic colorectal[5], breast[6] and fibrosarcoma cell lines[7] from lowly tumorigenic cell lines of similar origin from their proton NMR spectra. The differences have been based on the altered lipid profile[5,6], higher choline metabolite content and more complex cell surface fucosylation of the highly tumorigenic cells[5,8]. The problems faced with resolution due to the heterogenous nature of tissue samples have been alleviated to some extent with the use of 2D techniques. Magnitude mode COSY[8] (correlated spectroscopy), PCOSY[9] (purged correlated spectroscopy) and TOCSY[10] with linear prediction have been used in cancer cells tissues and cellular extracts. The TOCSY or homonuclear polarization transfer by isotropic mixing has become a popular method for 2D-correlation spectroscopy. An earlier report[10] of ^{1}H NMR 2D TOCSY at 600 MHz on *ex vivo* smooth muscle tumour tissue was found to identify a large number of metabolites in intact tumour tissue and was found to show advantages in sensitivity over the PCOSY method. In this paper, one-dimensional and two-dimensional TOCSY (without linear prediction) experiments have been recorded on tissues.

Tissue samples were acquired from untreated patients with soft tissue tumours. Fresh specimens were frozen in liquid nitrogen in the operating room itself. Of the 13 samples analysed, 6 were obtained at biopsy and the others from patients undergoing surgical resection, the specimen being selected by the pathologist. The specimens were transported on dry ice to Mumbai/Bangalore for the NMR experiments. The measurements were recorded on the 500 MHz-AMX Bruker at TIFR and 500 MHz-DRX Bruker machine at SIF, IISc, Bangalore. Routine histopathological analysis was performed on the tissue bits. A small portion of the tissues used for spectroscopy was graded histopathologically based on cellularity, vascularity, pleomorphism, mitotic activity, cell type and necrosis by a pathologist. An overview of the type of tumour, grade, site and histological features is listed in Table 1. Prior to the experiment, the specimen was washed in PBS/D_2O (phosphate buffered saline in deuterium oxide) and then placed in a 5 mm NMR tube. About 400 µl of PBS/D_2O was used to cover the sample. Presaturation was used for residual water suppression. The 1D spectra were acquired with a spectral width of 4000 Hz using 16 K data points, 256 accumulations, a relaxation delay of 1.5 s (the spectra would not be fully relaxed). A 90° pulse of 8.8 µs was applied. Peaks were referenced to TSPS (3-trimethylsilyl propanesulfonate).

The phase sensitive TOCSY experiments were performed using the MLEV-17 composite spin lock pulse with a mixing time of 85 ms with water suppression by presaturation during the relaxation delay of 1.5 s. A continuous wave pulse of 45 Hz was used for presaturation of the water signal. The sweep width was 6000 Hz in both dimensions. A total of 1 K data points were collected with 512 t_1 increments. 32 scans per t_1 increment were recorded. The total experimental time was 7 hours. Quadrature detection in f_1 was achieved using TPPI. All experiments were processed using standard Bruker XWINNMR software. The data set was zero filled to 1 K × 1 K data points, 90° phase shifted squared sine window function were applied to both dimensions. All spectra are in the pure absorption mode. The final 1 K × 1 K real data set had a resolution of 5.87 Hz per point. The one-dimensional spectra were collected before and after the 2D to check for sample stability.

The *ex vivo* 1D NMR spectra of four tissues – a fibroma, an intermediate grade fibrosarcoma, a high-grade fibrosar-

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Table 1. Characteristics of tissues examined by 500 MHz NMR

Age (yr)	Sex	Clinical status	Site	Type	Grade	Characteristics/miotic activity
75	M	Recurrence	Thigh	Malignant fibrous histiocytoma (MFH)	III, ng I	1–3/hpf, myxoid, pleomorphic nuclei
62	M	New	Thigh	Malignant fibrous histiocytoma (MFH)	III, ng I	1–5/10 hpf
55	M	New	Arm	Liposarcoma	II, ng II	0–1/hpf
54	M	New	Hand	Spindle cell sarcoma	II–III, ng II	0–2/hpf
37	F	New	Thigh	Spindle cell sarcoma	II, ng II	1–3/10 hpf
40	F	New	Leg	Liposarcoma	I, ng III	Mitotic figures rare, Cellular
55	F	New	Leg	Fibrosarcoma	II, ng II	Few mitotic figures, Pleomorphic Cellular
47	F	Recurrence	Mandible	Fibrosarcoma	III, ng I	Scattered mitotic activity
75	M	New	Thigh	Leiomyosarcoma	II, ng II	1–2/10 hpf
71	M	New	Shoulder	Leiomyosarcoma	I–II, ng II	Spindle shaped, pleiomorphic nuclei, 1–2/10 hpf mitotic activity
55	M	New	Chest	Fibroma	Benign	NA
50	M	New	Arm	Fibroma	Benign	NA
45	F	New	Abdomen	Lipoma	Benign	NA

ng, nuclear grade; NA, not applicable; hpf, high power field.

