Pergamon

0731-7085(94)E0026-W

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 1, pp. 65–71, 1995
Elsevier Science Ltd
Printed in Great Britain
0731-7085/95 $9.50 + 0.00

HDL subfractions as altered in cancer patients

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Abstract: Previous studies from the authors' laboratories have shown that cancer patients are characterized by lower levels of high-density lipoprotein cholesterol (HDL–C) compared with those of normal subjects. HDLs are a complex class of lipoproteins which can be divided mainly into two categories, HDL_2 and HDL_3, that have not only different lipid and protein composition but also different functions. Therefore, for a better understanding of the metabolism of HDL during tumour growth, the different subfractions of HDL (HDL_2 and HDL_3) were analysed in the serum of neoplastic patients using a rapid and simple high-performance liquid chromatography (HPLC) method for the analysis. The results obtained showed that serum from neoplastic patients exhibits a peculiar pattern in the distribution of HDL subfractions, consisting of a sharp decrease in HDL_3 and a consequent increase of the normal HDL_2/HDL_3 ratio. It is suggested that evaluation of the HDL subfractions may be of clinical relevance for cancer status and that due to its simplicity, short analytical time and small sample volume required, the HPLC technique used in this study can be easily applied to routine analysis in cancer patients.

Keywords: *HPLC; gel filtration; lipoproteins; gastrointestinal tumours; lung tumours; haematological neoplasms.*

Introduction

During the last few decades, cholesterol metabolism and its production, transport and distribution in the body has been extensively studied mainly in relation to the atherosclerotic process. More recently, however, the role of cholesterol metabolism has also been questioned in cancer, not only from a scientific and experimental point of view but also for its medical importance in human tumour pathology [1–3].

An inverse correlation between serum cholesterol and cancer incidence and mortality has been reported by several epidemiological studies [4–6]. The results of these studies, however, were sometimes conflicting; only a few studies [3, 7] were related to the distribution of the different lipoproteins in cancer patients. Cholesterol metabolism has been previously studied in the authors' laboratory in different experimental models of neoplastic cell proliferation [8, 9] as well as in different types of human neoplasms [10–12]. A constant finding of these studies was that tumour growth is characterized by specific patterns of cholesterol metabolism, namely an increase in cholesterol content in growing tissues, and a corresponding reduction of high density lipoprotein cholesterol (HDL–C) in the plasma compartment. The authors suggested that the reduced levels of serum HDL–C may be a consequence of the cancer process, probably mediated by the utilization of cholesterol for new membrane biogenesis and by the accumulation of esterified cholesterol in tumour tissues. From the results, however, the possibility cannot be excluded that low HDL concentrations *per se* may be responsible for the intracellular alterations of cholesterol metabolism observed during tumour growth.

HDLs represent a class of lipoproteins involved in the transport of the excess of cholesterol from the tissues to the liver for excretion (reverse cholesterol transport). In humans HDLs form a heterogeneous group which can be divided into subfractions, mainly HDL_2 and HDL_3, with different lipid and protein composition [13]. Therefore, for a better understanding of the mechanisms by which HDLs are reduced during cell proliferation, it seemed interesting to study the possible variations of HDL subfractions in patients with different types of neoplasms.

Several methods for the separation of HDL_2 and HDL_3 subfractions have been described,

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mostly based on density-gradient ultracentrifugation [14].

Density-gradient ultracentrifugation enabled HDL subfractions to be resolved and quantified satisfactorily, but the cost of the equipment, the time needed, and the large sample required for the analysis precluded its use in routine clinical practice. In 1980, Okazaki *et al.* [15] developed a simple and rapid technique for the separation of lipoproteins based on their molecular weight, using high-performance liquid chromatography (HPLC) with gel permeation columns (GPC). This method, that needed a preliminary 24-h ultracentrifugation for the separation of the total lipoprotein fraction, was useful for many clinical applications such as investigations of changes in patients with coronary heart disease and the diagnosis of the different types of hyperlipidaemia [16].

In the present study, HDL_2 and HDL_3 subfractions were separated by HPLC with GPC using the total HDL fraction obtained from sera of patients with different types of tumours and from sera of healthy subjects, for an appropriate comparison. The HDL fraction was obtained after precipitating the very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) with phosphotungstic acid and magnesium ions. The aim was to verify whether low HDL–C levels, previously observed in cancer patients, are associated with consistent changes in HDL subfractions.

