INSTITUTE OF PHYSICS PUBLISHING PHYSICS IN MEDICINE AND BIOLOGY

Phys. Med. Biol. **48** (2003) N239–N246 PII: S0031-9155(03)63055-1

NOTE

Characterization of cirrhosis and hepatocellular carcinoma using low-angle x-ray scattering signatures of serum

Wael M Elshemey[1], Omar S Desouky[2], Mohammed S Mohammed[3], Anwar A Elsayed[1] and Motawa E El-houseini[3]

[1] Biophysics Department, Faculty of Science, Cairo University, Egypt
[2] Radiation Physics Department, National Center for Radiation Research and Technology, IAEA, Egypt
[3] Cancer Biology Department, National Cancer Institute, Cairo University, Egypt

E-mail: Biophysics20@yahoo.com

Received 5 May 2003
Published 12 August 2003
Online at stacks.iop.org/PMB/48/N239

Abstract

The diagnosis of hepatocellular carcinoma (HCC) usually occurs at late stages in the disease when there are few effective treatment options. The measurement of the concentration of tumour markers in the serum of patients is a complementary tool frequently used for the interpretation of diagnostic imaging results. It is also used as a prognostic tool for the detection of cancer. Unfortunately, the sensitivity of tumour markers is still low and many times it yields normal results for cirrhotic and HCC patients. In the current work, the detection possibility of the structural changes in serum proteins accompanying cirrhosis and HCC is investigated using a low-angle x-ray scattering (LAXS) technique. The results show that there are significant differences in the LAXS profiles of cirrhosis and HCC lyophilized serum samples compared to normal. The changes in shape, total counts and position of the first scattering peak at 4.8°, which was previously reported to be sensitive to the structural changes in protein, showed the most characteristic deviations from normal serum. The present results are promising and would offer a potentially helpful complementary tool for monitoring cirrhosis and HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is an extremely prevalent malignancy that is the fourth most common worldwide. It is an important contributor to the mortality of those with chronic liver diseases in both developing and developed countries (Aziz and Wu 2002). Early detection of cancer offers the best chance for cure; this is why screening for HCC is recommended on a

regular basis. Screening may involve blood tests to detect the level of tumour markers in serum and radiological tests including CT, MRI and ultrasound imaging. Unfortunately, most known tumour markers (e.g. alpha-fetoprotein) are neither specific for a single individual tumour nor sensitive enough for screening (Burtis and Ashwood 2001).

The development of HCC in the liver is associated with genetic changes, which are translated and transcribed into proteins exhibiting phenotypic changes (McCance and Huether 1994). Produced in liver, structural changes in the serum proteins of HCC patients were also identified (Yamashita *et al* 1993, Johnson *et al* 2000).

The present work is an attempt to detect the structural changes in serum proteins accompanying HCC. The proposed technique, low-angle x-ray scattering (LAXS), has previously shown remarkable sensitivity towards structural changes in lyophilized human serum. Furthermore, a scattering peak was found specific to the induced changes in the structure of serum proteins (Elshemey *et al* 2001, Desouky *et al* 2001). The sensitivity of LAXS towards the molecular structure of biological samples was utilized by Kidane *et al* (1999) to offer characteristic signatures for normal and neoplastic breast tissues and the development of enhanced imaging techniques (Chapman *et al* 1997, Leclair and Johns 1999, 2001). It was also used in the characterization of tissues (Evans *et al* 1991, Elshemey *et al* 1999) and even for the detection of irradiated spices (Desouky *et al* 2002).

The sensitivity of LAXS towards the molecular structure of biological samples is attributed to the nature of coherent scattering of low-energy x-rays from biological samples. At low scattering angles, x-ray photons coherently scattered from the molecules of the medium interfere together (molecular interference effect) resulting in a scattering distribution characterized by one or two distinct peaks (Kosanetzky *et al* 1987).

In this work, in addition to the measurement of LAXS from the serum of normal individuals and HCC patients, LAXS from cirrhotic patients is also measured. In many cases cirrhosis is found to be a pre-malignant condition (Okuda and Tabor 1997) and it is thus important to investigate the possibility of its characterization using the current technique. A number of parameters measured from the scattering profiles of different samples are presented in a table for the purpose of characterization.