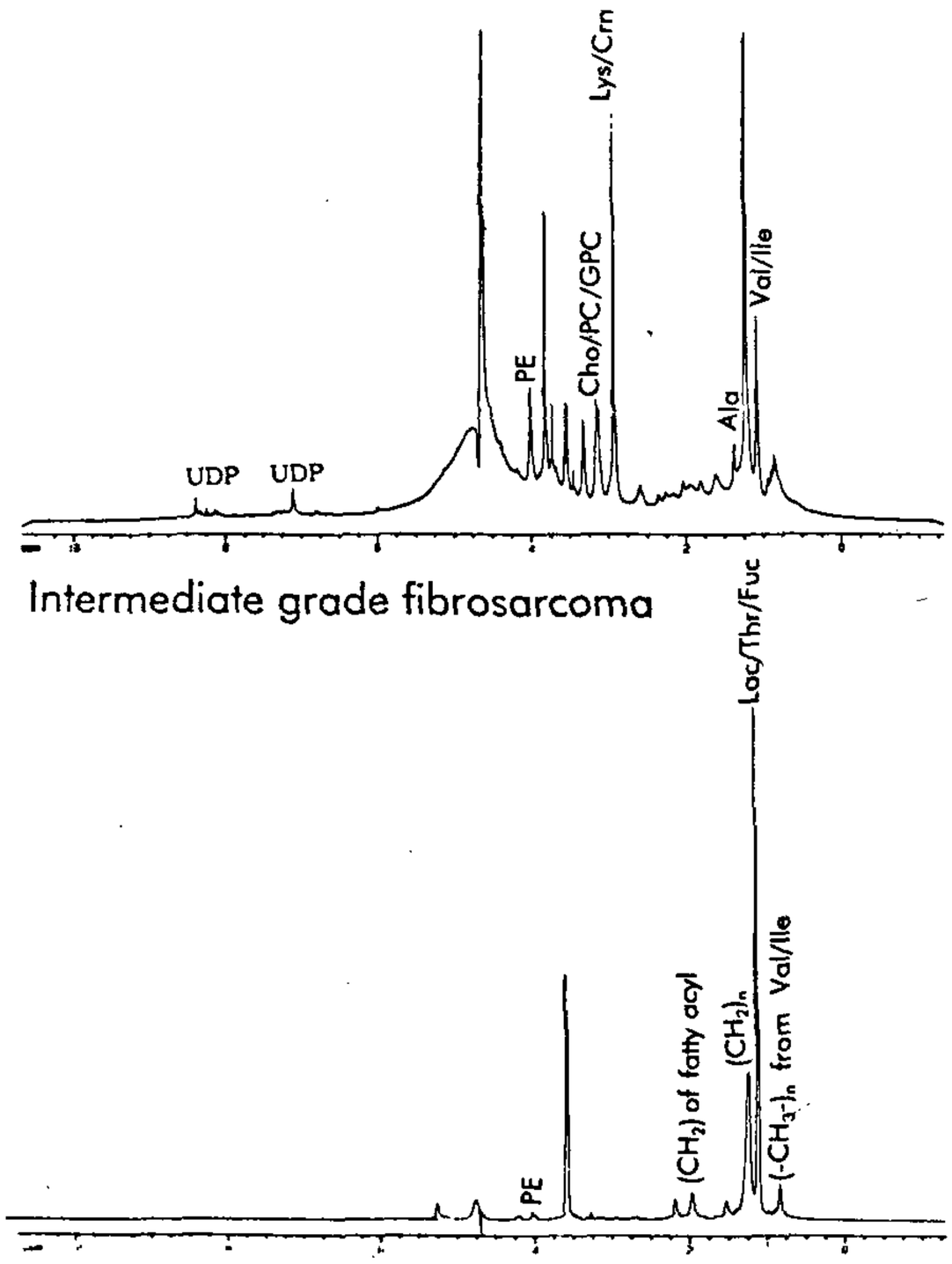

Figure 1 *a*. 500 MHz ^{1}H NMR spectra of a fibroma and intermediate-grade fibrosarcoma in PBS/D_2O. 256 transients were accumulated in the FID. The spectra are plotted on a constant vertical scale.