Materials and Methods

Patients

A first series of studies was carried out on sera from 22 patients (aged 35–69 years) with two different types of solid tumours: 12 were male patients with newly diagnosed, untreated lung cancer and 10 were male patients with newly diagnosed untreated gastrointestinal cancer. A second series of studies was performed on sera from 41 patients (30 male and 11 female) (aged 22–71 years), with different types of haematological neoplasms: 11 were patients with chronic lymphocytic leukaemia (CLL), seven with non-Hodgkin's disease (NHD), six with multiple myeloma (MM), five with chronic myelogenous leukaemia (CML), four with acute lymphoblastic leukaemia (ALL) and eight with other types of haematological malignancies. Control samples were obtained from 24 volunteers (aged 30–50 years) who were not receiving prescription medication and had no known medical problems.

Blood samples were taken from all subjects after they had fasted for at least 12 h. At the time of sampling no subject was being treated with drugs affecting lipid metabolism. Serum was separated by centrifugation at room temperature (3000***g*** for 15 min) within 2 h, then stored at −40°C until the analysis, which in all cases was performed within 2 months.

For control of accuracy a special control serum for lipid and apoproteins prepared exclusively from blood donors tested individually was used (Precinorm L, Boehringer Mannheim, Germany).

Total cholesterol and HDL–C determinations

Total cholesterol concentrations in serum were determined using an enzymatic colorimetric method (CHOD–PAP method, Boehringer Mannheim, Germany).

The serum HDL–C values were determined after selective precipitation of serum LDL and VLDL with phosphotungstic acid and magnesium ions. Serum (0.2 ml) was mixed with 0.5 ml of a solution containing phosphotungstic acid (0.55 mM) and magnesium chloride (25 mM). After standing for 10 min at room temperature, the mixture was centrifuged for 10 min at 5000 rpm and at room temperature. After centrifugation, the supernatant containing the HDL fraction was separated within 2 h and the cholesterol content determined by the CHOD–PAP method. This procedure yielded a clear total HDL fraction contaminated by only small amounts of proteins [17].

Separation of HDL subfractions by HPLC

For the separation of HDL subfractions by HPLC the HDL fraction preisolated by precipitation with phosphotungstic acid and magnesium ions was used.

The analyses were carried out on a Series 4 Perkin–Elmer Liquid Chromatograph equipped with a LC-85B Perkin–Elmer variable wavelength UV detector and a II Series 3396 Hewlett–Packard integrator. One hundred and seventy-five microlitres of HDL fraction was injected and sodium chloride (pH 6.96; 0.15 M) was used as eluant. The columns were gel permeation chromatography (GPC) columns filled with microspheres of hydrophilic polymers with an aqueous support based

on a chemically modified silica (TSK GEL, type SW; Toyo Soda, Tokyo) suitable for separation of small size lipoproteins such as HDL [15, 16]. Each column was 600 × 7.5 mm i.d. For better resolution of HDL subfraction peaks, a combination of GPC columns (G4000SW + G3000SW × 2) was used. To increase the lifespan of the columns a guard column was inserted. The columns were balanced with HDL_2 and HDL_3 standard prepared by ultracentrifugation according to the method of Havel *et al.* [18].

The elution volume of each HDL subfraction peak, obtained using the supernatant of human sera after removal of other lipoproteins, is consistent with that of the reference standards. Proteins in HDL subfractions were monitored by absorbance at 280 nm.

To calculate the percentage distribution of HDL subfractions, areas under the scanning curves were integrated by dropping vertical lines corresponding to each subpopulation size limits. Each value was expressed as a percentage of the total area. The HDL_2/HDL_3 ratio was calculated from the relative areas of the corresponding fractions.

The statistical significance of the results was calculated using Student's *t*-test.

Results and Discussion

As reported in Table 1 total and HDL–C levels were determined in patients with solid tumours (lung and gastrointestinal tumours) as well as in patients with different types of haematological neoplasms. HDL–C was significantly reduced ($P < 0.01$) in both absolute and relative terms in the subjects, for all types of tumours. No significant changes in total cholesterol were observed (Table 1). These data are in agreement with those of previous studies in patients with the same types of tumours [10–12].

As previously pointed out, HDLs are a complex class of lipoproteins divided mainly into HDL_2 and HDL_3 subfractions having different lipid and protein composition and also different physiological roles. Numerous studies on the regulation of the HDL_2 and HDL_3 levels in human plasma indicated that HDL_3 may be the precursor of HDL_2. HDL_3 is converted in HDL_2 by enrichment of cholesterol derived from peripheral tissues and/or from the breakdown of triglyceride-rich lipoproteins (chylomicrons and VLDL) [13, 19]. Consequently the second step of the investigation was the separation and quantitative determination of HDL_2 and HDL_3.