2. Materials and methods

2.1. Collection and preparation of samples

Blood samples were collected from patients at the National Cancer Institute (NCI) of Cairo for a period of one year. This allowed the collection of 51 samples, among which nine samples were healthy individuals (five males and four females), 13 samples were diagnosed as cirrhotic patients (seven males and six females) and 29 as HCC patients (24 males and five females). The sex ratio of the randomly collected HCC patient's samples is not far from the international incidence ratio of 4:1 male to female (Carr *et al* 1997). The distribution of the age of the collected samples for the three groups is presented in figure 1. One can notice that the number of HCC samples for patients below 40 years of age is very small and starts to increase for samples of patients above 40 years up to 69 years of age. This distribution is a fine representation of the universal age distribution of the disease (Okuda and Tabor 1997). Among the 29 HCC patients, seven were classified as grade I, 18 as grade II and four as grade III.

Samples were collected using the venipuncture technique in glass test tubes and were left to clot for a period of 30 min at 37 °C. Samples were then centrifuged at 3000 rpm for 10 min. The supernatant serum was collected and stored at −80 °C (Wayne 1991, Burtis and Ashwood

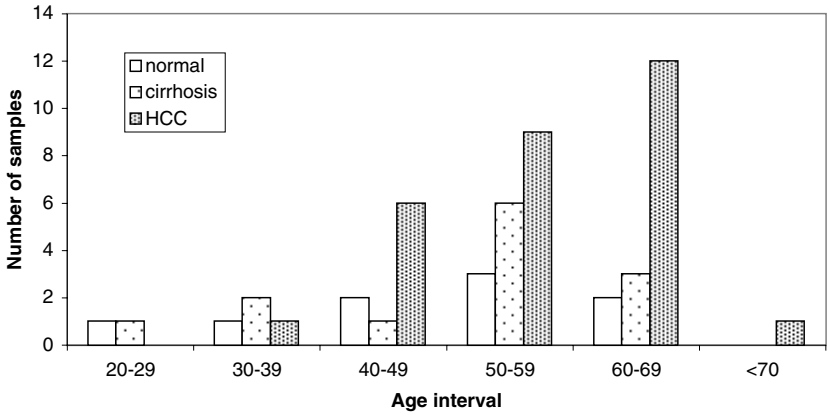

Figure 1. Age distribution for normal, cirrhosis and HCC samples used in this study.

1999). It was previously reported that the storage of a biological sample at −80 °C for a long period of time does not affect its scattering signature (Kidane *et al* 1999). After collection, samples were lyophilized at −50 °C (6.4 mbar) for a period of 6 h using a freeze dryer (Edwards, UK) and kept in dry, well-sealed polystyrene tubes at −80 °C. Before measurements, samples were left to warm up to room temperature.

2.2. X-ray scattering measurements

A Shimadzu powder x-ray diffractometer working in reflection geometry is used in the present work. The device, working at 40 kV and 30 mA, uses a Cu target to produce an 8.047 keV, highly collimated x-ray beam. Scattering angles from 2° up to 30° are scanned in steps of 0.25°. Powder samples of lyophilized serum were smeared on a rough glass slide mounted vertically on a rotating sample holder. Rotation was in a (θ–2θ) mode. Scattering data are collected using a scintillation detector employing a sodium iodide crystal, graphite monochromator and interfaced to the computer.

2.3. Calculation and statistical analysis of the characterization parameters

A number of characterization parameters are calculated from the measured LAXS profiles and presented in table 1. The way these parameters are calculated is illustrated in figure 2. The $FWHM_1$ and $FWHM_2$ parameters are the full width at half maximum in degrees of the first and second scattering peaks at 4.8° and 10.5°, respectively. A base line is first plotted for each peak and the FWHM is calculated as shown in figure 2. The values I_1 and I_2 represent the maximum amplitude of the first and second scattering peaks, respectively (figure 2). Figure 2 also illustrates the values A_1 and A_2, which represent the amplitudes of the rising and falling edges of peak 1, respectively.

The statistical analysis of the measured characterization parameters is carried out using the Statistical Package for the Social Sciences (SPSS) version 7.5. Data were represented as mean ± standard error of the different parameters (table 1). The mean values of the different parameters are compared using the analysis of variance (ANOVA) test which utilizes the individual values (used to calculate the mean) of each characterization parameter. When significant, the ANOVA test is followed by Duncan's multiple range test in order to point out the significant differences between the three investigated groups.