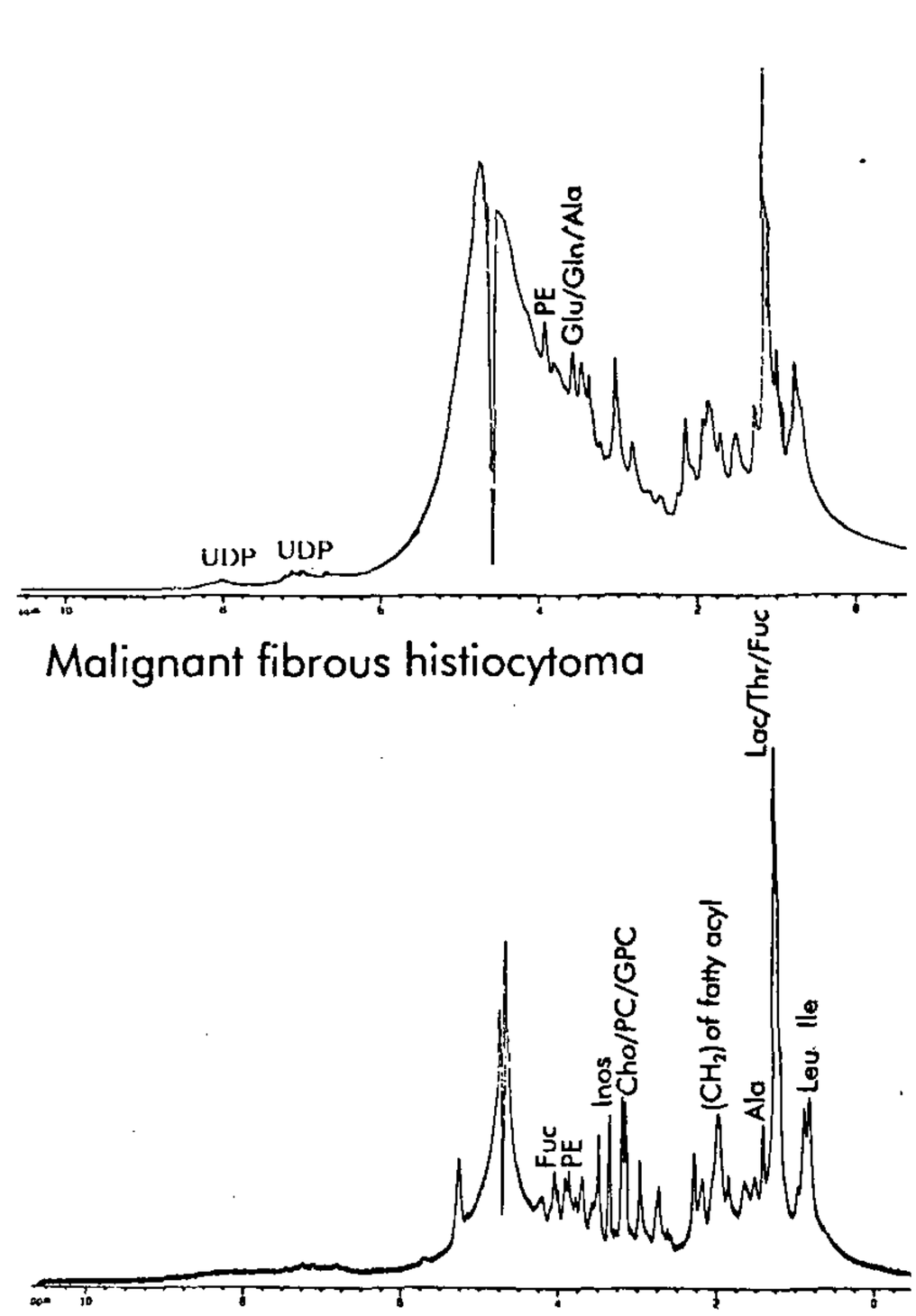

Figure 1 *b*. 500 MHz ^{1}H NMR spectra of a high-grade fibr coma and a malignant fibrous histiocytoma in PBS/D_2O. 256 sients were accumulated in the FID. The spectra are plotted constant vertical scale.

coma and a malignant fibrous histiocytoma are shown in Figure 1 *a*, *b*. The 1D spectra are dominated by resonances from lipids and amino acids. Qualitatively there are clear differences between the benign fibroma, intermediate gr and high-grade fibrosarcoma. The high-grade fibrosarc spectrum has many more resonances than the intermed

