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(54) **METHOD OF ASSAYING LEUKOCYTE ELASTASE INHIBITOR FOR THE IN VITRO DIAGNOSIS OF COLORECTAL CANCER**

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None
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a method for the in vitro diagnosis of colorectal cancer by determining the presence of the Leukocyte Elastase Inhibitor tumor marker in a biological sample taken from a patient suspected of having colorectal cancer. Said method can be used for early diagnosis, screening, therapeutic follow-up and prognosis, and also for relapse diagnosis in relation to colorectal cancer.

15 Claims, 12 Drawing Sheets

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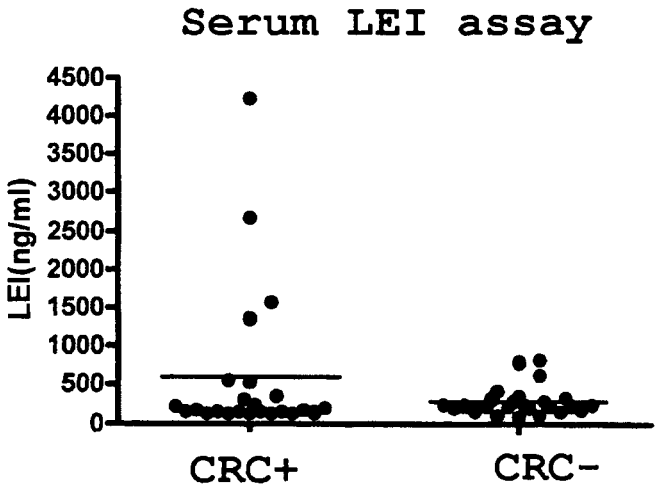


Figure 1

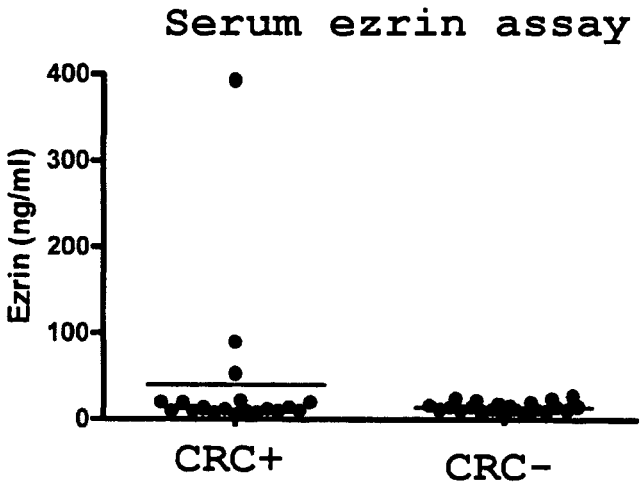


Figure 2

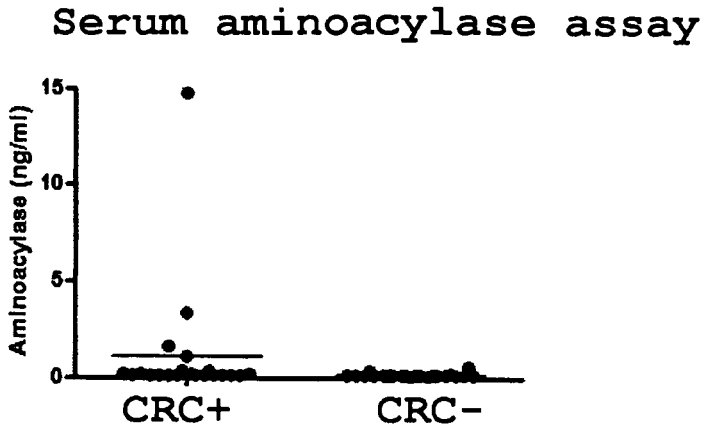


Figure 3

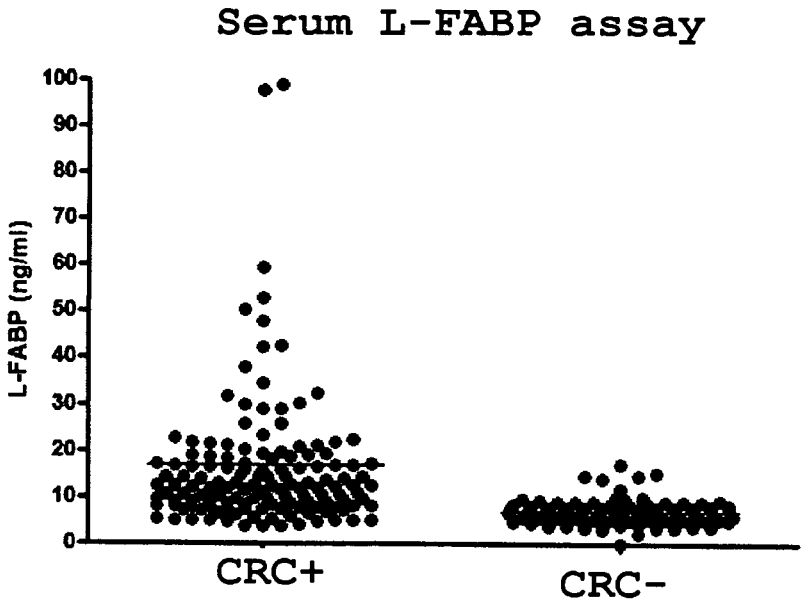


Figure 4

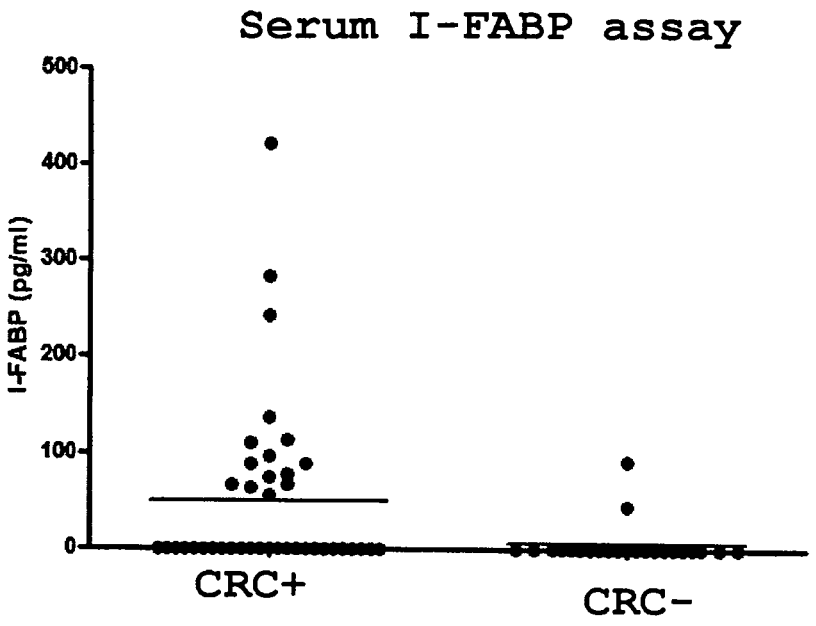
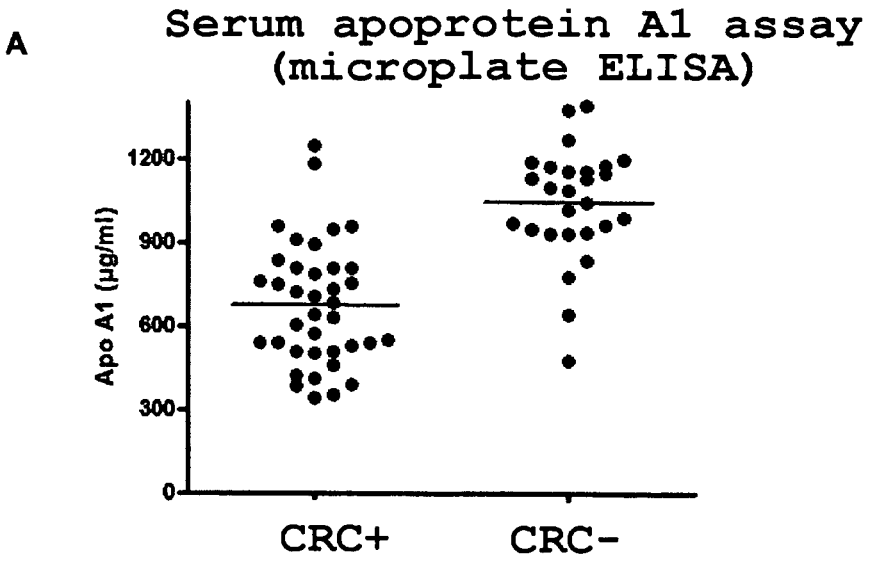


Figure 5


A
Serum apoprotein A1 assay
(microplate ELISA)
Apo A1 (µg/ml)
1200
900
600
300
0
CRC+
CRC-

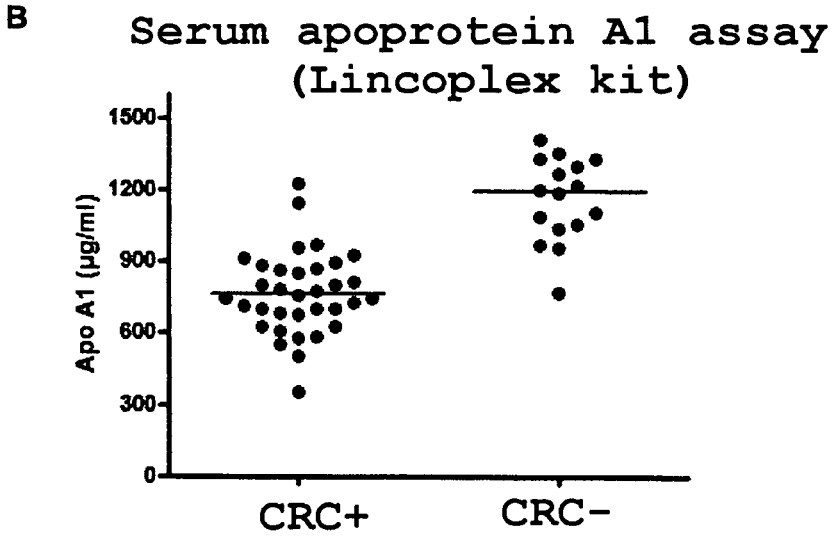

B
Serum apoprotein A1 assay
(Lincoplex kit)
Apo A1 (µg/ml)
1500
1200
900
600
300
0
CRC+
CRC-