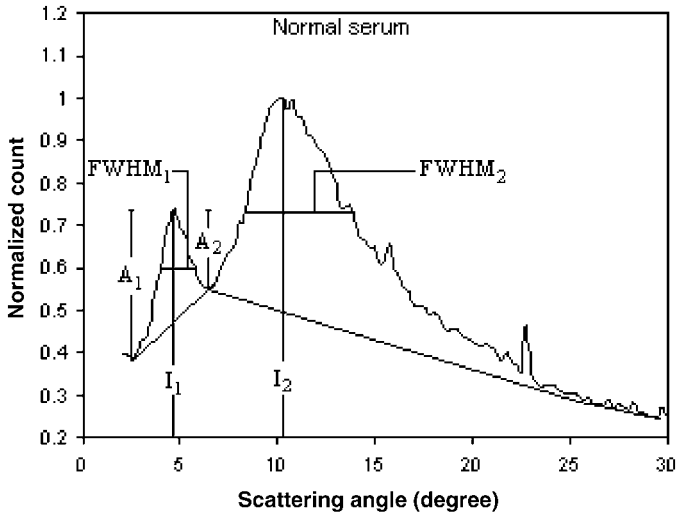

Figure 2. Calculation of the different characterization parameters from the scattering profile of normal serum.

Table 1. Mean values of the measured characterization parameters for low-angle x-ray scattering from normal, cirrhotic and HCC serum samples.

	Normal serum ($n = 9$)	Cirrhotic serum ($n = 13$)	HCC serum ($n = 29$)	F-ratio	P-value
$FWHM_1$ (deg)	1.96 ± 0.13^{a}	1.95 ± 0.22^{a}	2.19 ± 0.20^{b}	4.907	0.0120
$FWHM_2$ (deg)	5.22 ± 0.14	5.24 ± 0.23	5.39 ± 0.28	1.902	0.1600[c]
Peak position 1 (deg)	4.78 ± 0.15^{a}	4.93 ± 0.22^{b}	5.08 ± 0.16^{b}	8.515	0.0005
Peak position 2 (deg)	$10.53 \pm 0.16^{a,b}$	10.45 ± 0.22^{a}	10.62 ± 0.13^{b}	3.878	0.0270
$I_1/I_2\%$	55.14 ± 2.32^{a}	$54.20 \pm 1.42^{a,b}$	53.10 ± 1.74^{b}	3.324	0.0440
$A_2/A_1\%$	45.64 ± 5.80^{a}	38.20 ± 3.82^{b}	33.80 ± 3.81^{b}	11.411	0.0001
Counts under peak 1	7.12 ± 0.22^{a}	6.99 ± 0.16^{a}	6.62 ± 0.19^{b}	20.112	0.0001

[a] Statistically classified group a.
[b] Statistically classified group b which is significantly different to group a.
[c] NS: non-significant.

3. Results and discussion

The average LAXS profiles for normal, cirrhotic and HCC samples are presented in figure 3. All graphs are normalized to unity at the second peak of scattering at 10.5° and a maximum of three-point average is used in plotting each graph. In addition to the presence of two relatively broad scattering peaks, figure 3 shows a number of sharp diffraction peaks which were previously reported to be due to the NaCl crystals present in serum (Desouky *et al* 2001).The differences in the amplitudes of these peaks are most probably due to the variations in the concentration of NaCl crystals in the serum of different individuals. At first glance, one can point out considerable differences in the characteristics of the first scattering peak at 4.8° for the three groups. Table 1 shows that the full width at half maximum of peak 1 ($FWHM_1$) for the HCC group is significantly greater than that for the normal and cirrhotic groups. The difference between the normal and cirrhotic groups is non-significant (table 1). There is a significant shift in the position of the first scattering peak from 4.78° for the normal group,

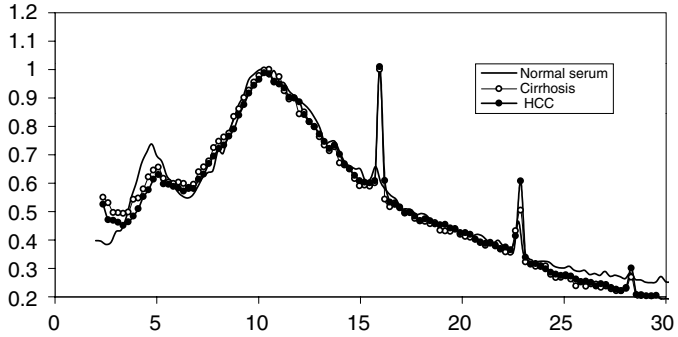

Figure 3. Average LAXS profiles for normal, cirrhosis and HCC lyophilized serum samples.