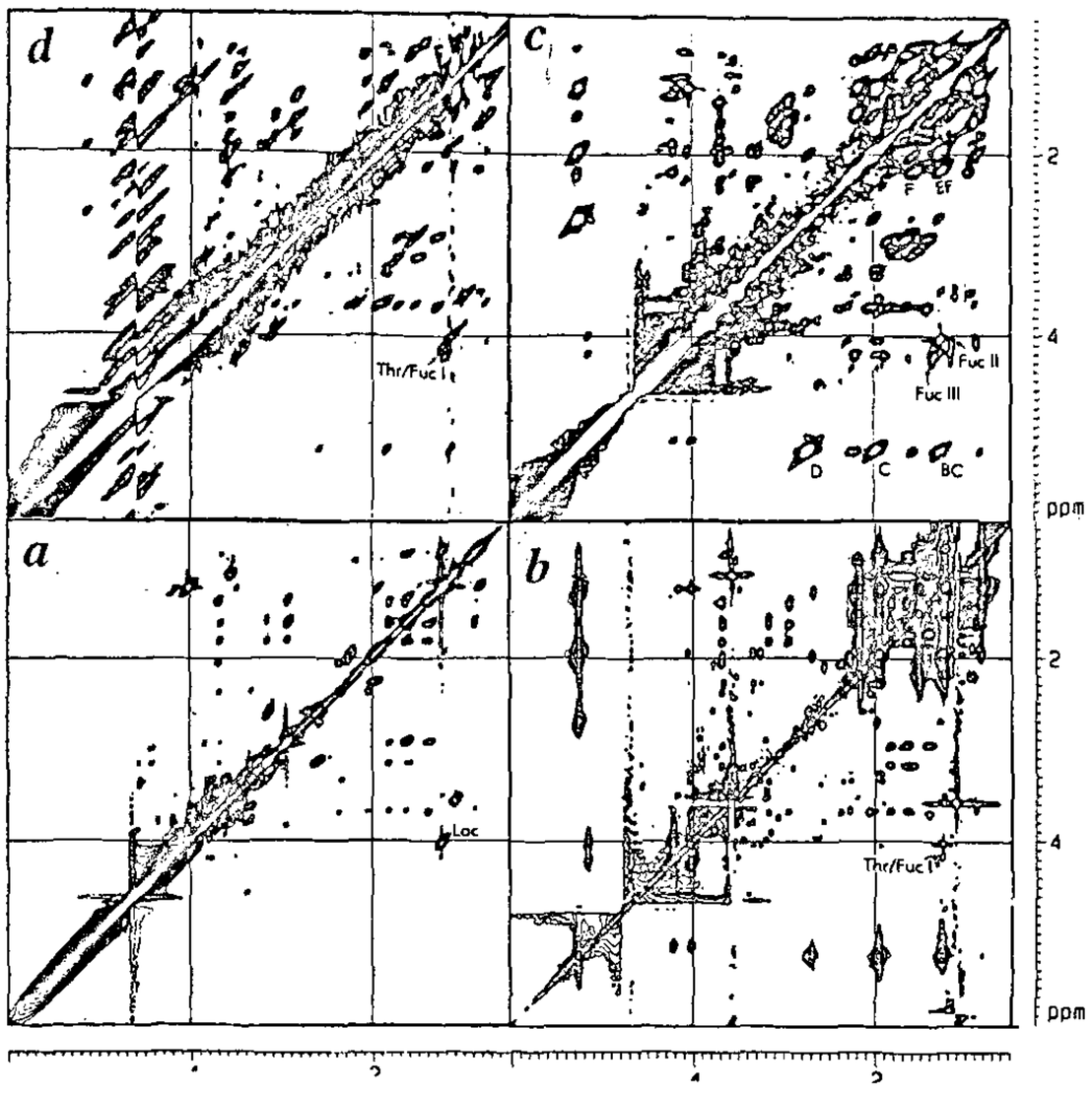

Figure 2. 500 MHz TOCSY spectra (7 hour acquisitions) of *a*, fibroma; *b*, intermediate grade fibrosarcoma; *c*, high grade fibrosarcoma; *d*, malignant fibrous histiocytoma tissue in PBS/D_2O. These are TOCSY spectra of the same samples used in Figure 1. All spectra are in the pure absorption mode. The plots use the same absolute intensity scaling and same contour levels.

grade spectrum. The MFH (malignant fibrous histiocytoma) spectrum is not as well resolved as that of high-grade fibrosarcoma. The high-grade fibrosarcoma differs from MFH in that the resonances from amino acids, carbohydrates, phospholipid precursors in the range 3.00–4.00 ppm are narrower and better resolved. Two MFH samples were run and both the spectra showed poor resolution when compared to other sarcoma tissues.

The TOCSY spectra of the fibroma, intermediate grade fibrosarcoma, high-grade fibrosarcoma and the MFH tissue are presented in Figure 2 *a–d*. These spectra have been obtained from the same tissue used for Figure 1 *a*, *b*. An expansion of the high grade fibrosarcoma spectrum is also provided (Figure 3) to indicate cross peak assignments. The most obvious differences in the transition from benign to malignant in the 2D spectra are in the intensity of cross peaks from triglyceride (A–G′), number of cross peaks from free amino acids, the intensity of $-CH_2-CH-$ cross peak from phospholipid and $-CH_3-CH-$ cross peaks from lactate, alanine, threonine and fucose. The sharper better resolved resonances from 3.00–4.00 ppm, in the high grade fibrosarcoma spectrum are also manifested in the 2D spectrum by increased cross peaks of lactate (1.33, 4.12 ppm), alanine (1.49, 3.79 ppm) and fucose (1.33, 4.27 ppm; 1.25, 4.28 ppm and 1.41, 4.30 ppm) (Figures 2 *c* and 3). There is also an increase in the cross peak of choline (3.50, 4.07).