Figure 6

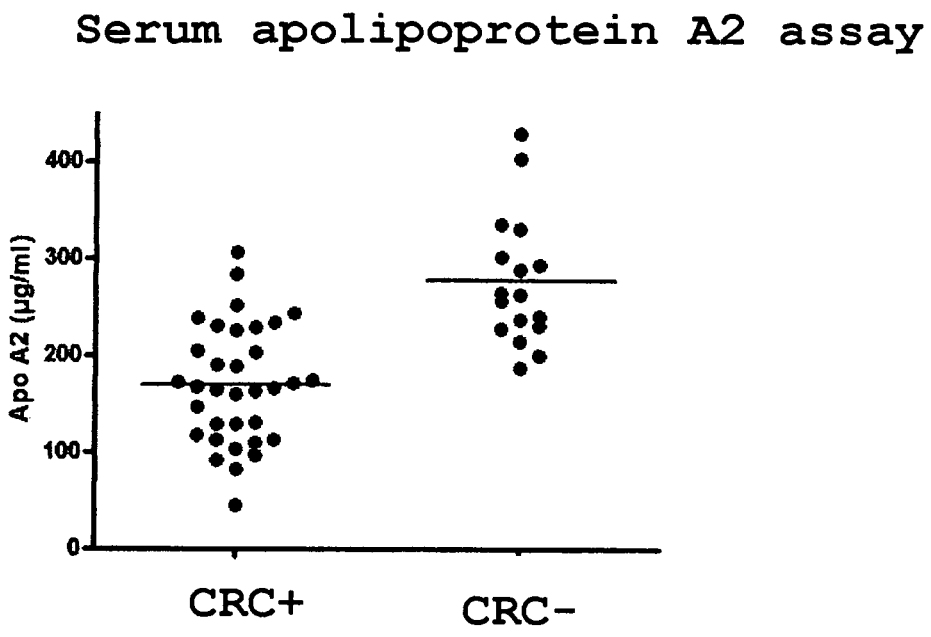


Figure 7

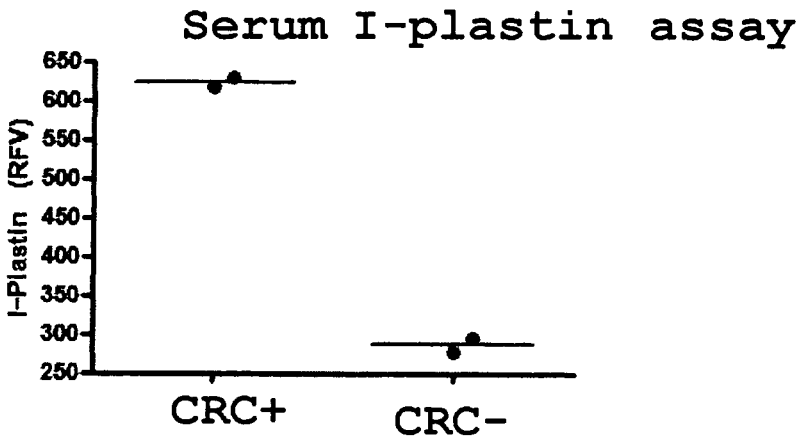


Figure 8

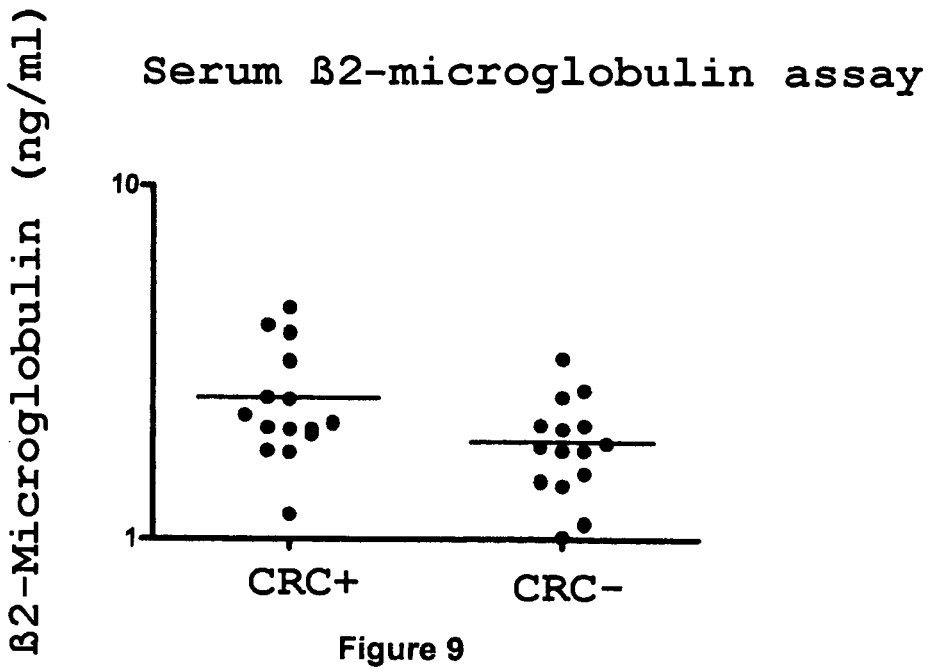


Figure 9

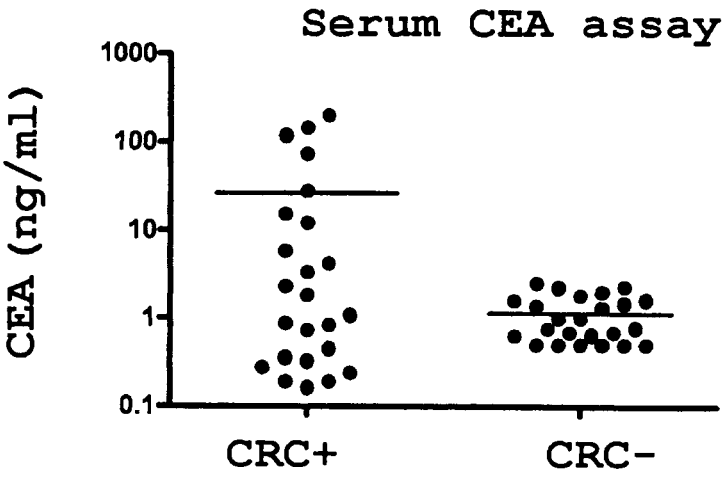


Figure 10

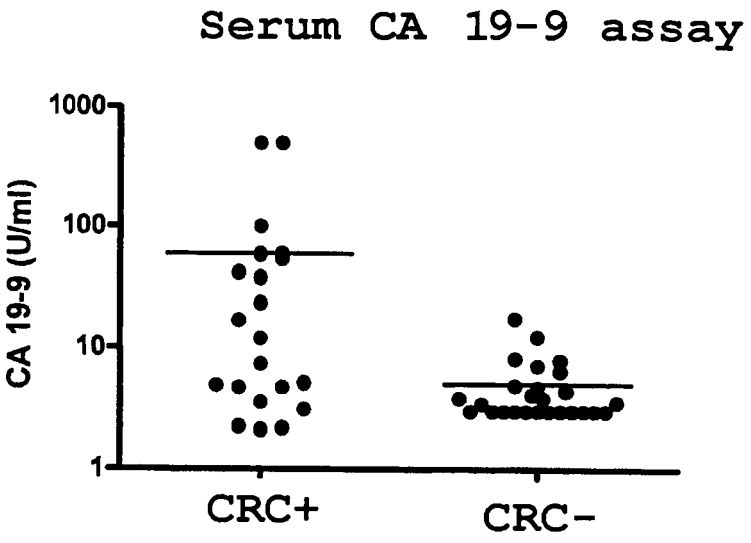


Figure 11

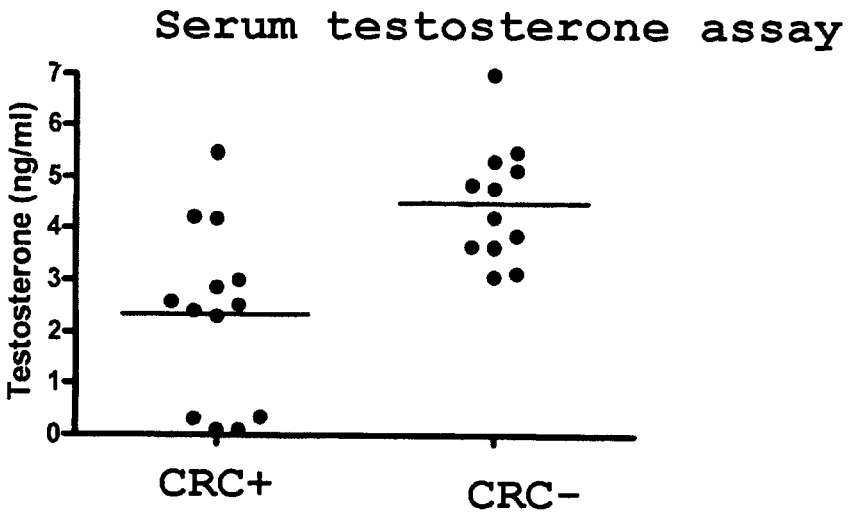


Figure 12

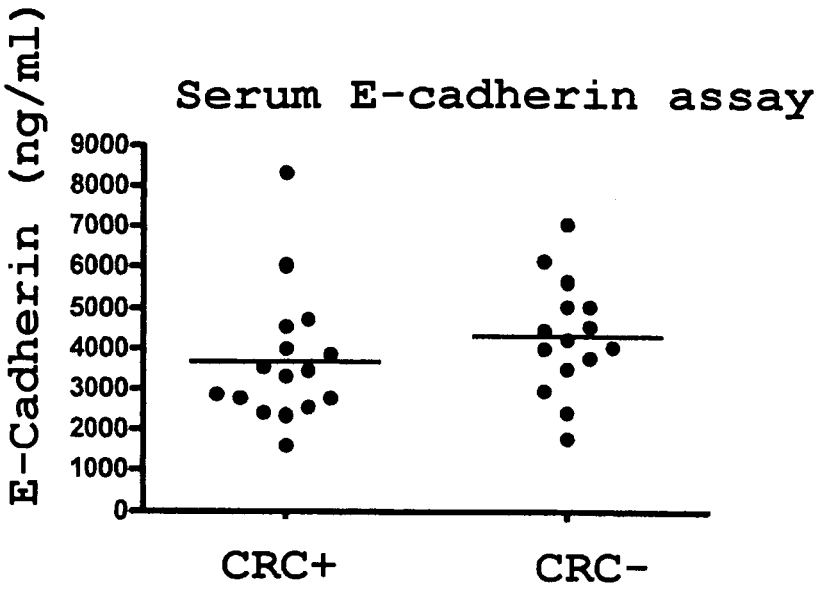


Figure 13

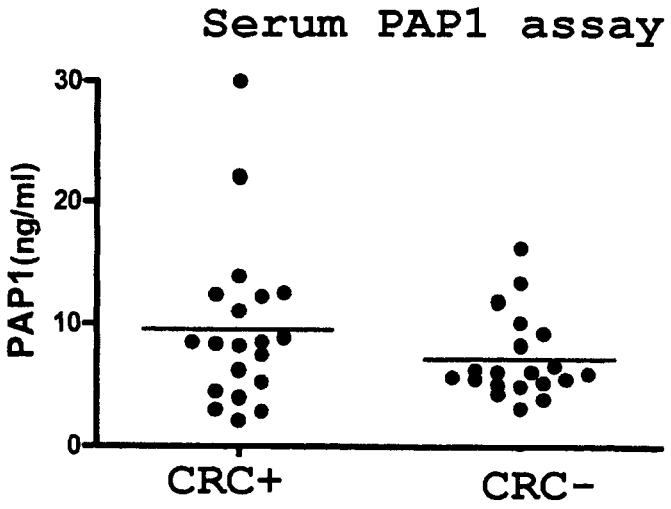


Figure 14

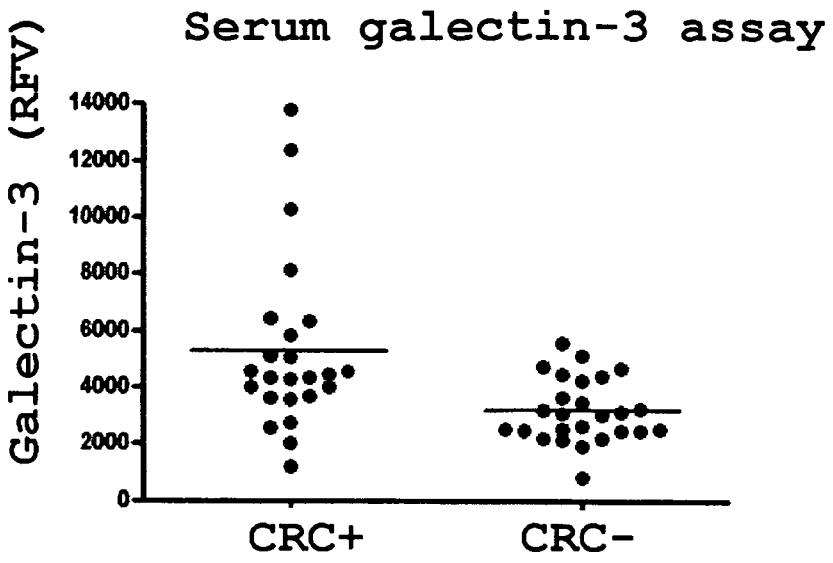


Figure 15

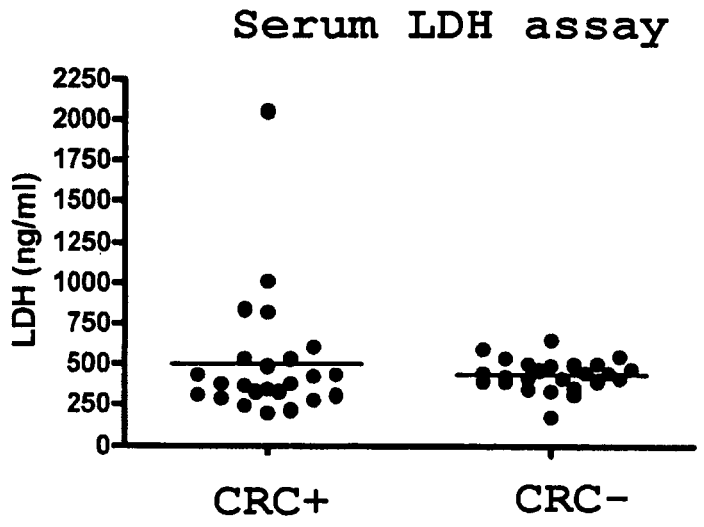


Figure 16

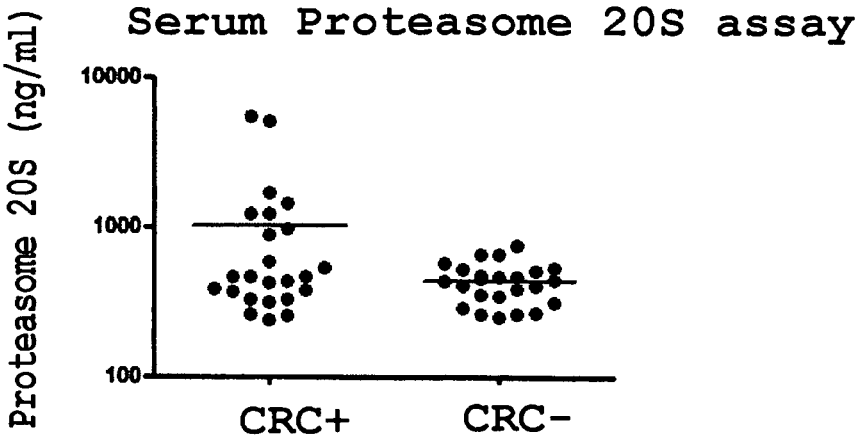


Figure 17

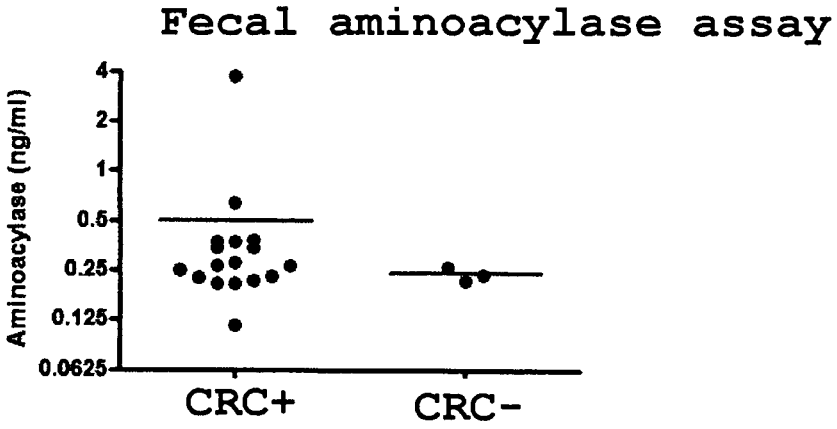


Figure 18

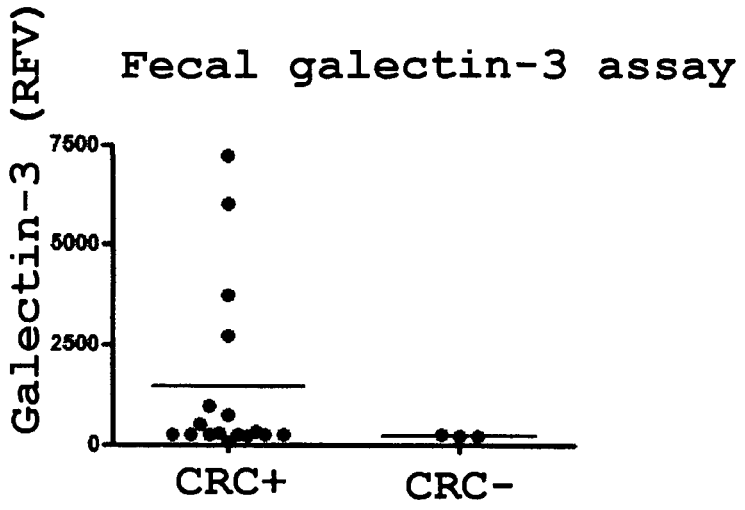


Figure 19

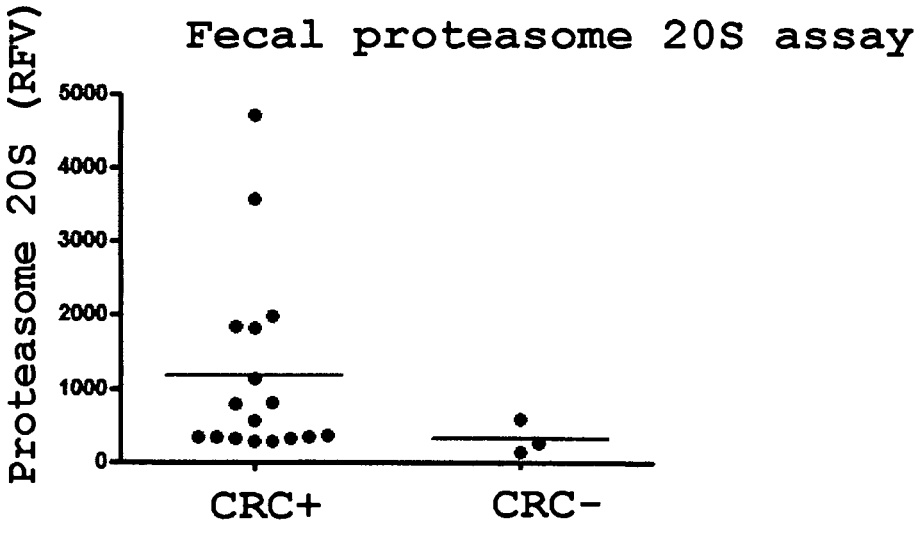


Figure 20

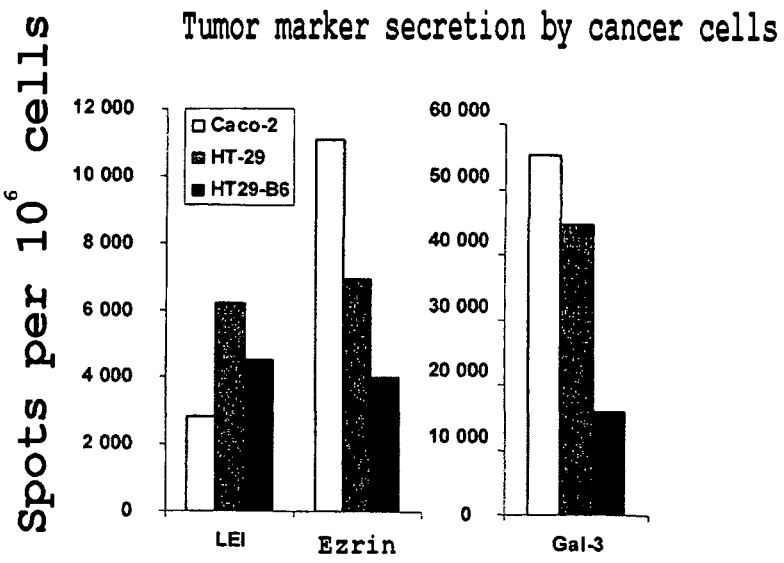

Tumor marker secretion by cancer cells
Spots per 10^6 cells
12 000
10 000
8 000
6 000
4 000
2 000
0
Caco-2
HT-29
HT29-B6
60 000
50 000
40 000
30 000
20 000
10 000
0
LEI
Ezrin
Gal-3

Figure 21

METHOD OF ASSAYING LEUKOCYTE ELASTASE INHIBITOR FOR THE IN VITRO DIAGNOSIS OF COLORECTAL CANCER

The present invention relates to the cancerology field. More particularly, the subject of the present invention is a method for the in vitro diagnosis of colorectal cancer in a human patient, by determining the presence of the Leukocyte Elastase Inhibitor (LEI) in a biological sample taken from this patient, it being possible for said method to be used both for early diagnosis, screening, therapeutic follow-up and prognosis, and for relapse diagnosis in relation to colorectal cancer.