to 4.93° for the cirrhotic group, up to 5.08° for the HCC group. The difference between the cirrhotic and HCC groups is non-significant (table 1). The ratio of the amplitudes of the first peak of scattering to the second peak of scattering $I_1/I_2\%$ shows a slight decrease in its value in cirrhotic and HCC groups compared to normal. A significant difference only exists between the normal and cirrhotic groups (table 1). The ratio $A_2/A_1\%$ represents the ratio of the heights of the rising edge (A_1) to the falling edge (A_2) of peak 1. This ratio is introduced in order to account for the distortion in the shape of the first scattering peak for cirrhotic and HCC groups compared to normal. This peak is, many times, characterized by the fusion of the falling edge of the first scattering peak into the second scattering peak resulting in a decrease in the height of A_2 relative to A_1. Despite the relatively high standard error, the ratio $A_2/A_1\%$ shows significant differences between the normal and HCC groups. The value of the ratio $A_2/A_1\%$ for the cirrhotic group falls in between the normal and HCC groups and is significantly different from the normal group (table 1). Figures 4(a)–(d) illustrate the differences in the shape of the first scattering peak for individual samples of HCC compared to normal. It shows that all HCC profiles have a distorted first scattering peak, yet the shape of the distortion may vary between individual HCC samples. A common feature is that they all show a decrease in the area under peak 1 compared to normal. This is well represented by the significant decrease in the 'counts under peak 1' parameter in table 1 for the HCC group compared to normal. Figures 4(e)–(f) present the LAXS profiles of two different cirrhotic samples showing the differences in the shape of peak 1 of the cirrhotic samples compared to normal. The 'counts under peak 1' parameter in table 1 also shows significant difference between the cirrhotic and the HCC groups. The value of this parameter for the cirrhotic group, like almost all other parameters of the cirrhotic group, lies in between the values for normal and HCC groups.

To summarize the previous results, a total of six characterization parameters yielded significant differences between two of the three groups. One parameter ($FWHM_2$) showed non-significant variations among the three groups (table 1). Five parameters showed significant differences between the normal and HCC groups ($FWHM_1$, peak position 1, $I_1/I_2\%$, $A_1/A_2\%$ and counts under peak 1). Two parameters yielded significant difference between the normal and cirrhotic groups (peak position 1 and $A_2/A_1\%$) and three parameters showed significant differences between cirrhotic and HCC groups ($FWHM_1$, peak position 2 and counts under peak 1).

The discussed differences in the characterization parameters of the first peak of scattering for the three investigated groups can be attributed to the reported sensitivity of this peak towards the structural alterations in protein. Elshemey *et al* (2001) showed that irradiation of