Five typical profiles of HDL subfractions obtained from sera of normal subjects are shown in Fig. 1. HDLs were separated into two peaks: peak 1, that corresponds to the high molecular size fraction (HDL_2); and peak 2, that corresponds to the low molecular size fraction (HDL_3). Elution times of peak 1 and peak 2 were 42–44 and 47–49 min, respectively. Other serum proteins having a lower molecular weight than HDL subfractions were detected at approximately 54–58 min (peak 3). The levels of the different peaks were slightly different for the various subjects; however in all the samples, the peak corresponding to HDL_3 was greater than that of HDL_2 (Fig. 1, a–e). A similar pattern was observed using the standard serum Precinorm L (Fig. 1, f–g). These data on the pattern of HDL in normal subjects are in agreement with those obtained by other authors using ultracentrifugation. All reported data clearly indicated that HDL_3 was the main HDL subfraction in the serum of a healthy population [13, 14, 20].

As reported in Figs 2–5 remarkable variations of HDL subfractions were obtained with sera from patients with solid tumours and haematological malignancies. Observed vari-

Table 1
Total and HDL cholesterol levels in patients with solid tumours and haematological neoplasms

	TC* (mg dl^{-1})	HDL–C† (mg dl^{-1})	$\frac{\text{HDL–C}}{\text{TC}} \times 100$
Normal subjects ($n = 24$)	177.71 ± 5.43	47.12 ± 4.59	26.54 ± 2.67
Precinorm L ($n = 7$)	176.36 ± 6.89	35.63 ± 2.49	19.88 ± 1.62
Solid tumours ($n = 22$)	154.96 ± 7.21	12.07 ± 1.63‡	8.54 ± 1.13‡
Haematological neoplasms ($n = 41$)	189.32 ± 8.44	20.44 ± 1.79‡	10.93 ± 0.95‡

*Total cholesterol.
†High density lipoprotein cholesterol.
‡$P < 0.01$ vs normal subjects.

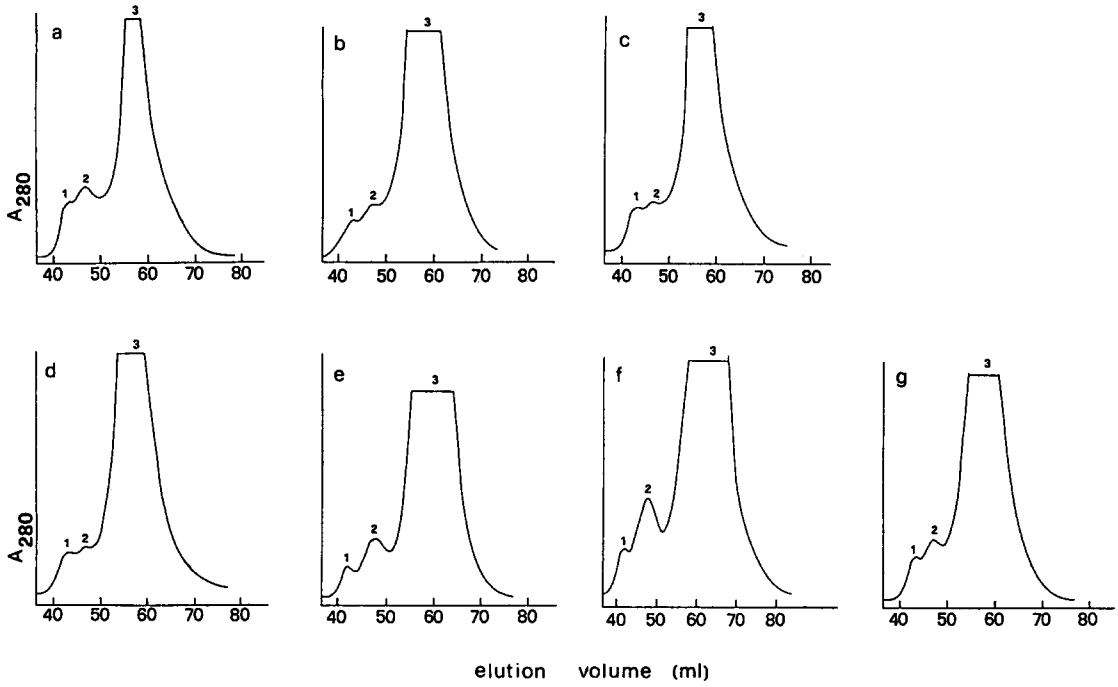

Figure 1
Elution pattern of HDL subfractions from normal subjects and from human standard serum (Precinorm L). Sample: a–e (normal subjects); f–g (Precinorm L). HPLC conditions are indicated under materials and methods. Peaks: 1, HDL_2; 2, HDL_3; 3, other protein fractions.