Colorectal cancer (CRC) is a major public health problem. The worldwide incidence thereof was estimated at 875 000 new cases in 1996[1]. Taking into account both sexes, it is the cancer that occurs most frequently in western countries, where it is generally classed among the first 3 most common causes of death due to cancer. The 5-year survival rate, all stages taken into account, is in the region of 60%.

Only early diagnosis offers the hope of a curative treatment. However, at the current time, there is no serological screening test nor specific diagnostic test which is early.

Screening for colorectal cancer is currently carried out in Europe with two distinct approaches: firstly, using a paraclinical test which consists in looking for the presence of blood in the stools (Faecal Occult Blood Test, FOBT, marketed, for example, under the name Hemoccult®). This technique has demonstrated its clinical usefulness. When it is used every 2 years in individuals between the ages of 50 and 74, it can reduce by 15 to 20% mortality due to colorectal cancer[2]. For this, it is necessary for more than half the population concerned to participate regularly in the screening and for a colonoscopy to be carried out in the event of a positive test, optionally followed by an appropriate treatment.

Nevertheless, this screening technique suffers from a certain number of handicaps:

- The major drawback of this test is its mediocre sensitivity, most especially for adenomas (precancerous dysplastic lesion) which, if they are large in size, will result in the development of cancer in 1 case out of 10.
- The test is also not very specific. The appearance of blood in the stools may be related to a nontumor condition: ulcerative colitis, hemorrhoids, fistulae, etc. In this case, an investigation by colonoscopy must be carried out, with the drawbacks described hereinafter.
- Finally, Hemoccult® tests are difficult to interpret; they must therefore be read in specialized centers, by qualified competent personnel.

Immunological tests specific for human hemoglobin (Feca EIA®, Heme Select®, etc.) have also been described. They probably constitute progress compared with Hemoccult®, but they essentially exhibit the same problems. Thus, InSure™, marketed by Enterix Inc., makes it possible to detect 87% of patients suffering from CRC and 47% of those having precancerous polyps. It is a test for detecting human hemoglobin in the stools, and more particularly the globin portion of this molecule.

A second screening strategy is the systemic performing of a colonoscopy after the age of 50, which makes it possible in theory to reduce mortality due to colorectal cancer. However, the acceptability of this examination in individuals who are in good health is too low for a screening policy using endoscopy to reduce mortality (the level of compliancy for colonoscopy in European countries having set up this screening strategy is around 2%). There is a not insignificant risk (0.1%) of perforation and bleeding of the colon and of death (1/10000), and it is also very expensive for public health. Furthermore, colonoscopy requires a very restrictive prior colonic preparation, which in large part explains the poor compliance.

Tumor markers that can be assayed by immunoassays have for a long time been described in the context of colorectal cancer. They are in particular the carcinoembryonic antigen (CEA) and CA19-9.

CEA is used for follow-up. It cannot be used for the screening or for the early diagnosis of colorectal cancer because its sensitivity and its specificity are insufficient. This is because this marker is expressed by other types of cancer, and in benign pathologies. Despite everything, it is possible to increase sensitivity without losing specificity by combining, with CEA, another tumor marker such as CA19-9 or CA72-4.

The causes of physiological variations in CA19-9 are rare, but other benign conditions (hepatobiliary conditions, pancreatic conditions), or malignant conditions may induce an increase in CA19-9. This marker, taken alone, is therefore also of no interest for diagnosis. Nevertheless, since its serum concentration is correlated with the size of the tumor and the presence of metastases, it may also enable a therapeutic follow-up or the early demonstration of relapses.

Commercially available tests have, moreover, been proposed, such as:

- Colopath®/ColorectAlert^{MD}, marketed by Ambrilia, is a rapid and relatively noninvasive screening test for CRC. Colopath® detects a plasmalogen (class of complex lipids which are part of phospholipids) in the rectal mucus of individuals with a colorectal pathological condition, whereas ColorectAlert^{MD} detects T-antigen, a complex sugar in the rectal mucus. The Colopath®/ColorectAlert^{MD} test involves the application of rectal mucus to a test strip, and the positive or negative result is based on a Schiff reaction. Ambrilia has studied 1787 individuals and demonstrated that Colopath®/ColorectAlert^{MD} detects 54% of cases of early-stage colorectal cancer and 49% of all stages combined.
- COLARIS, marketed by Myriad Genetics, is a test for detecting, in the blood, mutations in the MLH1 and MSH2 genes for screening for hereditary nonpolyposis colon cancer (HNPCC syndrome). The result of the test is available in 3 weeks. Myriad uses the most sensitive and most specific sequencing techniques currently available. The test is expensive.
- DR-70® marketed by AMDL, is a test to screen for various types of cancer (lung, colon, breast, liver, stomach, etc.). It is not therefore specific for CRC. The principle of said test is based on the double sandwich ELISA technique (assaying of the DR-70 antigen). Revealing is carried out by enzymatic reaction (antibodies coupled to biotin and to streptavidin). A colored reaction indicates the presence of cancer.

The applicants have now demonstrated, surprisingly, a novel tumor marker which is released by colonic tumors out of the cancerous tissues and is characteristic of these tumors, such that it can be detected both in biological samples remote from the tumors and in the tumors themselves.

Thus, a first subject of the present invention is a method for the in vitro diagnosis of colorectal cancer by determining the presence of Leukocyte Elastase Inhibitor in biological samples taken from patients suspected of having colorectal cancer, and preferably remote from the tumors.

The present invention also relates to the use of this method both for early diagnosis, screening, therapeutic follow-up and prognosis, and for relapse diagnosis in relation to colorectal cancer.

The method of the invention therefore makes it possible to diagnose colorectal cancer specifically and early by means of a simple test consisting in searching for the presence of Leukocyte Elastase Inhibitor in a biological sample taken from a patient, which is preferably remote from the potential tumor. This is because the Applicants have shown, unexpectedly, that colonic tumors not only specifically secrete Leukocyte Elastase Inhibitor, but especially release it out of the cancerous tissue, as will be demonstrated in greater detail hereinafter, and that its concentration in the biological sample in which the method of the invention is carried out is increased compared with the reference values determined for healthy patients.

The determination, in a biological sample which may or may not be remote from the tumor, of the presence of Leukocyte Elastase Inhibitor then makes it possible to conclude with respect to the pathological condition sought. One of the advantages of the method of the invention therefore lies in the possibility of using a sample remote from the potential tumor as a diagnostic sample, thereby enabling a simple and noninvasive diagnosis, whereas a tissue diagnosis requires a biopsy taken invasively. In fact, the study of tissue markers, for example on a tissue section (immunohistochemistry), may be of prognostic interest, but is of no interest for screening for or diagnosing colorectal cancer.

The Leukocyte Elastase Inhibitor tumor marker (Swiss Prot No. P30740, also known as LEI, Serpin B1, Monocyte/neutrophil elastase inhibitor, M/NEI or EI) was sequenced in 1992[3]. LEI specifically inhibits proteases having elastase-type or chymotripsin-type properties by formation of a complex that cannot be dissociated under the action of SDS[4]. LEI thus inhibits three of the major proteases produced by neutrophils: Leukocyte Elastase, Proteinase-3 and Cathepsin G. These proteases enable the immune system to defend the organism by proteolysis of extracellular or phagocytosed substrates. However, when these proteases are in excess, they are responsible for inflammatory reactions. LEI could therefore have a role in regulating and limiting the inflammatory action induced by cell proteases. However, LEI has never been described as potentially being useful as a marker in relation to cancer, and in particular colorectal cancer, and as potentially being assayed in a biological sample remote from the tumor.

The expression "determining the presence of a tumor marker" is intended to mean determining the presence of the protein or of its messenger RNA, or detecting a modification on its gene in the coding or noncoding sequences, such as methylations.

The expression "release by colonic tumors" is intended to mean the active or passive secretion or the release, whatever the mechanism, of the tumor marker by the tumor cells themselves or by the neighboring nontumor cells following lesions or modifications of cell phenotype resulting from the tumor development.

The expression "biological sample in which the method of the invention is carried out" is intended to mean any biological sample capable of containing the tumor marker of interest. By way of example of a biological sample not remote from the tumor, mention may be made of solid samples such as the tissue originating from the tumor, from biopsies of this tumor, from lymph nodes or from metastases of the patient, and cells purified from these solid samples. By way of example of a biological sample remote from the tumor, mention may be made of biological fluids such as whole blood or derivatives thereof, for example serum or plasma, urine, saliva and effusions, bone marrow and stools, and cells purified from these liquid samples. Blood or derivatives thereof and also stools, effusions and cells purified from these liquid samples are preferred.

The method of the invention may be improved by detecting, in addition to LEI, at least one other tumor marker, where appropriate also released by colonic tumors out of the cancerous tissues. Thus, the combination of at least two markers makes it possible to improve the specificity and the sensitivity of the test for the diagnosis of colorectal cancer.

Thus, another subject of the invention also consists in determining the presence of at least one other tumor marker chosen from the following two groups of markers, considered alone or in combination:

group A: Ezrin, Aminoacylase 1, Liver Fatty Acid-Binding Protein, Intestinal Fatty Acid-Binding Protein, Apolipoprotein AI, Apolipoprotein AII and I-Plastin, some of these markers being new markers identified by the applicant,

group B: markers having an additional diagnostic interest, namely: Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Keratin type II Cytoskeletal 8, Keratin type I Cytoskeletal 18, Keratin type I Cytoskeletal 19, Epithelial Cadherin, CEA, Villin, CA19-9, CA 242, CA 50, CA 72-2, Testosterone, TIMP-1, Cripto-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, the detection of DNA fragments in the blood having specific alterations to their methylation profile, for instance methylated DNA of the AXL4 gene (aristaless-like homeobox-4 gene methylation) or the methylated DNA of the septin-9 gene, the detection of specific alterations in fecal DNA fragments, such as specific mutations of fecal DNA or specific alterations of the methylation profile of fecal DNA, the detection of human fecal hemaglobin.

The method of the invention may therefore be improved by detecting at least two markers, one being LEI, the other being another tumor marker chosen from group A, namely Ezrin, Aminoacylase 1, Liver Fatty Acid-Binding Protein, Intestinal Fatty Acid-Binding Protein, Apolipoprotein AI, Apolipoprotein AII and I-Plastin.

The "newly described tumor marker" is intended to mean the protein or the messenger RNA or specific modifications of the corresponding gene, such as mutations or methylations.

The Ezrin marker (Swiss Prot No. P15311, also known as p81, Cytovillin or Villin-2) is a protein which provides binding between the cell membrane and the Actin filaments of the cytoskeleton of the cell, in particular in the microvilli of intestinal epithelial cells[5]. W. G. Jiang and S. Hiscox[6] have shown that the Interleukins IL-2, IL-8, IL-10, etc. can inhibit the expression of ezrin in the HT29 human colorectal cancer cell line. The same authors[7] have shown that the inhibition of Ezrin expression in the HT115 and HRT18 colorectal cancer cell lines reduces the adhesion between cells and increases the mobility and the invasive behavior of the cells. They have concluded that Ezrin regulates cell/cell and cell/matrix adhesions by interacting with the cell adhesion molecules E-Cadherin and beta-Catenin. They have suggested that Ezrin could play an important role in con-

trolling the invasive potential of cancer cells. Moreover, T. Xiao et al.[8] have used an ELISA assay to quantify the plasma Ezrin of patients with lung cancer. However, they have not observed any differences compared with control individuals. The applicant has shown, for its part, surprisingly, that this protein is a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote or not from the tumor.

The Aminoacylase 1 marker (Swiss Prot No. Q03154, also known as EC 3.5.1.14, N-Acyl-L-Amino Acid Amidohydrolase or ACY-1) is part of the Aminoacylase family. They are enzymes which catalyze the hydrolysis of acylated amino acids so as to give fatty acids and amino acids[9]. An immunochemical assay for Aminoacylase enzymatic activity was developed as early as 1975 by K. Lorentz et al.[10] and was used to assay various tissues and sera[11]. The study showed an increase in Aminoacylase activity in the case of hepatic pathological conditions but not in the case of colon cancer. Moreover, the Aminoacylase 1 gene has been identified on chromosome 3p21.1[12]. The 3p21.1 region is reduced to homozygosity in a small cell lung cancer, and in this case, the Aminoacylase expression is repressed or undetectable[13]. Similarly, S. Balabanov et al.[14] have shown that the Aminoacylase expression is repressed in the case of kidney cancer. The applicant has shown, for its part, surprisingly, that this protein is a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote or not from the tumor.

The Liver Fatty Acid-Binding Protein marker (Swiss Prot No. P07148, also known as L-FABP, FABP1, FABPL, Z-protein or sterol transporter protein) belongs to the FABP family which comprises nine isoforms. Each isoform is named according to the tissue in which it was first detected. These isoforms have a shared function and similar three-dimensional structures, but their sequence homology is not high. L-FABP was sequenced in 1985[15]. It is a small protein of 15 kDa that is abundant in the cytosol and that has the ability to bind to free fatty acids and also to bilirubin. Some recent studies appear to indicate that impairments in expression of the L-FABP protein could induce a tumorigenesis process. For prostate cancer, the level of expression of L-FABP mRNAs in tumor tissue biopsies was 10 times higher than in the normal tissue[16]. For colon cancer, several teams have identified a decrease in the expression of L-FABP protein in the tumor tissue compared with normal colonic mucosa, using 2-dimensional electrophoresis techniques[17]. This result has also been confirmed by immunohistochemistry techniques. In addition, the L-FABP protein is a prognostic liver resection marker in patients with colorectal cancer having metastasized to the liver[18]. The applicant has shown, for its part, surprisingly, that this protein is a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote from the tumor.