Cross peak volumes for triglyceride, choline, phosphocholine (PC), phosphoethanolamine (PE), ethanolamine, glycerophosphocholine (GPC), alanine, valine and fucose (as a block) were calculated. The calculated volumes relative to that of alanine cross peak are given in Table 2. The total volume of fucose block relative to a reference cross peak for all those samples in which the fucose cross peaks are seen are presented in the histogram in Figure 4. All the acyl chain cross peaks from triglyceride are present in the fibrosarcoma (intermediate grade and high grade), MFH and liposarcoma and lipoma but not so in the SCS tissues. Of the two fibromas analysed, Figure 2 *a*, (case k) lacked 'C' and 'D' from triglyceride, while the other had all the cross peaks from triglyceride (case l, spectrum not shown). As this fibroma (case l) and the lipoma (case m) did not have any identifiable alanine cross peak, relative values for identified cross peaks in these two spectra have not been given in Table 2.

Proton NMR studies on mouse fibrosarcoma cells[7] and human fibrosarcoma cells (communicated manuscript) had indicated that alterations in signals arising from NMR visible lipids and fucosylated antigens vary in intensity with increasing tumorigenicity of the cell lines. The present study was undertaken to determine whether the spectral characteristics are extendable to sarcoma tissues.

The advantages of the TOCSY over the PCOSY are in the number of cross peaks generated, the 'net' coherence transfer produced can be arranged to create pure absorption mode spectra with positive intensity peaks rather than differential coherence transfer which causes spectra with equal positive and negative intensities. More importantly, in TOCSY, the oscillatory exchange established proceeds through the entire coupling network, so that there can be net magnetization transfer from one spin to another even without direct coupling. Thus, there are more number of cross peaks in the TOCSY spectra from tissues relative to the PCOSY spectra from cells, and cross peaks from triglyceride-like BC and EF which connect protons more than three bonds apart are characteristic of a TOCSY spectrum[10].

An increase in volume of cross-peaks from triglyceride, amino acids, phospholipids is seen from benign to tumour tissue, of which increase from amino acids is not metabolically significant. Elevated levels of triglyceride signals have been reported in MRS studies of epithelial tissues and colorectal tissue[11]. It was thought that increased levels of triglyceride relate to the motility/migratory and hence metastatic capacity of cells. If the total volume of cross peaks from fatty acyl chains of triglyceride in the 2D spectra of soft tissue tumours were to be compared, our results show that between low grade and intermediate grade liposarcoma the increase in triglyceride is about 7.74 times (which is probably due to the type of cells from which these tumours arise,