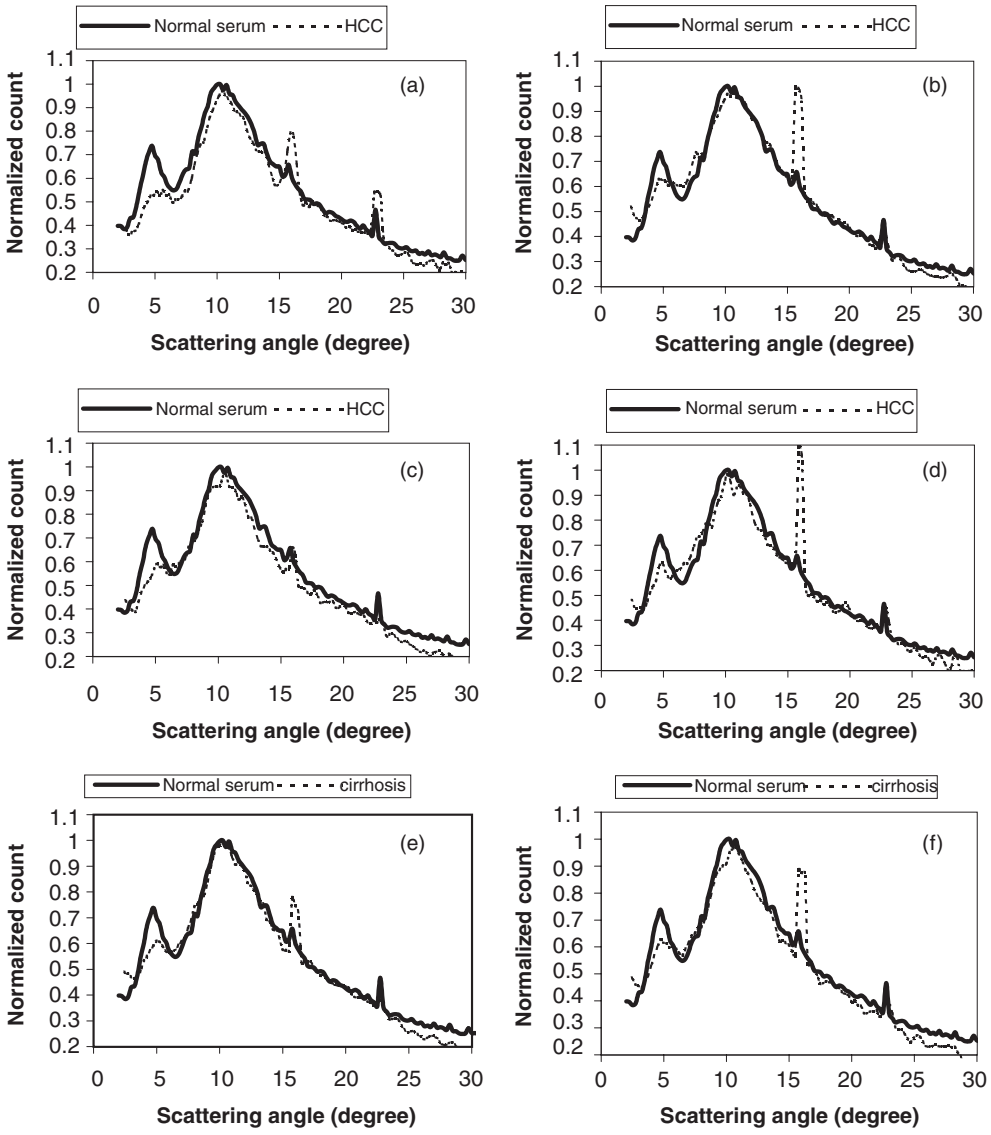

Figure 4. (a)–(d) LAXS profiles for individual lyophilized serum samples of HCC compared to average normal. (e)–(f) LAXS profiles for individual lyophilized serum samples of cirrhosis compared to average normal.

serum results in a distorted first peak of scattering. This was further reinforced by the work of Desouky *et al* (2001) which shows that the first peak of scattering is present in almost all biological samples containing protein and is absent otherwise. It also shows that the alteration of the tertiary structure of protein results in the distortion of the first peak of scattering. Since all serum proteins are produced in the liver, the structural alterations in the produced proteins in the case of HCC (Yamashita *et al* 1993, Johnson *et al* 2000) due to the associated genetic changes (McCance and Huether 1994) will be expressed in serum.

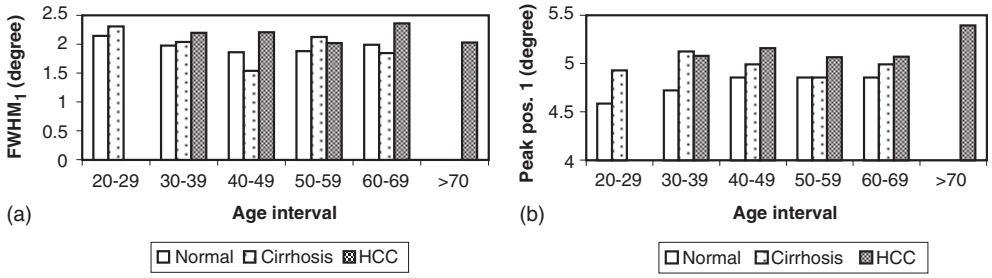

Figure 5. The variation with age of the $FWHM_1$ (a) and peak position 1 (b) parameters for normal, cirrhotic and HCC samples.

It should be mentioned that the scattering profiles of different stages of HCC were quite similar and that it was not possible in this study to further characterize the individual stages of HCC using LAXS.

In order to rule out the possibility that any of the observed differences in the measured characterization parameters are due to the difference in the age distribution of the three groups (normal, cirrhosis and HCC), the variation with age of the different parameters is investigated. The results show that there is no special trend with age in any of the characterization parameters for the three groups. Figures 5(a) and (b) show the variation with age for the $FWHM_1$ and peak position 1 parameters.