The Intestinal Fatty Acid-Binding Protein marker (Swiss Prot No. P12104, also known as I-FABP, FABP-2 or FABPI) was sequenced in 1987[19]. It is a small protein of 15 kDa that is abundant in the cytosol and that has the ability to bind to free fatty acids and also to bilirubin. The I-FABP protein is expressed in the enterocytes of the small intestine and may constitute approximately 2% of the protein content of this cell type. At the tissue level, the duodenum and the jejunum contain significantly higher amounts of I-FABP than the colon (jejunum: 4.8 μg/g, colon: 0.25 μg/g)[20]. I-FABP could not be detected in the plasma samples of healthy individuals. On the other hand, in certain pathological contexts such as intestinal ischemia, Crohn's disease or primary biliary cirrhosis, it is possible to demonstrate an increase in the plasma I-FABP concentration in certain individuals[20]. For prostate cancer, it has been shown that the level of expression of I-FABP mRNA in biopsies of tumor tissue is 7 times higher than in normal tissue[16]. In the model of induction of a colorectal tumor with azoxymethane in the rat, the level of expression of I-FABP mRNA is reduced by 2.92 to 3.97 times when the animals have a diet that reduces the incidence of cancer (soya proteins or whey hydrolysate)[21]. The applicant has shown, for its part, surprisingly, that this protein is a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote from the tumor.

The Apolipoproteins are a family of proteins constituted of polar amino acids, which allow the transport of lipids in the blood by formation of a hydrophilic macromolecular complex called a lipoprotein. For each of the human plasma Apolipoproteins, there are isoforms derived from genetic polymorphism and/or from post-translational modifications, the presence of which in the blood can be associated with certain pathological conditions[22]. The plasma concentration of Apolipoproteins is not insignificant, of the order of those mg/ml[23].

The Apolipoprotein AI marker (NCBI No. 490098, also known as Apo A-I, Apo AI and Apo A1) is a protein of 243 amino acids and of 28 kDa. It is essentially synthesized by the liver and the intestine. This protein has been shown to be underabundant in the sera of patients suffering from colorectal cancer compared with healthy individuals, by SELDI-TOF[24]. However, it is specified in this article that patients with CRC are distinguished from healthy individuals by combining Apo AI with other protein markers. Moreover, this article specifies that the assaying of Apo AI by turbidimetric immunoassay, carried out by another team, does not confirm the underabundance of this protein in the sera of patients having CRC[25]. Hachem et al.[26] have, for their part, assayed Apo AI in sera of patients having had liver cancer following colorectal cancer metastases. The applicant has shown, for its part, surprisingly, that assaying by immunoassay makes it possible to demonstrate a decrease in the concentration of this protein in patients having colorectal cancer, contrary to what was put forward by Engwegen et al.[24], who were able to demonstrate this decrease only by implementing the SELDI-TOF technique. The assaying of Apo AI by immunoassay in biological samples is a good method for the diagnosis of colorectal cancer, said samples being remote from the tumor, insofar as the assaying by immunoassay that is carried out is not turbidimetry as used by the team of Zhang et al.[25].

The Apolipoprotein AII marker (Swiss Prot No. PO2652, also known as ApoA II, Apo-AII and Apo A2) is a 17380-Da protein composed of two polypeptide chains of 77 amino acids each, linked by a disulfide bridge. Like Apolipoprotein AI, Apolipoprotein AII is essentially synthesized by the liver and the intestine. Hachem et al.[26] have also assayed, in addition to Apo AI, Apo AII in sera of patients having had liver cancer following colorectal cancer metastases. However, the results are not significant and do not enable a conclusion to be drawn as to the pathological condition sought. The applicant has shown, for its part, surprisingly, that the decrease in the concentration of this protein in patients having colorectal cancer makes it a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote from the tumor.

The I-Plastin marker (Swiss Prot No. Q14651, also known as intestine-specific Plastin or Plastin 1) belongs to the family of human Plastins of which three representatives are

known: I-Plastin, L-Plastin and T-Plastin. Some authors call Plastins "Fimbrins", yet other authors reserve the name Fimbrin for I-Plastin. The Plastins are proteins that bind to Actin so as to form the cytoskeleton (cell skeleton). They are 70 kDa proteins that are relatively well-conserved throughout eukaryotic evolution. They exhibit strong tissue specificity, only one isoform at a time is present in normal tissues[27]. The use of Plastins with respect to cancer has already been described in U.S. Pat. No. 5,360,715, which proposes a method for determining whether a cell is hematopoietic or neoplastic i.e. cancerous. This method claims the assaying of L-Plastin and of T-Plastin at the cellular level, and more particularly the assaying of the mRNA thereof. However, despite these properties, no prior study has been carried out to evaluate the importance of Plastins in relation to the diagnosis of colorectal cancer using a serum or stool sample. Furthermore, I-Plastin has never even been envisioned as a potential cancer marker[28]. The applicant has shown, for its part, surprisingly, that this protein is a good marker in biological samples taken from a patient having colorectal cancer, said samples being remote or not from the tumor.

The concentration of the tumor marker chosen from group A will, depending on the marker under consideration, be increased or decreased in the biological sample in which the method of the invention is carried out, compared with the reference values determined for the healthy patients.

The method of the invention may also be improved by combining the detection of the LEI and of one other tumor marker chosen from group B, namely: the markers Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Keratin type II Cytoskeletal 8, Keratin type I Cytoskeletal 18, Keratin type I Cytoskeletal 19, Epithelial Cadherin, CEA, Villin, CA19-9, CA 242, CA 50, CA 72-2, Testosterone, TIMP-1, Cripto-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally Controlled Tumor Protein, (Pro)defensin-A5, the detection of DNA fragments in the blood having specific alterations to their methylation profile, for instance methylated DNA of the AXL4 gene (aristaless-like homeobox-4 gene methylation) or methylated DNA of the septin-9 gene, the detection of specific alterations in fecal DNA fragments, such as specific mutations of fecal DNA or specific alterations of the methylation profile of fecal DNA, and the detection of human fecal hemoglobin. Of course, the method of the invention may also implement the detection, in the same assay, of LEI, of at least one tumor marker chosen from group B and of at least one other tumor marker chosen from group A.

The Beta2-Microglobulin marker (Swiss Prot No. P61769, also known as β2-Microglobulin, β2M) is a low-molecular-weight (11 to 12 kDa) protein found at the surface of most nucleated human cells. The serum β2-Microglobulin level increases in certain patients suffering from cancer, without this increase being specific, or correlated with the nature of the tumor, its stage or the severity of the disease. A significant increase is also observed in other diseases, such as lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, malignant diseases of the lymphoid system (multiple myeloma, B-cell lymphoma), certain viral diseases (hepatitis or AIDS), and in hemophiliac patients. Since β2-Microglobulin is filtered by the renal glomeruli and reabsorbed by the proximal convoluted tubules, its concentration in the blood may be modified in the case of renal pathological conditions. The assaying of β2-Microglobulin is thus most commonly reserved for the diagnosis of renal pathological conditions, or for the follow-up of infection with the acquired immunodeficiency virus. However, this marker is known as a tumor marker, in particular for colon cancer.

The Proteasome 20S marker (also known as Prosome) is the central structure of the proteasome, which is itself a molecular complex responsible for the intracellular degradation of ubiquitinated proteins[29]. The Proteasome is a molecular complex of 700 kDa, constituted of 28 subunits associated in 4 rings of 7 subunits. In humans, 7 alpha units (α1, α2, α3, α4, α5, α6 and α7) and 10 beta units (β1, β2, β3, β4, β5, β6, β7, β1i, β2i and β5i) are known. By virtue of its catalytic properties, the Proteasome plays a central role in the mechanisms of cell proliferation, growth, regulation and apoptosis, and therefore in the cancerization pathways. Proteasome inhibition with Bortezomib (Velcade) is a recognized treatment for multiple myeloma. Phase II or III therapeutic trials are ongoing for hematological cancers or tumors. T. Lavabre-Bertrand et al.[30] have shown that the serum level of Proteasome can increase on the occasion of certain pathological conditions, in particular in the case of cancers (myeloma, lymphoma and solid tumors).

The Galectin-3 marker (Swiss Prot No. P17931, also known as Gal-3, Galactose-Specific Lectin 3, MAC-2 antigen, IgE-Binding Protein, 35 kDa Lectin, Carbohydrate Binding Protein 35, CBP 35, Laminin-Binding Protein, Lectin L-29, L-31, Galactoside-Binding Protein or GALBP) is a lectin capable of binding to beta-galactoside structures of N-acetyllactosamine type. It is a protein with multiple functions involved in various biological functions, including the adhesion of tumor cells, proliferation, differentiation, angiogenesis, apoptosis, metastatic cancer progression[31]. Various studies have shown that Gal-3 can form complexes with numerous molecules: CEA, IgE, Laminin, Mucin, Mac-2BP, LAMP1, LAMP2, Fibronectin, etc. A serum assay of Gal-3 has been described by I. Iurisci et al.[32]. Gal-3 was captured on microplates coated with Mac-2-binding protein (a Gal-3-binding protein) and then revealed with an anti-Gal-3 rat antibody. This study showed elevated serum of Gal-3 in the case of gastrointestinal cancers, breast cancer, lung cancer, ovarian cancer, melanomas and non-Hodgkin lymphomas.

The L-Lactate Dehydrogenase Chain B marker (Swiss Prot No. P07195, also known as LDH-B, LDH Heart Unit or LDH-H) is a protein that can form complexes in the form of homotetramers. This protein can also form complexes with the L-Lactate Dehydrogenase Chain A protein (Swiss Prot No. P00338, also known as LDH-A, LDH muscle unit or LDH-M) in the form of heterotetramers. The serum dose and/or the serum enzymatic activity of the tetrameric complexes, called LDH, increase(s) in the bloodstream proportionally to the tumor mass for many solid tumors. Its use is recommended in combination with human chorionic gonadotrophin (beta-hCG) and placental alkaline phosphatase for the follow-up of seminal vesicle cancers. LDH is considered to be a marker of importance for the prognosis of lymphomas, of leukemia and of colon cancer[33].

The Calreticulin marker (Swiss Prot No. P27797, also known as CRP55, Calregulin, HACBP, ERp60 or grp60) is a multifunctional protein. It is a lectin capable of interacting transiently with virtually all the monoglycosylated proteins of the endoplasmic reticulum. D. J. McCool et al.[34] have thus shown that Calreticulin is involved in maturation of the colonic mucin MUC2. A method for the diagnosis of CRC which uses assaying of Calreticulin in a tissue, the stools or a body fluid is described in patent application WO 03/065003.

The Regenerating Islet-Derived Protein 3 Alpha marker (Swiss Prot No. Q06141, also known as Reg III-alpha, Pancreatitis-Associated Protein 1 or Pancreatis Associated Protein I (PAP 1)) is a protein that is weakly expressed in the healthy pancreas. It is overexpressed during the acute phases of pancreatitis and in certain patients suffering from chronic pancreatitis. In this case, it appears in the pancreatic fluid and in the bloodstream[35]. Y. Motoo et al.[36] have shown, by ELISA assay, that the level of PAP 1 in the blood increases in certain patients having colon cancer, stomach cancer, liver cancer or pancreatic cancer, and also in the case of renal insufficiency. To to this, they used the ELISA assay (PANCEPAP) marketed by the company Dynabio (La Gaude, France).

The Tumor-Associated Calcium Signal Transducer 1 marker (Swiss Prot No. P16422, also known as Major gastrointestinal tumor-associated protein GA733-2, Epithelial cell surface antigen, EpCAM, Epithelial glycoprotein, EGP, Adenocarcinoma-associated antigen, KSA, KS 1/4 antigen, Cell surface glycoprotein Trop-1 or CD326 antigen), was characterized in 1979 by virtue of its ability to be recognized by an antibody directed against colorectal cancer cells[37]. This protein is known by various names, as indicated above, but the most common use is to call it EpCAM. It is a transmembrane protein expressed at the basolateral surface of cells, in certain epithelia and many cancers[38]. As early as 1982, Herlyn et al.[39] showed that the injection of an anti-EpCAM monoclonal antibody could inhibit tumor growth in patients having colorectal cancer. These results resulted in the development of an antitumor treatment based on an anti-EpCAM antibody called Edrecolomab. This treatment is marketed under the name Panorex™. Moreover, H. Abe et al.[40] have shown, by ELISA assay, that a soluble form of EpCAM, called MK-1, is increased in the bloodstream in 10% of cancer patients studied.

The Cytokeratins are part of the proteins that make up the intermediate filaments of the cytoskeleton of epithelial cells. Currently, more than 20 human Cytokeratins have been identified. The Cytokeratins 8 (Swiss Prot No. P05787, also known as Cytokeratin-8, CK-8, Keratin-8 or K8), 18 (Swiss Prot No. P05783, also known as Cytokeratin-18, CK-18, Keratin-18 or K18) and 19 (Swiss Prot No. P08727, also known as Cytokeratin-19, CK-19, Keratin-19 or K19) are the most abundant in epithelial cells and are useful tools for the diagnosis of cancer pathologies[41]. This clinical importance is linked to the release of Cytokeratins by epithelial cells in the apoptotic or proliferation phase. In the case of apoptosis, this release occurs in the form of soluble fragments which seem to appear under the proteolytic action of caspases. Undegraded Cytokeratin forms have never been described in the bloodstream. The three Cytokeratin assays most commonly used clinically are the tissue polypeptide antigen (TPA) assay, the tissue polypeptide specific antigen (TPS) assay and the CYFRA 21-1 assay. TPA is a broad-spectrum test which measures Cytokeratins 8, 18 and 19. The TPS and CYFRA 21-1 assays are more specific and measure, respectively, fragments of Cytokeratin 18 and of Cytokeratin 19. These 3 assays detect soluble Cytokeratin fragments that may be present on their own or in the form of protein complexes. TPA, TPS or CYFRA-21-1 have been used for the therapeutic follow-up of colorectal cancers, breast cancers, lung cancers, bladder cancers, ovarian cancers, pancreatic cancers, prostate cancers and certain ENT cancers. The assaying of soluble Cytokeratin fragments in the blood in fact has a clinical value in screening for relapses or evaluating the response to the therapy used (radiotherapy, chemotherapy, hormone treatment). Regular assaying makes it possible in particular to evaluate the progression of the tumor mass. The amount of soluble blood Cytokeratins also has a prognostic aspect with respect to the tumor stage and to the formation of metastases. Currently, the blood assay for Cytokeratin that is most commonly used is CYFRA 21-1. It is highly recommended for the follow-up of patients having non-small cell lung cancer. Various commercially available assays exist for TPA (AB Sangtec Medical Co., Byk-Roland, etc.), TPS (IDL Biotech AB, BEKI Diagnosiss, etc.) and CYFRA-21-1 (Roche Diagnosiss, CIS Bio-International, Fujirebio Diagnosiss, etc.). Moreover, H. Kim et al.[42] have shown that assaying fecal Cytokeratin 19 (DiNonA Inc.) may be useful in screening for gastrointestinal diseases, in combination with a fecal occult blood assay. Finally, the use of Cytokeratin 20 (Swiss Prot No. P35900, also known as Keratin, type I Cytoskeletal 20, CK-20, Keratin-20, K20, or IT protein) as a marker in colorectal cancer is described in patent application US 2002/0160382.