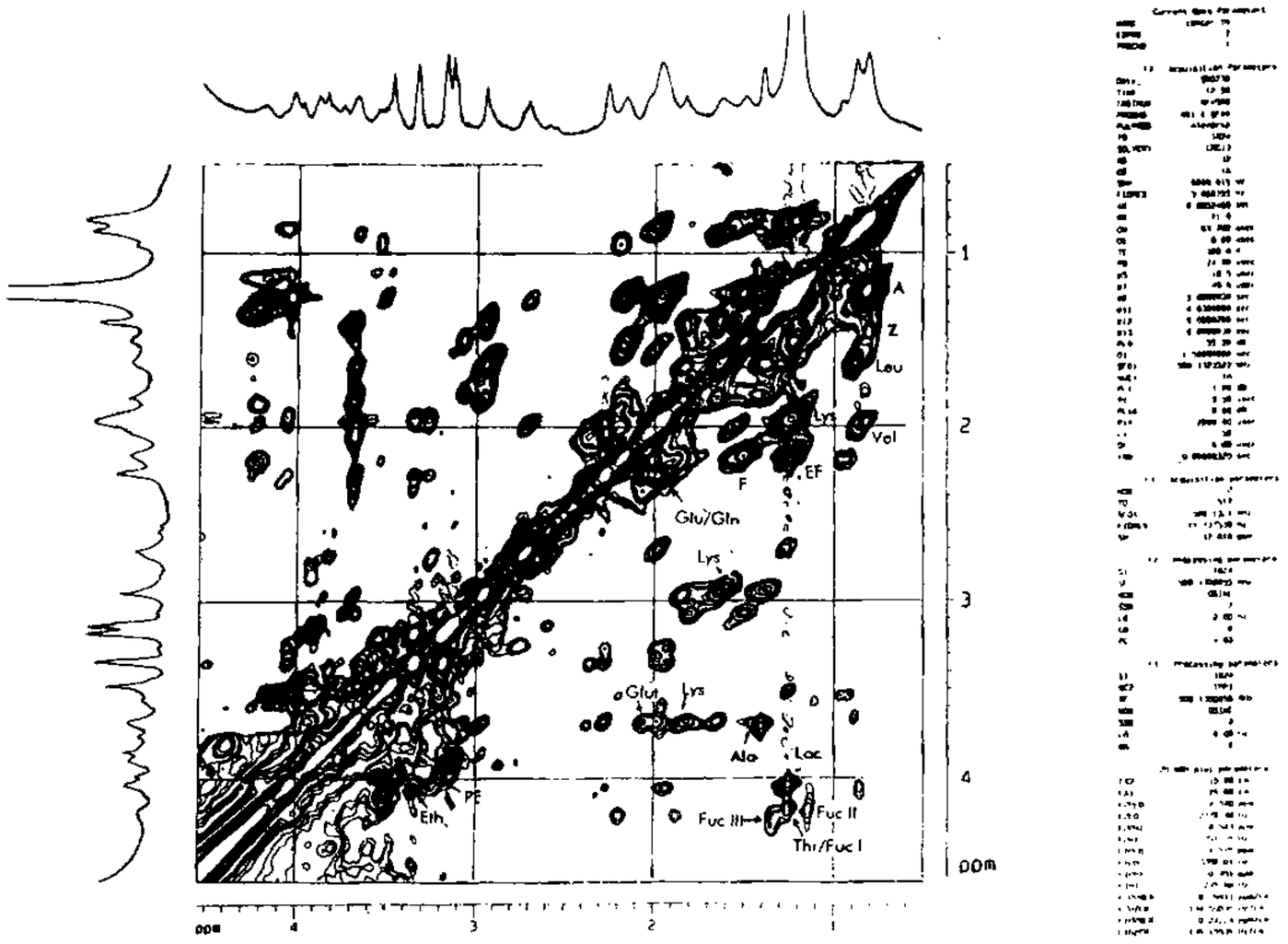

Figure 3. Expansion of the high-grade fibrosarcoma spectrum in Figure 2 *c*.

Table 2. Metabolite volumes calculated from the spectra of soft tissue tumours

Metabolite		Fibroma ($n = 2$)	Fibro sarcoma ($n = 1$ (ig)	Fibro sarcoma (hg) ($n = 1$)	Lipo sarcoma (lg) ($n = 1$)	Lipo sarcoma (ig)($n = 1$)	MFH ($n = 2$)	SCS ($n = 2$)	Leiomyo sarcoma (n = 2)
Triglyceride	A	2.22	5.67	8.09	5.47	115.3	3.237	5.8	4.1
	B	0.299	4.88	6.70	3.46	120.4	1.492	n.d.	1.2
	C	n.d	2.6	2.74	11.2	34.6	0.593	n.d	0.6
	D	n.d	1.23	6.42	13.4	10.47	0.360	n.d	0.4
	E	2.97	2.86	4.66	3.35	76.10	2.98	6.25	3
	F	0.395	2.02	2.54	13.33	31.74	0.677	n.d	0.8
Phospholipids									
Cho		n.d	0.89	1.34	n.d	1.91	0.830	5.3	0.790
Eth		n.d	0.1	2.63	n.d	6.13	1.58	8.37	1.7
PC		n.d	0.047	0.93	n.d	0.0477	1.106	8.33	1.2
GPC		n.d	0.102	0.33	n.d	0.910	1.19	4.26	1.3
PE		n.d	0.07	2.32	n.d	4.65	1.186	8.4	1.32
Fucose (as a block)		n.d	1.752	3.135	n.d	1.023	0.989	2.79	0.77
Lactate		13.12	3.29	1.86	n.d	15.8	4.64	2.7	3.5

These are relative metabolite intensities from the 2D spectra. Relative intensity of $x = \frac{(x \text{ cross peak volume})}{(\text{volume of ala cross peak})}$

MFH, malignant fibrous histiocytoma; SCS, spindle cell sarcoma; lg, low grade; ig, intermediate grade; hg, high grade; n.d not detected.
TOCSY spectra of the high grade fibrosarcoma and intermediate grade liposarcoma tissues were taken twice. The values for the lipoma (case m) and one of the fibroma (case I) could not be included in this table for want of the ala reference cross peak.

namely fat cells). Between intermediate and high-grade fibrosarcoma tissue the increase is 1.617 fold. The cross peaks from lipoma could not be compared for want of a suitable reference cross peak within the spectrum. In the 2D spectra of fibrosarcoma cells, cross peaks C and D from triglyceride could not be identified whereas the TOCSY spectra from fibrosarcoma tissue have all the cross peaks attributable to triglyceride. One reason for this could be the sensitivity of the TOCSY over the PCOSY technique, or it may be possible that these are contributions from the supporting matrix tissue.