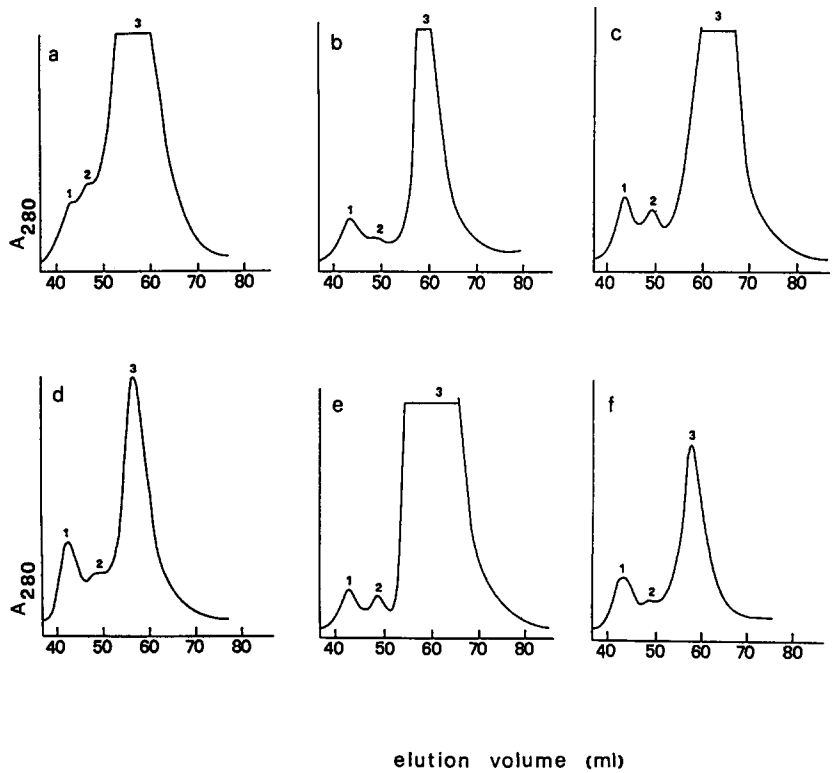

Figure 2
Elution patterns of HDL subfractions from human serum of patients with gastrointestinal cancer. Sample: a (reference obtained with normal subjects); b–f (patients with gastrointestinal cancer). HPLC conditions and peaks as in Fig. 1.