The Epithelial Cadherin marker (Swiss Prot No. P12830, also known as E-Cadherin, Uvomorulin, Cadherin-1, CAM 120/80 or CD324 antigen) is a transmembrane protein that mediates calcium-dependent cell adhesion. It is specifically expressed in epithelial cells, where it is involved in maintaining their phenotype. The cytoplasmic domain of E-Cadherin binds to β-Catenin, which is itself bound to the actin filament networks of the cytoskeleton. This E-Cadherin/β-Catenin binding plays an essential role in stabilizing cell/cell adhesions of the epithelial tissue. The loss of E-Cadherin can therefore reduce cell adhesion and increase the invasive capacity of cancer cells. A reduction in expression of E-Cadherin or of β-Catenin is generally associated with greater aggressiveness and dedifferentiation of the tumor, in particular with respect to gastrointestinal cancers. F. Roca et al.[43] have thus shown that patients having colorectal cancer and underexpressing E-Cadherin have a more unfavorable prognosis than patients having a normal expression level. As early as 1983, Damsky et al.[44] showed that a soluble form of E-Cadherin could be released by the MCF-7 breast cancer cell line. This soluble form corresponds to the cleavage of the extracellular portion of E-Cadherin. Later, M. Katayama et al.[45] showed that the soluble form of E-Cadherin could be released into the bloodstream in the case of cancer, and C. Willmanns et al.[46] showed that the increase in the amount of E-Cadherin in the blood is correlated with the tumor stage in colorectal cancers. A commercially available kit is, moreover, proposed by the company Takara BioChemicals (Tokyo, Japan).

The assaying of CEA (carcinoembryonic antigen) for the diagnosis of colorectal cancer has been proposed since 1965 by P. Gold and S. Freedman[47], but an assay for this marker in the blood has poor sensitivity for the diagnosis of colorectal cancers at a relatively nonadvanced stage. The assaying of serum CEA is thus especially recommended for evaluating the risk of liver metastases[48] and for therapeutic follow-up. In addition, it is a marker that is not very specific for colorectal cancer; it may in fact be increased in many other cancers (lung, breast, etc.). On the other hand, the assaying of fecal CEA appears to be more sensitive and more specific than the assaying of serum CEA or than the assaying of fecal blood[49]. However, this assaying is not yet proposed routinely.

The reactive antigenic determinants 1116-NS-19-9, more commonly called CA19-9 (carbohydrate antigen 19.9), are carried by high-molecular-weight proteins[50]. The assaying of CA 19-9 in the blood is more specific than that of CEA. The CA 19-9 level in the blood increases in the event of colorectal cancer, of pancreatic cancer and of liver cancer

(cholangiocarcinoma), but also in the event of noncancerous pathological conditions (cholangitis, etc.). Its use in combination with CEA is recommended both at the time of diagnosis of a cancer and for follow-up of the pathological condition.

J. Holmgren et al.[51] have shown that the amount of CA 50 antigen in the serum is increased in the case of colorectal cancer. The CA 50 antigen is defined by its ability to be recognized by a specific monoclonal antibody.

As regards the CA 72 marker, T. L. Klug et al.[52] have shown that the amount of CA 72 antigen in the serum is increased in the case of colorectal cancer. The CA 72 antigen is defined by its ability to be recognized by a specific monoclonal antibody.

Similarly, P. Kuusela et al.[53] have shown that the amount of CA 242 antigen in the serum is increased in the case of colorectal cancer. The CA 242 antigen is defined by its ability to be recognized by a specific monoclonal antibody.

The assaying of Testosterone for the diagnosis of colorectal cancer has been proposed in men by M. Holland et al.[54]. These authors have shown a fall in the blood Testosterone level in the case of colorectal cancer.

As regards the TIMP-1, or tissue inhibitor of matrix metalloproteinase type-1, marker, patent application US 2007/0020707 describes in particular the assaying of TIMP-1 for the diagnosis of colorectal cancer by assaying in a body fluid.

F. Model et al.[55] showed, in July 2006, during the World Congress on Gastrointestinal Cancer, that it was possible to detect methylated forms of the septin-9 gene in the plasma of patients having colorectal cancer.

M. P. Ebert et al.[56] have shown that the ALX4 gene, or aristaless-like homeobox-4 gene, is more often methylated in the sera of patients having colorectal cancer than in control sera ($P<0.0001$). Using a threshold value of 41.4 pg/mL, they have obtained a sensitivity of 83.3% and a specificity of 70%.

Villin is described as a blood marker for the diagnosis of colorectal cancer in patent application FR2581456.

C. Bianco et al.[57] have shown that the amount of Cripto-1 in the serum is increased in the event of colorectal cancer.

The assaying of Intelectin-1 (Swiss Prot No. Q8WWA0, also known as Intestinal lactoferrin receptor, Galactofuranose-binding lectin, Endothelial lectin HL-1 or Omentin) for the diagnosis of colorectal cancer has been described in patent application US 2003/0082533.

The use of Protein Disulfide Isomerase (Swiss Prot No. P07237, also known as EC 5.3.4.1, PDI, Prolyl 4-hydroxylase subunit beta, Cellular thyroid hormone-binding protein or p55), of Translationally-Controlled Tumor Protein (Swiss Prot No. P13693, also known as TCTP, p23, Histamine-releasing factor, HRF or Fortilin) and of (Pro)defensin-A5 (Swiss Prot No. Q01523), as markers in colorectal cancer, is described respectively in patent applications EP1724586, US 2003/0172388 and US 2006/0179496. The term "(Pro) defensin" is intended to mean the precursor, namely the Prodefensin before cleavage, the propeptide, namely the N-terminal moiety after cleavage of the Prodefensin, and the mature protein, namely Defensin, corresponding to the C-terminal moiety after cleavage.

Finally, the assaying of human fecal hemoglobin is known practice and can be implemented as previously described.

The concentration of the tumor marker chosen from group B will, depending on the marker under consideration, be increased or decreased in the biological sample in which the method of the invention is carried out, compared with the reference values determined for healthy patients.

Preferably, the tumor marker(s) of group B is (are) chosen from: the markers: Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Epithelial-Cadherin, CEA, CA19-9, Testosterone, TIMP-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, and detection of human fecal hemoglobin.

Of course, the method of the invention may also include the detection of any other marker for colorectal cancer that is known to those skilled in the art.

As indicated above, the tumor marker(s) of interest is (are) detected in the form of a protein, or in the form of a messenger RNA, or by alteration of the corresponding DNA (mutation or modification of methylations).

The determination of the presence, in the biological sample, of the "protein" tumor marker of interest can be carried out by any method for determining the presence of a protein in a sample, known to those skilled in the art, such as, for example, a biochemical test, including an immunoassay, or by mass spectrometry.

The biochemical test may be any test widely known to those skilled in the art involving molecular interactions, i.e. reactions between said tumor marker and one or more binding partner(s) specific or not specific for said tumor marker.

Preferably, the biochemical test is an immunoassay known to those skilled in the art, involving immunological reactions between the tumor marker, which is the antigen, and one or more specific binding partner(s), namely the antibodies directed against this antigen.

The binding partners specific or not specific for the tumor marker(s) sought in the method of the invention are any partner capable of binding to this or these marker(s). They are said to be specific when they are capable of binding to these markers with a high specificity, or even a specificity of 100%. They are said to be nonspecific when their specificity of binding to these markers is low and they are then capable of binding to other ligands, such as proteins. By way of example, mention may be made of antibodies, antibody fractions, receptors and any other molecule capable of binding to this marker.

The binding-partner antibodies are, for example, either polyclonal antibodies or monoclonal antibodies.

The polyclonal antibodies may be obtained by immunization of an animal with the tumor marker concerned, followed by recovery of the desired antibodies in purified form, by taking the serum from said animal, and separation of said antibodies from the other serum constituents, in particular by affinity chromatography on a column to which an antigen specifically recognized by the antibodies, in particular said marker, is attached.

The monoclonal antibodies can be obtained by the hybridoma technique, the general principle of which is recalled hereinafter.

Firstly, an animal, generally a mouse, is immunized with the tumor marker of interest, the B lymphocytes of said animal then being capable of producing antibodies against said antigen. These antibody-producing lymphocytes are subsequently fused with "immortal" myeloma cells (murine in the example) so as to produce hybridomas. Using the heterogeneous mixture of cells thus obtained, a selection of cells capable of producing a particular antibody and of multiplying indefinitely is then carried out. Each hybridoma is multiplied in the form of a clone, each resulting in the production of a monoclonal antibody of which the properties

of recognition with respect to said tumor marker may be tested, for example, by ELISA, by one-dimensional or two-dimensional Western blot, by immunofluorescence, or by means of a biosensor. The monoclonal antibodies thus selected are subsequently purified, in particular according to the affinity chromatography technique described above.

The monoclonal antibodies may also be recombinant antibodies obtained by genetic engineering, by means of techniques well known to those skilled in the art.

Examples of anti-Leukocyte Elastase Inhibitor antibodies are known and are available in particular in the Abcam catalog, rabbit anti-LEI polyclonal antibody, Cat. No. Ab47731. An anti-LEI monoclonal antibody, clone ELA-1, has been described in the article by R. Yasumatsu et al.[58].

Examples of anti-Ezrin antibodies are known and are available in particular in the Abcam catalog, anti-Ezrin monoclonal antibody, clone 3C12, Cat. No. Ab4069 and rabbit anti-Ezrin polyclonal antibody, Cat. No. Ab47418.

Examples of anti-Aminoacylase 1 antibodies are known and are available in particular in the Abnova catalog, anti-Aminoacylase 1 monoclonal antibody, clone 4F1-B7, Cat. No. H00000095-M01, and in the Abcam catalog, chicken anti-Aminoacylase 1 polyclonal antibody, Cat. No. Ab26173.

Examples of anti-Liver Fatty Acid-Binding Protein antibodies are known and are available in particular in the Abcam catalog, anti-L-FABP monoclonal antibody, clone 6B6, Cat. No. Ab10059, and rabbit anti-L-FABP polyclonal antibody, Cat. No. Ab7807.

Examples of anti-Intestinal Fatty Acid-Binding Protein antibodies are known and are available in particular in the R&D Systems catalog, anti-1-FABP monoclonal antibody, clone 323701, Cat. No. MAB3078, and in the Abcam catalog, rabbit anti-1-FABP polyclonal antibody, Cat. No. Ab7805.

Examples of anti-Apolipoprotein AI antibodies are known and are available in particular in the Biodesign Meridian Life Sciences catalog, anti-Apo AI monoclonal antibody, clone 4A90, Cat. No. H45402M and goat anti-Apo AI polyclonal antibody, Cat. No. K45252P.

Examples of anti-Apolipoprotein AII antibodies are known and are available in particular in the US Biological catalog, anti-Apo AII monoclonal antibody, clone 1402, Cat. No. A2299-31C and in the Biodesign Meridian Life Sciences catalog, goat anti-Apo AII polyclonal antibody, Cat. No. K74001P.

Examples of anti-I-Plastin polyclonal antibodies are known and are available in particular in the Santa Cruz Biotechnology catalog. The rabbit polyclonal antibody H-300 (Cat. No. sc-28531) reacts with I-Plastin, L-Plastin and T-Plastin. The applicant has developed monoclonal antibodies directed against I-Plastin.

Examples of anti-Beta2-Microglobulin, anti-CEA, anti-CA 19-9 and anti-Testosterone antibodies are known and are in particular used in the applicant's assay kits, respectively Vidas® β2-Microglobulin, Vidas® CEA, Vidas® CA19-9™ and Vidas® Testosterone.

Examples of anti-Proteasome 20S antibodies are known and are available in particular in the Affinitiy Research Products catalog.

Examples of anti-Galectin-3, anti-L-Lactate Dehydrogenase Chain B, anti-Calreticulin, anti-Tumor-Associated Calcium Signal Transducer 1, anti-Keratin type II Cytoskeletal 8, anti-Keratin type I Cytoskeletal 18, anti-Keratin type I Cytoskeletal 19, anti-Epithelial-Cadherin, anti-Villin and anti-TIMP-1 antibodies are known and are available in particular in the Abcam catalog.

Examples of anti-Regenerating Islet-Derived Protein 3 Alpha antibodies are known and are in particular used in the Dynabio assay kits (La Gaude, France).

Examples of anti-CA 242, anti-CA 50 and anti-CA 72-4 antibodies are known and are available in particular in the Fujirebio catalog.

Examples of anti-Intelectin-1 antibody are known and are available in particular in the Alexis Biochemicals catalog, anti-Intelectin-1 monoclonal antibody, clone Saly-1, Cat. No. ALX-804-850-C100 and rabbit anti-Intelectin-1 polyclonal antibody, Cat. No. ALX-210-941.

Examples of anti-Protein Disulfide Isomerase antibodies are known and are available in particular in the Abcam catalog, anti-PDI monoclonal antibody, clone RL77, Cat. No. Ab5484 and rabbit anti-PDI polyclonal antibody, Cat. No. Ab3672.

Examples of anti-Cytokeratin 20 antibodies are known and are available in particular in the Abcam catalog, anti-Cytokeratin 20 monoclonal antibody, clone Ks20.8, Cat. No. Ab962 and rabbit anti-Cytokeratin 20 polyclonal antibody, Cat. No. Ab36756.

Examples of anti-TCTP antibodies are known and are available in particular in the Abnova catalog, anti-TCTP monoclonal antibody, clone 3C7, Cat. No. 157H00007178-M01 and anti-TCTP polyclonal antibody, Cat. No. 157H00007178-A01.

Examples of anti-Defensin-A5 antibodies are known and are available in particular in the Santa Cruz Biotechnology catalog, anti-Defensin-A5 monoclonal antibody, clone 8C8, Cat. No. sc-53997 and in the Alpha Diagnosis International Inc. catalog, rabbit anti-Defensin-A5 polyclonal antibody, Cat. No. HDEFA51-A.

The binding partners which are specific or not specific for the tumor marker(s) sought in the method of the invention may be used as a capture reagent, as a detection reagent or as capture and detection reagents.

The visualization of the immunological reactions, i.e. of the tumor marker/binding partner binding, may be carried out by any means of detection, such as direct or indirect means.

In the case of direct detection, i.e. without the involvement of a label, the immunological reactions are observed, for example, by surface plasmon resonance or by cyclic voltametry on an electrode bearing a conductive polymer.

The indirect detection is carried out by means of labeling, either of the "revealing reagent" binding partner, or of the tumor marker of interest itself. In the latter case, this is then described as a competition method.

The term "labeling" is intended to mean the attachment of a label reagent capable of directly or indirectly generating a detectable signal. A nonlimiting list of these label reagents comprises:

- enzymes which produce a signal that can be detected, for example, by colorimetry, fluorescence or luminescence, such as horseradish peroxydase, alkaline phosphatase, β-galactosidase or glucose-6-phosphate dehydrogenase,
- chromophores such as fluorescent or luminescent compounds or dyes,
- radioactive molecules such as ^{32}P, ^{35}S or ^{125}I, and
- fluorescent molecules such as Alexa or phycocyanins.

Indirect detection systems may also be used, such as, for example, ligands capable of reacting with an antiligand. Ligand/antiligand pairs are well known to those skilled in the art, this being the case, for example, of the following pairs: biotin/streptavidin, hapten/antibody, antigen/antibody, peptide/antibody, sugar/lectin, polynucleotide/sequence

complementary to the polynucleotide. In this case, it is the ligand which carries the binding partner. The antiligand may be directly detectable by means of the label reagents described in the previous paragraph, or may itself be detectable by means of a ligand/antiligand.