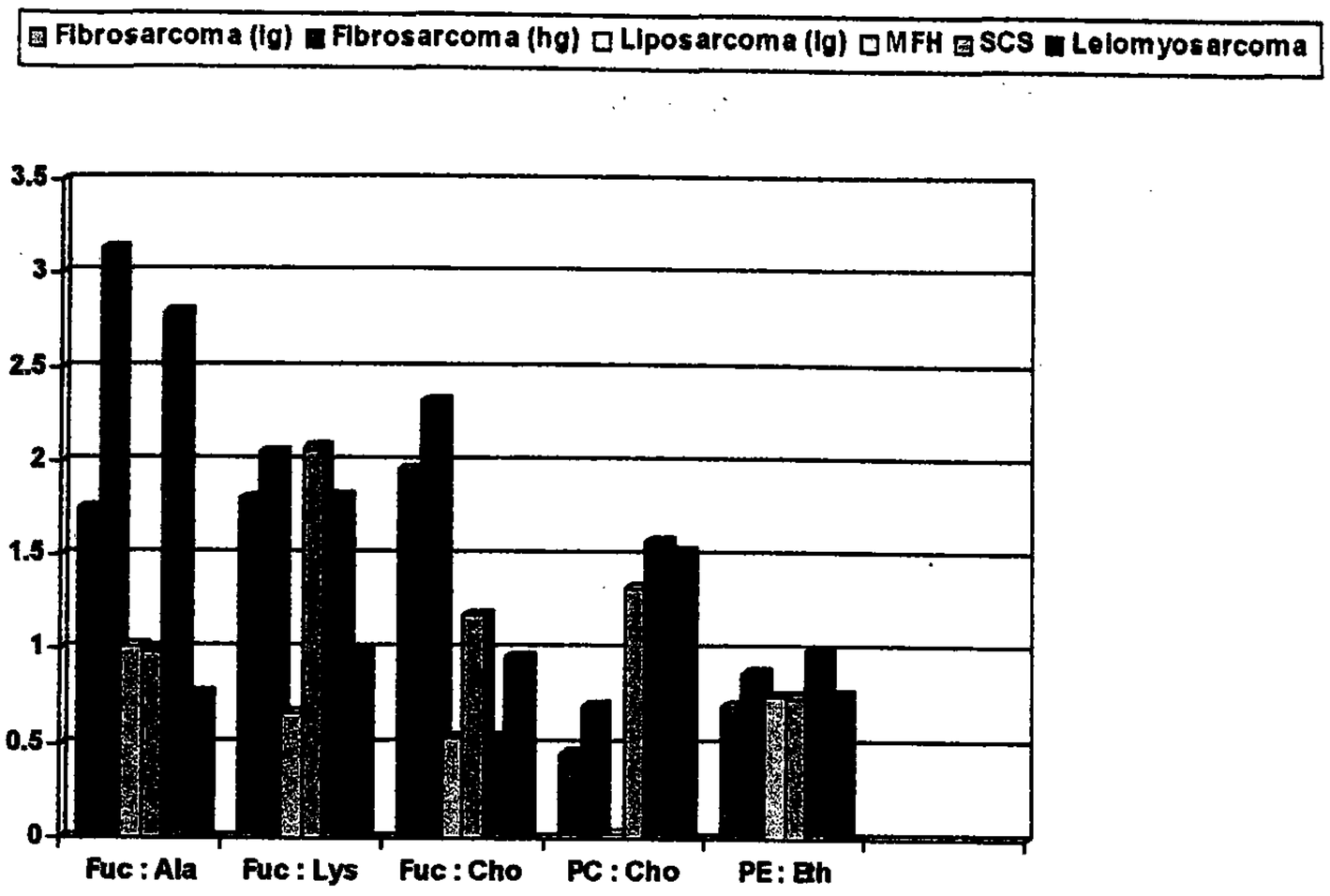

Figure 4. Cross peak volume ratios in human sarcoma tissues for fucose : alanine, fucose : lysine, fucose : choline, phosphocholine : choline and phosphoethanolamine : ethanolamine are presented. The tissues for which this data is available include the intermediate and high-grade fibrosarcomas, intermediate grade liposarcoma, malignant fibrous histiocytoma, spindle cell sarcomas and leiomyosarcomas. ig, intermediate grade; hg, high grade; MFH, malignant fibrous histocytoma, SCS, spindle cell sarcoma.

The presence of phospholipid pathway metabolite cross peaks only in tumour tissue and not in the fibroma/lipoma shows that an increase in these metabolites is specific of tumour metabolism. Spectra from higher-grade tissues (nuclear grade I and nuclear grade II) have increased levels of choline, ethanolamine, PC, PE, and GPC relative to lower grade tissue (nuclear grade III) as can be seen from Table 2. The elevated levels of ethanolamine and PE in the tissue spectra relative to what has been seen in cells can be explained by the fact that the availability of ethanolamine *in vivo* is more. Moreover ethanolamine being an inhibitor of choline kinase, the levels of choline metabolites in tissues is lesser. This data falls in line with other studies where increased PC and decreased PE have been seen in cells and increased PE with decreased PC *in vivo*[12]. It is speculated that a sustained activation of specific phospholipases, together with the rephosphorylation of ethanolamine and choline could be responsible for the high levels of PC/PE in tumour cells and tumour tissue. This provides a long-term source of diacylglycerol and phosphatidic acid, which are second messengers for cellular proliferation[13]. The high grade MFH, intermediate grade SCS and leiomyosarcoma tissues are all characterized by high PC:Cho ratios (Figure 4) than fibrosarcoma and liposarcomas. SCS tissues also have a high fuc:ala ratio differentiating them from MFH and leiomyosarcoma. MFH with a high fuc:lys ratio differs from the leiomyosarcoma. Thus, the appearance of the spectrum is much influenced by the type of tissue and its grade.