An important observation about the measured characterization parameters is that the cirrhotic group acquired values which are always midway between normal and HCC groups (table 1). This remark would be of interest in the prognosis of HCC, knowing that in 50% of HCC, cirrhosis was reported as a pre-malignant condition regardless of the etiology (Aziz and Wu 2002).

4. Conclusion

It was possible to provide characteristic LAXS profiles for the lyophilized serum samples of normal, cirrhotic and HCC groups. The observed differences were attributed to the structural changes in serum proteins accompanying cirrhosis and HCC. The first peak of scattering provided a number of useful characterization parameters for such purpose. The characterization parameters for cirrhosis looked as if they reflect an intermediate step for the transformation from normal to malignant state. This would suggest a possible application of the current technique in the screening of HCC in order to detect the malignancy in an early and treatable stage.

References

Aziz K and Wu G Y 2002 *Cancer Screening: A Practical Guide for Physician* (Totowa, NJ: Humana) pp 111–28

Burtis C A and Ashwood E R 1999 *Tietz Textbook of Clinical Chemistry* 3rd edn (Philadelphia, PA: Saunders) pp 42–72

Burtis C A and Ashwood E R 2001 *Tietz Fundamentals of Clinical Chemistry* 5th edn (Philadelphia, PA: Saunders) pp 390–413

Carr B I, Flickinger J C and Lotze M T 1997 Cancer of the liver *Cancer Principles and Practice of Oncology* 5th edn ed V T De Vita, S Hellman and S A Rosenberg (Philadelphia, PA: Lippincott Raven) pp 1087–114

Chapman D, Thomlinson W, Johnston R E, Washburn D, Pisano E, Gmur N, Zhong Z, Menk R, Arfelli F and Sayers D 1997 Diffraction enhanced x-ray imaging *Phys. Med. Biol.* **42** 2015–25

Desouky O S, Ashour A H, Abdullah M I and Elshemey W M 2002 Low-angle x-ray scattering from spices *Radiat. Phys. Chem.* **64** 267–71

Desouky O S, Elshemey W M, Selim N S and Ashour A H 2001 Analysis of low-angle x-ray scattering peaks from lyophilized biological samples *Phys. Med. Biol.* **46** 2099–106

Elshemey W M, Desouky O S and Ashour A H 2001 Low-angle x-ray scattering from lyophilized blood constituents *Phys. Med. Biol* **46** 531–9

Elshemey W M, Elsayed A A and El-Lakkani A 1999 Characteristics of low-angle x-ray scattering from some biological samples *Phys. Med. Biol.* **44** 2907–15

Evans S H, Bradley D A, Dance D R, Bateman J E and Jones C H 1991 Measurement of small-angle photon scattering for some breast tissues and tissue substitute materials *Phys. Med. Biol* **36** 7–18

Johnson P J, Poon T C, Hjelm N M, Ho C S, Blake C and Ho S K 2000 Structures of disease specific serum alpha fetoprotein isoforms *Br. J. Cancer* **83** 1330–7

Kidane G, Speller R D, Royle G J and Hanby A M 1999 X-ray scatter signatures from normal and neoplastic breast tissues *Phys. Med. Biol* **44** 1791–802

Kosanetzky J, Knoerr B, Harding G and Neitzel U 1987 X-ray diffraction measurement of some plastic materials and body tissues *Med. Phys.* **14** 526–32

Leclair R J and Johns P C 1999 Analysis of spectral blur effects in x-ray scatter imaging *Med. Phys.* **26** 1811–6

Leclair R J and Johns P C 2001 X-ray forward-scatter imaging: experimental validation of model *Med. Phys.* **28** 210–9

McCance K L and Huether S E 1994 *Pathophysiology: The Biological Basis for Disease in Adults and Children* (Saint Louis, MO: Mosby) pp 334–56

Okuda K and Tabor E 1997 *Liver Cancer* (Pearson Professional Limited: New York) pp 13–29

Wayne P A 1991 *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture Approved Standard H3-A3* 3rd edn (National Committee for Clinical Laboratory Standards)

Yamashita K, Taketa K, Nishi S, Fukushima K and Ohkura T 1993 Sugar chains of human cord serum alpha fetoprotein characteristics of N-linked sugar chains of glycoproteins produced in human liver and hepatocellular carcinoma *Cancer Res.* **53** 2970–5