These indirect detection systems may result, under certain conditions, in an amplification of the signal. This signal amplification technique is well known to those skilled in the art, and reference may be made to the prior patent applications FR98/10084 or WO-A-95/08000 by the applicant or to the article by Chevalier et al.[59].

Depending on the type of labeling used, those skilled in the art will add reagents that make it possible to visualize the labeling.

By way of example of immunoassays as defined above, mention may be made of "sandwich" methods such as ELISA, IRMA and RIA, "competition" methods and direct immunodetection methods such as immunohistochemistry, immunocytochemistry, Western blotting and Dot blotting.

Mass spectrometry can also be used for detecting, in the biological fluid, the tumor marker(s) sought in the method of the invention. The principle of spectrometry is widely known to those skilled in the art and is described, for example, in Patterson, S.[60].

To do this, the biological sample, which may or may not have been pretreated, is passed through a mass spectrometer and the spectrum obtained is compared with that of the tumor marker(s) sought in the method of the invention. An example of pretreatment of the sample consists in passing it over an immunocapture support comprising one of the binding partners for the tumor marker(s) sought in the method of the invention, for example an antibody directed against the tumor marker(s) sought in the method of the invention. Another example of pretreatment of the sample may be prefractionation of the biological sample in order to separate the proteins of the sample from one another. In techniques well known to those skilled in the art, the predominant proteins of the sample may, for example, first of all be depleted.

By virtue of recent technological advances, it has also become possible to quantify proteins in complex biological media using tandem mass spectrometry (MS/MS) carried out using a triple quadripole analyzer operating in the MRM (multiple reaction monitoring) mode. This operating mode has a double selectivity (two analyzers, parent ion selection and product ion selection) and the detection sensitivity is improved compared with other scanning modes. The technical feasibility of this approach was recently demonstrated by Anderson and Hunter[61] who succeeded in detecting proteins of which the concentration is of the order of a hundred or so ng/ml in plasma, after immunodepletion of the most abundant proteins.

The determination of the presence, in the biological sample, of the "mRNA" tumor marker of interest may be carried out by any method for determining the presence of mRNA in a sample, namely either direct detection of the mRNA, or indirect detection of the mRNA, or any other method for determining the presence of an RNA in a sample, known to those skilled in the art.

The term "direct detection of the mRNA" is intended to mean the demonstration of the mRNA itself in the biological sample.

The direct detection of the mRNA in the biological sample may be carried out by any means known to those skilled in the art, such as, for example, by hybridization with a binding partner specific for the mRNA, where appropriate after amplification by the PCR or NASBA technique.

The term "hybridization" is intended to mean the process during which, under suitable conditions, two nucleotide fragments bind to one another with stable and specific hydrogen bonds so as to form a double-stranded complex. These hydrogen bonds form between the complementary bases adenine (A) and thymine (T) (or uracil (U)) (referred to as an A-T bond) or between the complementary bases guanine (G) and cytosine (C) (referred to as a G-C bond). The hybridization of two nucleotide fragments may be complete (reference is then made to complementary nucleotide fragments or sequences), i.e. the double-stranded complex obtained during this hybridization comprises only A-T bonds and C-G bonds. This hybridization may be partial (reference is then made to sufficiently complementary nucleotide fragments or sequences), i.e. the double-stranded complex obtained comprises A-T bonds and C-G bonds which make it possible to form the double-stranded complex, but also bases that are not bonded to a complementary base. The hybridization between two nucleotide fragments depends on the operating conditions that are used, and in particular the stringency. The stringency is defined in particular as a function of the base composition of the two nucleotide fragments, and also by the degree of mismatching between two nucleotide fragments. The stringency can also depend on the reaction parameters, such as the concentration and the type of ionic species present in the hybridization solution, the nature and the concentration of denaturing agents and/or the hybridization temperature. All these data are well known and the appropriate conditions can be determined by those skilled in the art. In general, depending on the length of the nucleotide fragments that it is desired to hybridize, the hybridization temperature is between approximately 20 and 70° C., in particular between 35 and 65° C., in a saline solution at a concentration of approximately 0.5 to 1 M. The binding partners which are specific or not specific for the mRNA are any partner capable of binding to this mRNA. By way of example, mention may be made of nucleic probes, amplification primers, and any other molecule capable of binding to this mRNA.

The term "hybridization probe" is intended to mean a nucleotide fragment comprising from 5 to 100 nucleic units, in particular from 10 to 35 nucleic units, having a hybridization specificity under given conditions so as to form a hybridization complex with the material specific for the target gene of interest. The hybridization probe may comprise a label enabling its detection.

For the purpose of the present invention, the term "amplification primer" is intended to mean a nucleotide fragment comprising from 5 to 100 nucleic units, preferably from 15 to 30 nucleic units, enabling the initiation of an enzymatic polymerization, such as, in particular, an enzymatic amplification reaction. The term "enzymatic amplification reaction" is intended to mean a process that generates multiple copies of a nucleotide fragment via the action of at least one enzyme. Such amplification reactions are well known to those skilled in the art and mention may in particular be made of the following techniques:

- PCR (polymerase chain reaction), as described in U.S. Pat. No. 4,683,195, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,800,159,
- NASBA (nucleic acid sequence-based amplification) with patent application WO 91/02818, and
- TMA (transcription mediated amplification) with U.S. Pat. No. 5,399,491.

The term "detection" is intended to mean either a physical method, or a chemical method with an intercalating dye such as SYBR® Green I or ethidium bromide, or a method of

detection using a label. Many detection methods exist for detecting nucleic acids[62]. The appropriate labels are as defined above.

For the purpose of the present invention, the hybridization probe may be a "detection" probe. In this case, the "detection" probe is labeled by means of a label as defined above. By virtue of the presence of this label, it is possible to detect the presence of a hybridization reaction between a given detection probe and the transcript to be detected.

The detection probe may in particular be a "molecular beacon" detection probe[63]. These "molecular beacons" become fluorescent during hybridization. They have a stem-loop structure and contain a fluorophore and a quencher group. The binding of the specific loop sequence with its complementary target nucleic acid sequence causes unfolding of the stem and the emission of a fluorescent signal during excitation at the appropriate wavelength.

The hybridization probe may also be a "capture" probe. In this case, the "capture" probe is immobilized or can be immobilized on a solid support by any appropriate means, i.e. directly or indirectly, for example by covalence or adsorption. The appropriate solid supports are known to those skilled in the art, and, by way of examples, mention may be made of synthetic materials or natural materials, latices, magnetic particles, metal derivatives, gels, etc. The solid support may be in the form of a microtitration plate, a membrane as described in application WO-A-94/12670 or a particle. It is also possible to immobilize several different capture probes on the support, each capture probe being specific for a target transcript. In particular, it is possible to use, as support, a biochip on which a large number of probes may be immobilized.

The immobilization of the probes on the support is also known to those skilled in the art, and mention may be made of a deposit of probes by direct transfer, microdeposition, in situ synthesis and photolithography.

The demonstration, in the biological sample, of the DNA modifications or anomalies in the gene encoding the tumor marker of interest may be carried out by any method for determining DNA alterations in a sample, namely either the direct detection of mutations, or the demonstration of alterations in the methylation profile of the loci of interest, or any other method for determining DNA alterations in a sample, known to those skilled in the art.

The mutations may include point substitutions of one nucleotide with another, deletions of one or more nucleotides and insertions of one or more nucleotides. The mutations may be located in the coding portion of the gene of the tumor marker of interest, or in the 5' and 3' noncoding portions, such as the transcription promoter region or the transcription termination region.

The strategies for demonstrating a mutation are based on molecular biology techniques and comprise steps of DNA extraction, amplification by PCR or another amplification technique, hybridization and/or sequencing. In the case of colorectal cancer, the following method has been successfully used to detect mutations in fecal DNA: concentration of the DNA by precipitation, enrichment in the target using capture oligonucleotides on magnetic beads, PCR amplification of the genes of interest, solid-phase sequencing for identifying point mutations[64]. The deletions were identified with respect to the difference in size between the expected reference fragment and the mutated fragment. Imperiale et al.[64] have described a panel of 21 mutations located in the K-ras, APC and p53 genes, which makes it possible to detect 16/31 of invasive cancers.

Other DNA markers used are the BAT-26 deletion, which is a marker for instability of microsatellites and highly amplifiable DNA called long DNA (L-DNA), which is not a specific marker but which appears to reflect the disorganized apoptosis of exfoliated tumor cells in the colonic lumen[65]. These markers are not satisfactory, either in terms of their sensitivity or in terms of their specificity.

As previously indicated, the DNA alterations may also correspond to a modification of the methylation profile of the gene corresponding to the tumor marker of interest. The modification of the methylation profile may correspond to a hypomethylation (decrease in the number of methylations) or to a hypermethylation (increase in the number of methylations). The altered units may be located in the coding portion of the gene of the tumor marker of interest, or in the 5' and 3' noncoding portions, such as the transcription promoter region or the transcription termination region.

The analysis of the DNA methylation may be carried out using techniques based on qualitative and/or quantitative PCR, such as MSP (methylation-specific PCR), bisulfite sequencing, digestion with a methylation-sensitive restriction enzyme coupled with PCR, COBRA (combined bisulfite restriction analysis) and Ms-SNuPE (methylation-sensitive single nucleotide primer extension). All these techniques have been reviewed comparatively and in detail in a methodology article[66].

In the literature, several hypermethylated genes have been reported in the case of colorectal cancer. By way of example, mention may be made of the ALX4 (aristaless-like homeobox-4) gene[56], the promoter region of the TPEF/HHP1 (transmembrane protein containing epidermel growth factor and follistatin domain) gene[67] or else the septin-9 gene[68].

When, in the method of the invention, at least two markers are detected, they may be demonstrated separately, for example using different immunoassay measurements, or else simultaneously, in a multiplex assay.

When, in the method of the invention, two markers of different nature are detected, for example a protein marker and an mRNA marker, two different detection methods, chosen from those described above, may be used. They may also be detected simultaneously, in the same detection medium and under the same reaction conditions, as described in patent application WO 03/104490. The steps of the detection method described in this patent application, which consists in simultaneously detecting hybridization and immunological reactions in a sample that may contain target analytes constituted of at least one nucleic acid and of at least one other ligand of different nature, consist in:

(i) depositing a known volume amount of the sample diluted in a reaction buffer, on a capture surface precoated with capture partners for said target analytes, said capture partners comprising at least one nucleic probe and at least one antiligand,

(ii) reacting at a temperature of between 15° C. and 60° C., and

(iii) visualizing the hybridization and immunological reactions thus obtained.

The biological sample may require a particular treatment because it may contain the tumor marker(s) sought in the method of the invention, as such, or else it may contain circulating tumor cells which contain the markers sought in

the method of the invention and/or circulating tumor cells which are capable of secreting the marker(s) sought in the method of the invention.

Thus, according to one embodiment of the invention, the biological sample is pretreated in order to isolate the circulating tumor cells contained in said fluid.

The expression "isolate circulating tumor cells" is intended to mean obtain a cell fraction enriched in circulating tumor cells.

The treatment of the biological sample in order to isolate the circulating tumor cells can be carried out by cell sorting in a flow cytometer, by enrichment on Ficoll, by enrichment with magnetic beads covered with specific antibodies, or by any other method of specific enrichment known to those skilled in the art.

In the case of blood as biological sample, the circulating tumor cells may be isolated by means of a technique of cell separation on Ficoll combined with depletion of the blood cells using anti-CD45 antibodies coupled to magnetic beads (Dynal Biotech ASA, Norway).

The detection of the tumor marker(s) sought in the method of the invention can then be carried out directly using circulating tumor cells isolated from the biological sample, for example by immunocytochemical labeling of these cells with an antibody against tumor marker(s) sought in the method of the invention, after having deposited the circulating tumor cells on a slide by cytospin. The detection of the tumor marker(s) sought in the method of the invention may also be carried out directly in the circulating tumor cells using the flow cytometry method as described in Métézeau et al.[69].

Under these conditions, said circulating cells can be treated under conditions which make it possible to block the tumor marker(s) sought in the method of the invention, inside said cells. Such a treatment is described by Mathieu et al.[70].

The detection of the tumor marker(s) sought in the method of the invention is then carried out after having made the cell membrane permeable so as to allow entry of the binding partners specific for the marker(s) sought in the method of the invention.

The direct detection of the tumor marker(s) used in the method of the invention, based on the circulating cells, may also be carried out by means of an ELISPOT method, for example by means of the method described in patent application WO 03/076942 filed by the applicant. This method is a method for detecting and/or quantifying circulating tumor cells of a biological sample, which are capable of releasing or secreting, in vitro, one or more tumor marker(s), comprising the steps consisting in:

(i) depositing an amount of said cells at the bottom of a culture surface to which at least one binding partner specific for said tumor marker(s) is attached,

(ii) culturing said cells under conditions such that they release or secrete said tumor markers, which are immunocaptured at the bottom of the culture surface,

(iii) removing the cells by washing,

(iv) adding at least one labeled conjugate specific for said tumor markers, and

(v) visualizing the labeling thus obtained.

The direct detection of the tumor marker(s) used in the method of the invention in the tumor cells may also be carried out in the culture medium of said cells after having cultured them under conditions such that they secrete tumor marker(s) used in the method of the invention.

The culture conditions for release or the expression of the tumor markers are conventional conditions such as 37° C. under a humid atmosphere and at 5% CO_2.

When the biological sample is a solid sample, the presence of the tumor marker(s) may also be shown in vivo, in situ in the tumors.

In order to show the presence of a tumor marker in a tumor in vivo, any imaging method known to those skilled in the art may be used. For this, a binding partner for said tumor marker may be coupled to an imaging tracer.

The term "coupling of the binding partners to an imaging tracer" is intended to mean the attachment of a tracer capable of being detected by any imaging method known to those skilled in the art, and of directly or indirectly generating a detectable signal. Thus, the tracer may be a radioactive tracer such as technetium-99. In this case, the organ which has the primary cancer or the metastases will bind the tumor marker and its tracer. The radiation emitted by the organ can be filmed with a special camera, for example a gamma-camera. The instrument collects the scintillations generated by the radioactive substance and thus makes it possible to visualize the organ.

In another method of the invention, the tracer may comprise a positron-emitting radioactive substance (fluorine 18). The images will then be acquired by a positron emission tomography system.

In another preferred method of the invention, the partner of the tumor marker(s) may be coupled to nanoparticles. By way of example, they may be supramagnetic nanoparticles; for example, anionic magnetic nanoparticles for use in direct cell labeling and in vivo detection by nuclear magnetic resonance imaging. They may also be gold nanoparticles.

By virtue of the methods of the invention which enable the detection of the tumor marker in vivo, the areas of the body where there has been binding of the binding partner for the tumor marker, cancers producing tumor marker, and in particular colorectal cancer, and also localizations of their remote metastases and involved lymph nodes, may be visualized.

The method of the invention may be used not only for early diagnosis but also for screening, therapeutic follow-up, prognosis and relapse diagnosis in relation to colorectal cancer, since only the cancer cells secrete LEI and this production depends on the grade of the cancer, which constitutes another subject of the invention.