In conclusion, the TOCSY spectra of malignant soft tissue sarcomas and benign fibroma and lipoma, allow the detection of triglycerides, amino acids, fucose moiety of tumour-associated antigens, phospholipid metabolites such as choline, PC and GPC. Based on the identification of the cross peaks and their intensity, a gradation could be achieved from benign to intermediate-grade and from intermediate-grade to malignant tumour tissue. The present findings differ from the earlier report on smooth muscle tumours[10] in that altered fucosylation has been found to relate with the grade of tumour. NMR observable altered fucosylation has so far been reported only in the spectra of human colorectal carcinomas. Notwithstanding this similarity, the sarcoma spectral pattern appears to be distinctly different from the adenocarcinoma spectra. This study was carried out to primarily evaluate if there are perceptible differences in the NMR spectra of tissues with histopathology. Only if there are obvious differences in *ex-vivo* spectra, would it become a starting ground for *in vivo* experimentation. Though *ex-vivo* studies are a starting point it is difficult (given tissue heterogeneity) to extrapolate them to the *in vivo* situation. Yet there are reports on *in vivo* spectroscopy being used to monitor therapy response in nude mice with osteosarcomas, by following changes in phosphocreatine and phosphomonoester (PC and PE) levels[14]. Extrapolation is promising and clearly requires comparative studies in tumour models in culture, tissues and experimental animals. The spectrum obtained from fibrosarcoma tissue resembled that ob-

tained from fibrosarcoma cells, meaning that despite the heterogenous nature of sarcoma tissue, the spectral pattern of the predominant cell type is maintained. Phospholipid metabolites, fucose block volume and triglyceride to a lesser extent, appear to reflect characteristics of the tissue, its grade and its type, thus functioning as 'NMR markers' in the study of sarcoma tissue.

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Receptor-C_k controls cholesterogenesis and DNA replication in HEP G2 cells

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The present study, addressed to understand the molecular link between LDL, cholesterogenesis and DNA replication in Hep G2 cell line, revealed that the inhibitory effect of LDL upon cellular DNA synthesis, cholesterol synthesis and apo B-specific LDL-receptor gene expression at the transcriptional level, was mediated entirely through the activation of Receptor-C_k and not through its conventional apo B-specific LDL-receptor pathway. Based upon these experiments, we propose that Receptor-C_k controls the cellular cholesterogenesis as well as DNA replication and hence, it may have a crucial role in hepatic pathophysiological process.

RECENT studies addressed to understand the paradoxical interaction of lipoproteins with human platelets revealed the existence of a unique and a novel cell surface 69 kDa glycoprotein designated as Receptor-C_k based upon its characteristic of having high affinity for cholesterol moiety in various types of lipoproteins as well as intrinsic tyrosine kinase activity responsible for intracellular signaling[1-3]. This Receptor-C_k was not only shown to be ubiquitously present in various human cells/organs but also was conspicuously and selectively absent in various types of leukemic cell lines as well as patients[3]. Further, Receptor-C_k-dependent signaling was also shown to regulate mevalonate pathway and apo B-specific LDL-receptor gene at the transcriptional level in human lymphocytes[4]. Since Hep G2 cell line also has the ability to express this Receptor-C_k (ref. 3) and apo B-specific LDL-receptor responsive to LDL, the present study was undertaken to understand the role of Receptor-C_k in the regulation of endogenous cholesterol synthesis, DNA synthesis and transcriptional expression of apo B-specific LDL-receptor gene in this cellular model.

Hep G2 cell line was obtained from the National Facility for Animal Tissue and Cell Culture, Pune, India. ^{3}H-thymidine and ^{3}H-water were purchased from Bhabha Atomic Research Center, Bombay. Nucleic acid kit was obtained from Boehringer Mannheim (Germany). Polyclonal monospecific antibody against Receptor-C_k (Ab-RC_k) was raised in our laboratory. Oligonucleotide probe for apo B-specific LDL-receptor gene was obtained from Molecular Medicine Unit, King's College London, UK.

Low density lipoprotein (LDL) fraction was obtained from the plasma of normal healthy human subjects by using the standard gradient ultracentrifugation method[5].

Receptor-C_k (69 kDa glycoprotein) was purified from human platelets to high purity by the method reported earlier[3] and rabbits were immunized with this preparation emulsified 1:1 with Freund's complete adjuvant given intraperitoneally. Two weeks later, the same amount of protein (25 μg) emulsified with Freund's incomplete adjuvant, was injected intraperitoneally. These rabbits then received weekly i.p. injection of the same preparation by booster dose 4 days before blood was drawn from the animals. The antibodies from serum were purified by precipitation with ammonium sulphate

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