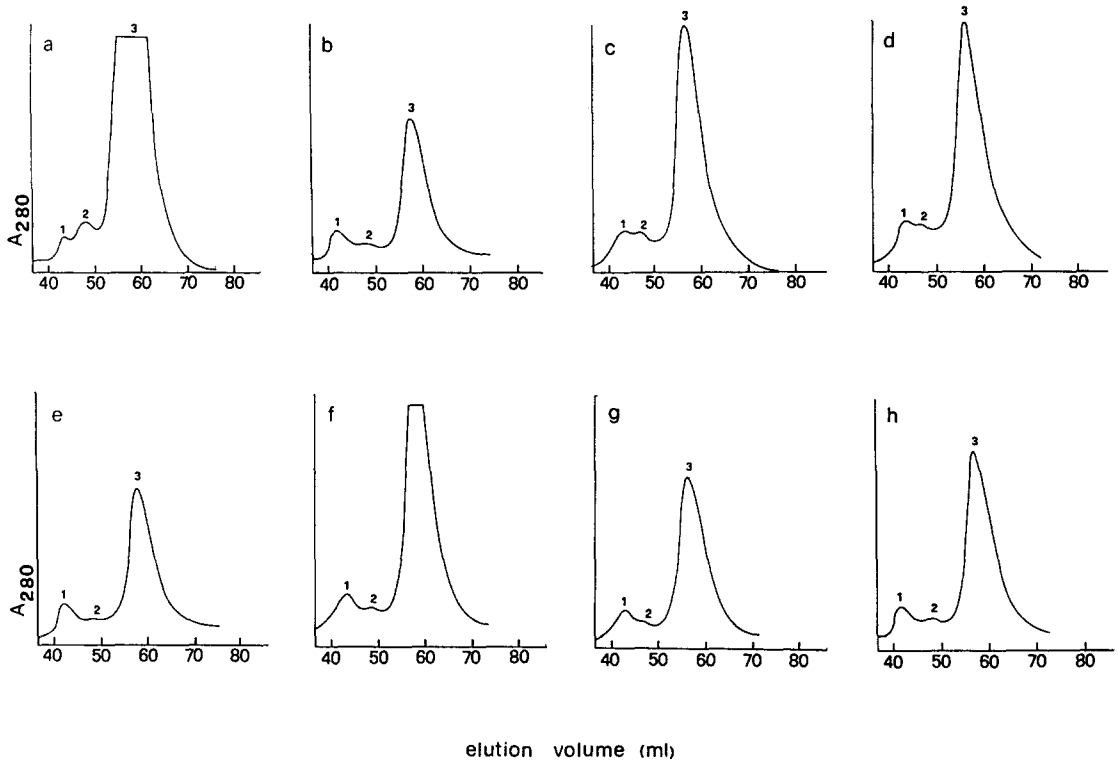

Figure 3
Elution pattern of HDL subfractions from human serum of patients with lung cancer. Sample: a (reference obtained with normal subjects); b–h (patients with lung tumours). HPLC conditions and peaks as in Fig. 1.

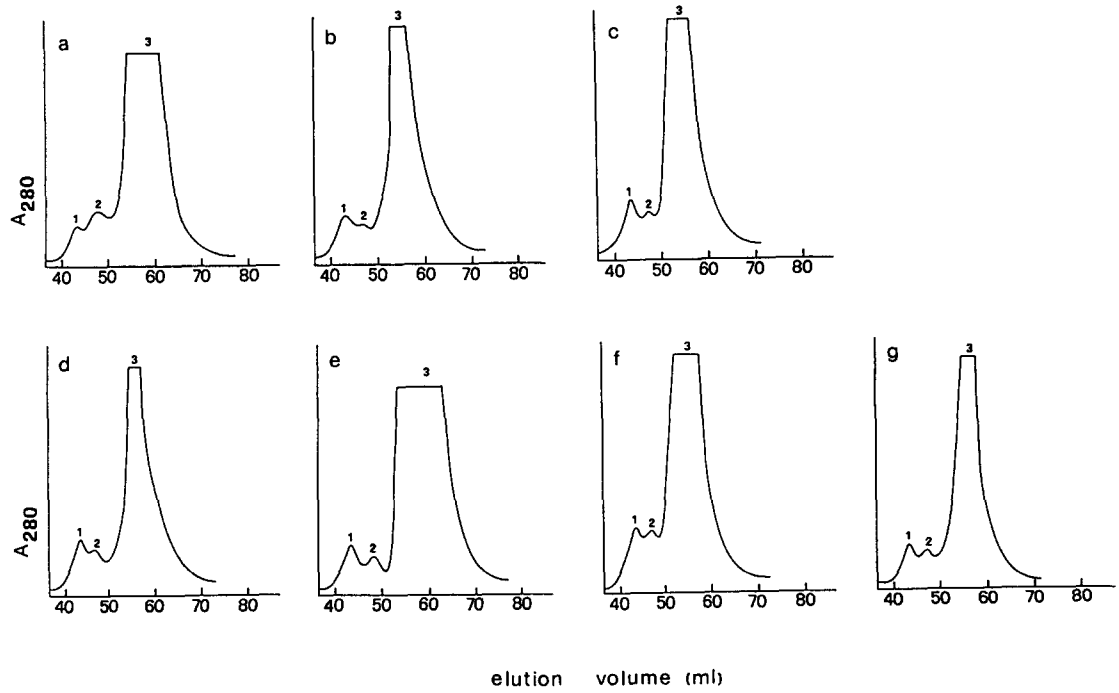

Figure 4
Elution pattern of HDL subfractions from human serum of male patients with different types of haematological neoplasms. Sample: a (reference obtained with normal male subjects); b, (ALL); c, (CLL); d, (CML); e, (MM); f, (Hodgkin's disease — HD); g, (NHL). HPLC conditions and peaks as in Fig. 1.