The invention will be understood more clearly by means of the following examples which are given by way of nonlimiting illustration, and also by means of the attached FIGS. **1** to **21**, in which:

FIG. **1** is a graph relating to the assaying by ELISA of LEI, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **2** is a graph relating to the assaying by ELISA of Ezrin, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **3** is a graph relating to the assaying by ELISA of Aminoacylase 1, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **4** is a graph relating to the assaying by ELISA of L-FABP, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **5** is a graph relating to the assaying by ELISA of I-FABP, in pg/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **6** is a graph relating to the assaying by ELISA of Apolipoprotein AI, in μg/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−), either by microplate ELISA (FIG. **6**A), or with the Linco-plex kit (FIG. **6**B),

FIG. **7** is a graph relating to the assaying, using the Linco multiplex kit, of Apolipoprotein AII, in μg/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **8** is a graph relating to the assaying by ELISA of I-Plastin, in RFV (relative fluorescence value), in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **9** is a graph relating to the assaying by ELISA of Beta2-Microglobulin, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **10** is a graph relating to the assaying by ELISA of CEA, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **11** is a graph relating to the assaying by ELISA of CA 19-9, in U/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **12** is a graph relating to the assaying by ELISA of Testosterone, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **13** is a graph relating to the assaying by ELISA of E-Cadherin, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **14** is a graph relating to the assaying by ELISA of PAP1, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **15** is a graph relating to the assaying by ELISA of Galectin-3, in RFV, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **16** is a graph relating to the assaying by ELISA of LDH, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **17** is a graph relating to the assaying by ELISA of Proteasome 20S, in ng/ml, in the serum of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **18** is a graph relating to the assaying by ELISA of Aminoacylase 1, in ng/ml, in the stools of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **19** is a graph relating to the assaying by ELISA of Galectin-3, in RFV, in the stools of patients having colorectal cancer (CRC+) and of healthy patients (CRC−),

FIG. **20** is a graph relating to the assaying by ELISA of Proteasome 20S, in RFV, in the stools of patients having colorectal cancer (CRC+) and of healthy patients (CRC−), and

FIG. **21** is a graphic representation of an ELISPOT assay for LEI, for Ezrin and for Galectin-3, in number of spots per 10^6 cancer cells of the Caco-2, HT-29 and HT29-B6 lines.

EXAMPLE 1: CLONING OF THE GENES ENCODING THE TUMOR MARKERS AND EXPRESSION OF THE RECOMBINANT PROTEINS

1. cDNA Amplification and Cloning

The Caco-2 colorectal cancer line is cultured in DMEM medium containing 2 mM of L-glutamine, without FCS (fetal calf serum) (all Gibco).

For the cloning of the LEI, L-FABP and Gal-3 genes, the messenger RNAs were extracted from a pellet of 10^8 Caco-2 cells using the Invitrogen FastTrack 2.0 kit (Cat. No. 45-0019) according to the protocol supplied by the manufacturer. The reverse transcription and PCR steps are carried out in a single step using 450 ng of Caco-2 mRNA with the Superscript III One Step RT-PCR System kit (Invitrogen Cat. No. 12574-018) using the Platinum Taq DNA polymerase enzyme according to the protocol supplied by the manufacturer. The PCR primers used for the gene amplification are given in Table 1.

TABLE 1

Genes and primers	Oligonucleotides
LEI	
OL215 (SEQ ID No. 1)	5'-ATGGAGCAGCTGAGCTCAGCAAAC-3'
OL216 (SEQ ID No. 2)	5'-CTAAGGGGAAGAAAATCTCCCCAA-3'
L-FABP	
Forward (SEQ ID No. 3)	5'-CGGAGCGTCTCCCATGAGTTTCTCCGGCAAGTA-3'
Reverse (SEQ ID No. 4)	5'-GAAATGCAGACTTGTCTAGATGCGCTTGCTGATG CGCTTGAAGACAATG-3'
Gal-3	
OL217 (SEQ ID No. 5)	5'-ATGGCAGACAATTTTTCGCTCC-3'
OL218 (SEQ ID No. 6)	5'-TTATATCATGGTATATGAAGCACTGG-3'

The DNA fragments obtained were cloned into the vector pCR2.1 TOPO (LEI and Gal-3) with the TA cloning kit (Invitrogen Cat. No. K4520-01) or the vector pCMV6-XL4 from Origene (L-FABP) after digestion with Bsm BI and Xba I. The plasmids were sequenced in order to verify that the cDNA indeed complies with the expected sequence.

For the cloning of the gene encoding Aminoacylase 1, the total RNA was extracted from a pellet of 10^8 Caco-2 cells using the RNA Easy Mini kit from Qiagen, according to the protocol supplied by the manufacturer. The reverse transcription is carried out using 10 ng of Caco-2 RNA, with the Superscript II enzyme (Invitrogen) according to the protocol supplied by the manufacturer. The reverse transcription primer is an oligo(dT).

The cDNA derived from this reaction was used as template in a PCR reaction using the AccuPrime pfx kit (Invitrogen Cat. No. 12344-024) according to the protocol supplied by the manufacturer. The PCR primers are:

ACY-1 fwd2 (SEQ ID No. 7:
5'-GCGAATTCTTTAAGAAGGAGATATACATATGACGAGCAAAGGTCCGG

AAGAGGAGCACCCATCG-3')
and

ACY-1 Rev (SEQ ID No. 8:
5'-GCAAGCTTCAGCTGTCACTGGGCAGGGC-3').

Under these conditions, it was possible to amplify a 1.3 kb fragment which was cloned into a cloning vector of the Zero Blunt TOPO PCR cloning kit type (Invitrogen Cat. No. K2820-20). This plasmid was sequenced in order to verify that the cDNA indeed complies with the expected sequence.

The following DNA fragment (SEQ ID No. 9) containing the I-FABP open reading frame was obtained by chemical synthesis, carried out by the company Geneart.

SEQ ID No. 9:
GGTACCGAATTCCGCGTTTGACAGCACTTGGAAGGTAGACCGGAGTGAAA

ACTATGACAAGTTCATGGAAAAAATGGGTGTTAATATAGTGAAAAGGAAG

CTTGCAGCTCATGACAATTTGAAGCTGACAATTACACAAGAAGGAAATAA

ATTCACAGTCAAAGAATCAAGCGCTTTTCGAAACATTGAAGTTGTTTTTG

AACTTGGTGTCACCTTTAATTACAACCTAGCAGACGGAACTGAACTCAGG

GGGACCTGGAGCCTTGAGGGAAATAAACTTATTGGAAAATTCAAACGGAC

AGACAATGGAAACGAACTGAATACTGTCCGAGAAATTATAGGTGATGAAC

TAGTCCAGACTTATGTGTATGAAGGAGTAGAAGCCAAAAGGATCTTTAAA

AAGGATTCTAGAGTCGACGAGCTC.

2. Expression Vector Construction

The genes encoding LEI and Galectin-3 were subcloned into the prokaryotic expression vector pMR78[71] and the L-FABP gene was cloned into the vector pET3d (New England Biolabs). The restriction sites necessary for the cloning were introduced by PCR using, as template, the plasmids pCR2.1 TOPO-LEI, pCR2.1 TOPO-Gal-3 and pCMV6-LFABP. The PCR enzyme is the Promega Pfu DNA polymerase, the PCR reaction was carried out according to the manufacturer's instructions, with the primers given in Table 2.

The PCR products containing the open reading frames encoding LEI or Galectin-3 were digested with the Eco RI and Hind III restriction enzymes. The fragments were introduced into the vector pMR78 restricted with the same enzymes (plasmids pMR-LEI and pMR-Gal-3). The vector pMR78 contains a 6-histidine sequence in frame with the protein to be expressed, which enables purification by metal-chelate affinity chromatography. The L-FABP PCR product was cloned into the vector pET3d, at the Nco I and Bam HI restriction sites.

For Aminoacylase 1, the TOPO cloning vector was directly digested with the Eco RI and Hind III restriction enzymes in order to generate a 1.3 kb fragment containing the acy1 open reading frame, which was introduced into the vector pStaby1 (Eurogentec). The recombinant plasmid is called pStaby1-ACY.

For I-FABP, the cloning vector provided by Geneart was digested with the Eco RI and Sal I restriction enzymes in order to generate an approximately 400 bp fragment containing the coding sequence, which was introduced into the vector pMRCH79 (derived from the vector pMR78, bioMérieux). The recombinant plasmid is called pMRCH-IFABP.

The plasmids pGEX-Ezrine and pGEX-1-plastin, which make it possible to express, respectively, Ezrin and I-Plastin fused with GST (glutathione S-transferase), were supplied by the Institut Curie.

3. Recombinant Protein Expression and Purification

The expression plasmids for producing the recombinant tumor markers are introduced into *E. coli* BL21 bacteria and derivatives (Stratagene). The cultures are carried out at ambient temperature with shaking. The precise culture conditions for each protein are reproduced in Table 3. IPTG is isopropyl-beta-D-1-thiogalactosidase.

The bacterial pellets are taken up in 2×PBS (phosphate buffered saline) buffer and passed through a cell disintegrator at 1.5 kbar (Constant System). The lysates are centrifuged at 3000 g for 30 min at 4° C. The supernatant contains the soluble proteins. The pellet contains the inclusion bodies. The buffer for solubilizing the inclusion bodies depends on the protein.

For LEI, the purification is carried out using the soluble fraction, on a column containing 5 mL of Ni-NTA-Sepharose resin (Qiagen) and the protein is eluted with 2×PBS containing 450 mM imidazole, pH 7.5.

TABLE 2

Genes and primers	Oligonucleotides
LEI	
OL228 (SEQ ID No. 10)	5'-ATGGGAATTCAGGAGCAGCTGAGCTCAGCAA-3'
OL229 (SEQ ID No. 11)	5'-CGATAAGCTTAAGGGGAAGAAAATCTCCCC-3'
L-FABP	
Forward (SEQ ID No. 12)	5'-GCTGGCCATGGGCAGCAGCCATCATCATCATCATCA CATGAGTTTCTCCGGCAAGTACCAAC-3'
Reverse (SEQ ID No. 13)	5'-GCACGGATCCTAGATGCGCTTGCTGATGCGCTTGAA GAC-3'
Gal-3	
OL230 (SEQ ID No. 14)	5'-ATGGGAATTCAGGCAGACAATTTTTCGCTCC-3'
OL231 (SEQ ID No. 15)	5'-CGATAAGCTTATATCATGGTATATGAAGCACTGG-3'

For Galectin-3, the inclusion bodies are solubilized in 2×PBS containing 1M urea, and passed over 5 mL of Ni-NTA-Sepharose resin (Qiagen) and the Gal-3 protein is eluted with 2×PBS containing 450 mM imidazole and 1M urea, pH 7.5.

For L-FABP, the purification is carried out using the soluble fraction, with the Ni-IDA kit from Macherey-Nagel.

TABLE 3

	Strain	Culture volume	IPTG induction	Purification
LEI	BL21	250 mL	0.1 mM	Ni-NTA
Gal-3	BL21-Codon plus (DE3)-RIPL	400 mL	0.5 mM	Ni-NTA
L-FABP	BL21	500 mL	0.1 mM	Ni-IDA
GST-Ezrin	BL21	250 mL	0.1 mM	GST
ACY-1	BL21-Codon plus (DE3)-RIPL	500 mL	0.1 mM	other

For GST-Ezrin, the purification is carried out using the inclusion bodies solubilized in 100 mM Tris buffer containing 8M urea and 10 mM DTT, by GST affinity chromatography. A column containing 5 mL of Glutathione Sepharose 4 fast flow gel (Amersham) is used. The equilibration and washing buffer is 2×PBS containing 0.05% Tween 20. The elution buffer is 50 mM Tris-HCl containing 20 mM reduced glutathione, pH 8.

For Aminoacylase 1, the soluble fraction of the culture is passed over an Amersham HiTrap Q FF column and the ACY-1 protein was eluted with 0.3M NaCl at pH 7.5. Since several other proteins were co-eluted under these conditions, the purification was continued on a hydrophobic-interaction column (HIC Phenyl HP, Amersham). The ACY-1 protein was eluted with 0.5M NaCl at pH 7.

The recombinant GST-1-Plastin protein was provided by the Institut Curie in purified form.

The recombinant Calreticulin protein was produced by the company Proteus Services for Industry (Dijon, France). The sequence encoding Calreticulin was obtained by chemical synthesis.

EXAMPLE 2: PRODUCTION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST THE TUMOR MARKERS

1. Animal Model

The immunization experiments were carried out in female BALB/c (H-2^d) mice that were 6 to 8 weeks at the time of the first immunization.

2. Immunogens and Immunizations

In order to increase the immune responses obtained in the mice and to be able to generate monoclonal antibodies, the tumor markers were produced in the form of recombinant proteins produced according to the procedures described in Example 1. The LDH protein was obtained from the company SciPac (Cat. No. 103-133). These proteins were mixed volume for volume with Freund's adjuvant (Sigma), prepared in the form of a water-in-oil emulsion and which is known to have a good immunogenic capacity. 3 mice were immunized for each tumor marker. The mice received 3 successive doses of 10 μg of the immunogens at 0, 2 and 4 weeks. All the injections were given subcutaneously. The first injection is given as a mixture with complete Freund's adjuvant, the following two are given as a mixture with incomplete Freund's adjuvant. Between D50 and D70 after the first injection, the humoral responses were restimulated with an intravenous injection of 100 μg of the recombinant protein.

3. Monitoring of the Appearance of the Humoral Response

In order to monitor the appearance of the antibodies, blood samples are taken regularly from the mice. The presence of the anti-tumor marker antibodies is tested using an ELISA. The protein of interest is used for capture (1 μg/well); after saturation, the antigen is reacted with various dilutions of the test sera (incubation at 37° C. for 1 h). The specific antibodies present in the serum are revealed with an AffiniPure goat anti-mouse IgG antibody conjugated to alkaline phosphatase (H+L, Jackson Immunoresearch, Cat no. 115-055-146), which binds to the antibodies being sought (0.1 μg/well).

4. Production of Monoclonal Antibodies

Three days after the final injection, for each tumor marker, one of the immunized mice was sacrificed. The blood and the spleen were taken. The splenocytes obtained from the spleen were cultured with Sp2/0-Ag14 myeloma cells in order for them to fuse and become immortalized, according to the protocol described by Köhler and Milstein[72,73]. After an incubation period of 12-14 days, the supernatants of the hybridomas obtained were screened in order to determine the presence of anti-tumor marker antibodies, using the ELISA assay described in point 3 of this example. When GST fusion proteins were used as immunogen, the clones directed against GST are eliminated by carrying out an ELISA screening with uncoupled GST for capture. The positive hybridoma colonies were subcloned twice according to the limiting dilution technique, which is well known to those skilled in the art.

5. Characterization of the Monoclonal Antibodies by Immunoblotting

The list of monoclonal antibodies obtained against the various tumor markers is given in Table 4. These monoclonal antibodies were analyzed by the Western blotting technique.