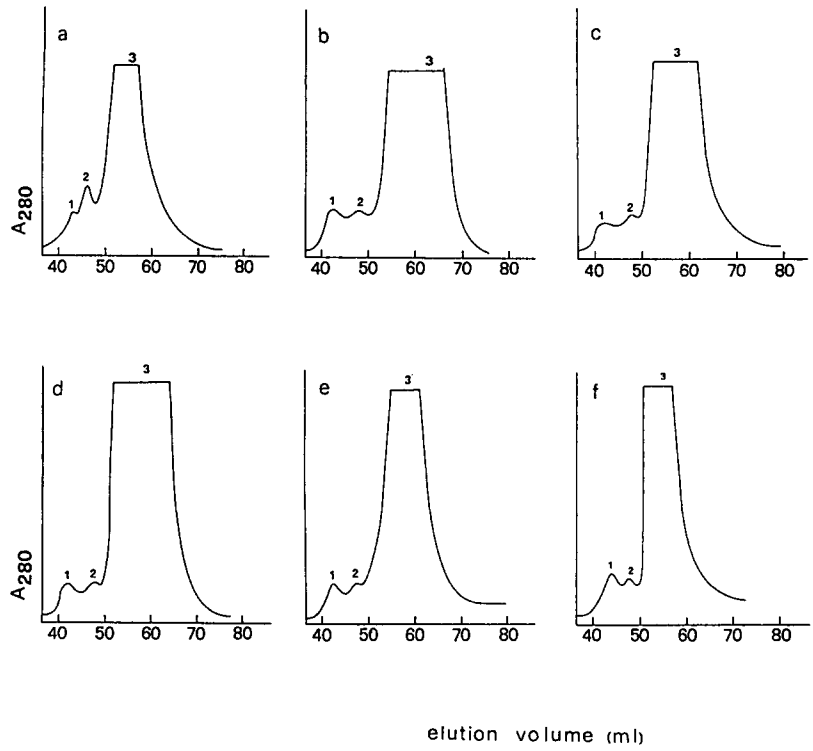

Figure 5
Elution pattern of HDL subfractions from human serum of female patients with different types of haematological neoplasms. Sample: a (reference obtained with normal female subjects); b, (ALL); c, (CLL); d, (CML); e, (MM); f, (acute non-lymphatic leukaemia — ANLL). HPLC conditions and peaks as in Fig. 1.

Table 2
HDL subfractions in patients with solid tumours and haematological neoplasms

	$\frac{HDL_2}{\text{Total area}} \times 100$	$\frac{HDL_2^*}{HDL_3^*}$
Normal subjects (n = 24)	29.55 ± 2.13	0.45 ± 0.04
Precinorm L (n = 7)	21.46 ± 2.89	0.30 ± 0.05
Solid tumours (n = 22)	59.82 ± 3.58†	2.00 ± 0.30†
Haematological neoplasms (n = 41)	49.96 ± 2.34†	1.21 ± 0.12†

* High density lipoprotein.
‡ P <0.01 vs normal subjects.

ations were mainly related to a sharp reduction of HDL_3, resulting in an inversion of the profile obtained with healthy subjects. The quantitation of HDL subfractions confirmed these results; a significant increase in the percentage of HDL_2 fraction as well as in the HDL_2/HDL_3 ratios was in fact observed in cancer patients when compared with a normal healthy population (Tables 1 and 2).

These results indicate that neoplastic patients may be characterized not only by a sharp decrease of HDL–C levels but also by an abnormal distribution of HDL_2 and HDL_3 in their HDL. No appreciable differences in these pattern variations were observed among patients with different types of tumours or between the sexes.

Conclusions

Although the data do not explain the exact mechanism at the basis of the changes in HDL metabolism in human cancer, they demonstrate that neoplastic patients exhibit a peculiar pattern in the distribution of HDL lipoproteins in the plasma compartment. Due to its simplicity, short analytical time and small sample volume required, the HPLC techniques

described in the present study can be easily applied in routine analysis as a possible clinical indicator for cancer status.

It remains to be established whether low HDL levels *per se* are a predisposing factor for human cancer development or whether they reflect a metabolic consequence of tumour growth. Studies are under investigation in order to evaluate HDL subfractions by HPLC in cancer patients, before any treatment and after remission of the disease following surgical or chemotherapy.

Acknowledgement — The authors wish to thank Mr Renato Mascia for technical assistance. This work was supported by grants from Centro Nazionale Ricerche, Associazione Italiana Ricerca Cancro, Ministero dell'Università e Ricerca Scientifica e Regione Autonoma della Sardegna.

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[*Received for review 10 February 1994*]