TABLE 4

Tumor markers	Monoclonal antibody name
Leukocyte Elastase Inhibitor (LEI)	21B10A5 and 10E1H1
Ezrin	4A7A6C1 and 4A9H5
Aminoacylase 1	2A7F6 and 11H7D9
I-plastin	3D11D10, 8C8C5, 3A3H2, 8G2D2
Calreticulin	5C10H10 and 11B6D11
L-lactate dehydrogenase chain B (LDH)	3F11E11 and 12F10G8
Galectin-3	12F8A12 and 14A5G1

5.1. Methodology

The Caco-2 and HT-29 line cell culture extracts are prepared by directly lyzing the cell pellet with 600 μl of an aqueous solution of 8.3M urea, 2M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 100 mM DTT, 2% Servalyte 4-9 (Serva, Heidelberg, Germany) and 0.1 g/l Orange G, and then treated according to the NuPAGE Novex gel sample preparation protocol (Invitrogen). To obtain the tissue extracts, tumor

and mucosal biopsies of patients GHBD001, GHBD004 and CLSP109 were dissected with a scalpel, and were then subjected to 10 cycles of extraction in the Medimachine system (Becton Dickinson) using 50-μm Medicons with 1 ml of PBS buffer containing 2.5 mM EDTA and protease inhibitors (tablets, Roche). These 10 ml of cell suspension are pooled, made up to 25 ml, and then centrifuged for 15 min at 600 g. The supernatant corresponds to the tissue extract which is treated according to the NuPAGE Novex gel sample preparation protocol. Reduced samples are used, at a final total protein concentration of 0.4 mg/ml. The deposit volume is 20 μl per well, on a NuPAGE Novex Bis-Tris 4-12% gel, with MOPS running buffer. After migration (at 200 V, for 1 hour) and transfer onto a PVDF membrane (at 400 mA, for 45 min), the quality of the transfer is assessed by staining with amido black.

The membranes are saturated with 5% skimmed milk (Régilait) in a solution of TNT (15 mM Tris, 0.14M NaCl, 0.5% Tween 20, pH 8) at ambient temperature for 1 hour. After saturation, the membranes are incubated for 1 hour with the various test antibodies diluted to 10 μg/ml in the saturating solution. After rinsing with TNT, the membranes are incubated for 1 hour at ambient temperature with an anti-mouse-horseradish peroxidase conjugate diluted to 1:5000, (Cat No. 115-035-062, Jackson Immunoresearch) in the saturating solution. After rinsing, the developing is carried out with the Substrate Supersignal West Dura Extended kit (Cat No. 34076, Pierce) according to the recommended information for use.

The chemiluminescence signal on the membranes was measured with the VersaDoc imaging system from Biorad. Based on the image of the Western blot, the volumes of the bands which correspond to the various tumor markers were evaluated with the QuantityOne software (Bio-Rad). The volume corresponds to the intensity of the chemiluminescence signal multiplied by the surface area of the band.

5.2. Results

The Western blotting results are reproduced in Table 5, which gives the volume of the bands corresponding to the tumor marker of interest for the Western blotting analyses, as a function of the various samples tested. These results show that the tumor markers tested are indeed expressed by the Caco-2 and HT-29 colon cancer lines, and also in the tissues, as shown with the extracts of tumor and mucosa, obtained from the patients. The intensity of the signal obtained with an antibody on a sample can be compared to the signals obtained with the other samples and the same antibody. The technique used makes it possible to confirm the presence or absence of the marker in the tissue (non-remote sample) and the specificity of the antibodies with respect to the markers. This technique was not used in this example in the remote samples because it would not make it possible to come to a conclusion regarding the presence or absence of the tumor marker in the remote samples, nor to determine whether the concentration of said tumor marker is increased or decreased in said samples. Furthermore, the experimental scheme used does not make it possible to compare the reactivity of one antibody with another.

TABLE 5

Tumor marker and antibody	Caco-2	HT-29	Tumor tissue GHBD001	Mucosal tissue GHBD004	Tumor tissue GHBD004	Mucosal tissue CLSP109	Tumor tissue CLSP109
LEI							
21B10A5	8365	7678	NT	60200	36506	NT	NT
10E1H1	0	0	NT	13357	6893	NT	NT
Ezrin							
4A9H5	7066	4742	NT	NT	NT	1588	2446
4A7A6C1	123436	116448	42480	15303	67439	NT	NT
Aminoacylase 1							
2A7F6	10687	4787	NT	NT	NT	4477	7238
11H7D9	217664	232005	36093	10513	30233	NT	NT
I-plastin							
3D11D10	136725	NT	NT	NT	NT	275477	246564
8C8C5	557	1110	4364	77	0	NT	NT
Calreticulin							
5C10H10	2842	3040	NT	NT	NT	2503	3294
11B6D11	3261	2937	NT	NT	NT	2070	2764
LDH							
3F11E11	45391	NT	NT	NT	NT	30411	13942
12F10G8	122907	154593	11841	15811	53285	NT	NT
Galectin-3							
12F8A12	245712	65790	18262	12961	7307	NT	NT
14A5G1	254531	120010	79833	98361	45872	NT	NT

NT: not tested.

5.3. Monoclonal Antibodies Directed Against I-Plastin

In the patient GHBD004, the 8C8C5 antibody does not light up, or only very weakly lights up, the band which corresponds to I-plastin. The presence of 1-plastin in these samples can be demonstrated using, for example, the 8G2D2 antibody, which has a better affinity for I-plastin in blotting.

Since I-plastin is a member of a family of proteins comprising 2 other isoforms (L-plastin and T-plastin) with which it has more than 70% homology, we tested all the clones of monoclonal antibodies obtained, for their reactivity with respect to the GST-plastin-L and GST-plastin-T proteins (provided by the Institut Curie). At the end of this

screening, we selected the clones 3D11D10, 8C8C5, 3A3H2 and 8G2D2 which do not exhibit any cross-reactivity with the other members of the family. These antibodies are indeed specific for the I-plastin isoform.

EXAMPLE 3: SERUM ASSAYS FOR THE TUMOR MARKERS

1. Patients and Specimens

Blood samples are collected from a network of 8 clinical centers distributed throughout France, in the context of 2 Huriet-law protocols.

In order to obtain serum, the blood sample is taken on a dry tube. In order to obtain plasma, the blood sample is taken on an EDTA tube. After coagulation, the tube is centrifuged for 10 min at 1000 g, and the serum is removed, aliquoted and stored at −80° C. The tube of plasma is directly centrifuged for 10 min at 1000 g, and the plasma is removed, aliquoted and stored at −80° C. The samples are completely documented for the clinical history of the patients.

2. Serum Assay for the LEI Tumor Marker

The LEI protein was assayed using the antibodies described in Example 2 and an ELISA assay using the Vidas® automated device (bioMérieux). To do this, the ELISA assay was constructed using the reagents of the Vidas® HBs Ag Ultra kit (bioMérieux, Cat. No. 30315). The reagents were used as described in the corresponding information sheet (ref. 11728 D-FR-2005/05), with the following modifications:

1. The cones were sensitized with the capture antibody 10E1H1 at a concentration of 10 μg/ml.
2. The content of the second well of the HBs Ag Ultra cartridge was replaced with 300 μl of revealing antibody 21B10A5, coupled to biotin, diluted to 1 μg/ml in the buffer of the second well of the Vidas® HBs Ag Ultra kit (buffer with goat serum and sodium azide at 1 g/l).
3. The serum, plasma or stool samples (50 μl) were diluted directly in the second well of the HBs Ag Ultra cartridge, pure or after a prior dilution to 1/20 in the buffer of the second well of the Vidas® HBs Ag Ultra kit (buffer with goat serum and sodium azide at 1 g/l).
4. The ELISA reaction was carried out using the Vidas® automated device and the protocol of the HBs Ag Ultra kit.
5. The results were obtained in the form of crude values after subtraction of the background noise (reading of the substrate before reaction).

A standard curve was established by assaying a range of concentrations of the tumor marker in the form of recombinant protein. The standard curve was plotted by reporting the concentration of the tumor marker along the x-axis and the signal read by Vidas® (RFV or Relative Fluorescence Value) along the y-axis. The concentration of tumor marker present in the body fluid to be assayed (blood, serum, plasma, stool) was calculated by reporting the concentration corresponding to the RFV signal read by Vidas®. The results of assaying the serum LEI in patients by ELISA on a Vidas automated device are given in Table 6.

TABLE 6

Pathological condition[a]	Sample identifier	Nature	Stage	TNM[b]	LEI ng/ml
CRC+	CLSP047/F0	Serum	I	T1N0M0	211.96
CRC+	CLSP080/F0	Serum	I	T2N0N0	133.49
CRC+	CLSP076/F0	Serum	II	T3N0M0	132.93
CRC+	GHBD020/F0	Serum	II	T3N0M0	232.71
CRC+	GHBD021/F0	Serum	II	T3N0M0	160.40
CRC+	GHBD023/F0	Serum	II	T3N0M0	153.67
CRC+	GHBD025/F0	Serum	II	T3N0M0	131.25
CRC+	GHBD029/F0	Serum	II	T3N0M0	157.59
CRC+	CBSE005/F0	Plasma	III	T3N1M0	4224.68
CRC+	CLSP050/F0	Serum	III	T2N1M0	120.60
CRC+	CLSP072/F0	Serum	III	T3N1M0	169.92
CRC+	CLSP078/F0	Serum	III	T3N1M0	535.09
CRC+	CLSP089/F0	Serum	III	T4N2M0	130.69
CRC+	CLSP090/F0	Serum	III	T1N1M0	317.94
CRC+	GHBD019/F0	Serum	III	T3N1M0	156.47
CRC+	CBSE008/F0	Serum	IV	T4N3M1	178.33
CRC+	CBSE021/F0	Serum	IV	T4N2M1	549.68
CRC+	CBSE026/F0	Serum	IV	T4N1M1	1356.05
CRC+	CLSP068/F0	Serum	IV	TxNxM1	1571.87
CRC+	CLSP156/F0	Serum	IV	T4N0M1	160.96
CRC+	CSEM005/F0	Serum	IV	T4N2M1	348.79
CRC+	CSEM005/F1	Serum	IV	T4N2M1	2664.06
CRC+	CLSP153/F0	Serum	III/IV	T3N2Mx	193.46
CRC+	CBSE003/F0	Serum	IV	T2N0M1	139.66
CRC−	N017197/F0	Serum			209.72
CRC−	N017250/F0	Serum			229.34
CRC−	N017349/F0	Serum			615.37
CRC−	N017357/F0	Serum			794.01
CRC−	N017373/F0	Serum			320.75
CRC−	N018640/F0	Serum			163.76
CRC−	N018667/F0	Serum			282.61
CRC−	N030068/F0	Serum			417.23
CRC−	N143574/F0	Serum			203.55
CRC−	N146628/F0	Serum			335.89
CRC−	N146978/F0	Serum			234.39
CRC−	N148324/F0	Serum			290.46
CRC−	N314199/F0	Serum			101.55
CRC−	N314324/F0	Serum			160.39
CRC−	N370510/F0	Serum			212.52
CRC−	N417852/F0	Serum			115.00
CRC−	N469695/F0	Serum			214.77
CRC−	N491079/F0	Serum			185.06
CRC−	N491124/F0	Serum			231.59
CRC−	N491722/F0	Serum			830.54
CRC−	N51869/F0	Serum			87.54
CRC−	N688038/F0	Serum			238.31
CRC−	N688118/F0	Serum			251.21
CRC−	N835827/F0	Serum			363.37
CRC−	N836221/F0	Serum			216.45

[a]CRC+: patients having colorectal cancer/CRC−: healthy individual
[b]TNM: stage of tissue invasion (T), lymph node invasion (N) and remote invasion (metastases, M)

The amounts obtained for the patients analyzed are reported in FIG. **1**. It may be noted, on this figure, that 3 sera of patients having stage IV colorectal cancer and 1 serum of a patient having stage III colorectal cancer show a clear increase in their amount of serum LEI.

3. Serum Assay for the Ezrin Tumor Marker

The Ezrin protein was assayed using the antibodies described in Example 2 and an ELISA assay using the Vidas® automated device (bioMérieux). To do this, the ELISA assay was constructed using the reagents of the Vidas® HBs Ag Ultra kit (bioMérieux, Cat. No. 30315). The reagents were used as described in the corresponding information sheet (ref. 11728 D-FR-2005/05), with the following modifications:

1. The cones were sensitized with the capture antibody 4A9H5 at a concentration of 30 μg/ml.
2. The content of the second well of the HBs Ag Ultra cartridge was replaced with 300 μl of revealing antibody

4A7A6C1, coupled to biotin, diluted to 1 μg/ml in the buffer of the second well of the Vidas® HBs Ag Ultra kit (buffer with goat serum and sodium azide at 1 g/l).

3. The serum, plasma and stool samples (50 μl) were diluted directly in the second well of the HBs Ag Ultra cartridge.

4. The ELISA reaction was carried out using the Vidas® automated device and the HBs Ag Ultra protocol, in which the step of incubating the sample with the capture and revealing antibodies had been taken to 100 cycles.

5. The results were obtained in the form of crude values after subtraction of the background noise (reading of the substrate before reaction).

The concentration of the tumor marker present in the body fluid to be assayed (blood, serum, plasma, stool) was calculated according to the procedure described in paragraph 2 regarding the assaying of LEI.

The amounts obtained for the patients analyzed are reported in FIG. **2**. It may be noted, in this figure, that 3 sera from patients having stage IV colorectal cancer show a clear increase in their amount of serum Ezrin.

4. Serum Assay for the Aminoacylase 1 Tumor Marker

The Aminoacylase 1 protein was assayed using the antibodies described in Example 2 and an ELISA assay using the Vidas® automated device (bioMérieux). To do this, the ELISA assay was constructed using the reagents of the Vidas® HBs Ag Ultra kit (bioMérieux, Cat. No. 30315). The reagents were used as described in the corresponding information sheet (ref 11728 D-FR-2005/05), with the following modifications:

1. The cones were sensitized with the capture antibody 2A7F6 at a concentration of 20 μg/ml.
2. The content of the second well of the HBs Ag Ultra cartridge was replaced with 300 μl of revealing antibody 11H7D9, coupled to biotin, diluted to 1 μg/ml in the buffer of the second well of the Vidas® HBs Ag Ultra kit (buffer with goat serum and sodium azide at 1 g/l).
3. The serum, plasma or stool samples (100 μl) were diluted directly in the second well of the HBs Ag Ultra cartridge.
4. The ELISA reaction was carried out using the Vidas® automated device and the HBs Ag Ultra protocol, in which the step of incubating the sample with the capture and revealing antibodies had been taken to 100 cycles.
5. The results were obtained in the form of crude values after subtraction of the background noise (reading of the substrate before reaction).

The concentration of the tumor marker present in the body fluid to be assayed (blood, serum, plasma, stool) was calculated according to the procedure described in paragraph 2 regarding the assaying of LEI.

The amounts obtained for the patients analyzed are reported in FIG. **3**. It may be noted, in this figure, that 1 serum from a patient having stage II colorectal cancer, 1 serum from a patient having stage III colorectal cancer and 2 sera from patients having stage IV colorectal cancer show a clear increase in their amount of serum Aminoacylase 1.

5. Serum Assay for the L-FABP Tumor Marker

We used an ELISA kit marketed by the company Hycult Biotechnology to assay the human L-FABP protein (Cat. No. HK404). This kit makes it possible to quantify the L-FABP protein in cell culture supernatants or in serum, plasma or urine, in order to determine the presence of lesions in the liver. We followed the procedure recommended by the manufacturer, with 2 modifications: the incubations were carried out at 37° C. and not at ambient temperature, the sera were diluted to $1/10^{th}$ before the assay. The assaying of the L-FABP protein can be carried out by alternative techniques, well known to those skilled in the art.

FIG. **4** gives the results of this assay. In the serum panel that we tested, 41 patients out of 141 having colorectal cancer have a serum L-FABP concentration of greater than 17 ng/ml, whereas, in the control group, no individual exceeds this value. Among these 41 patients, are 8 patients with stage I colorectal cancer, 8 with stage II colorectal cancer, 13 with stage III colorectal cancer and 12 with stage IV colorectal cancer. The mean serum L-FABP concentration observed for 141 patients with colorectal cancer is 16.6±1.3 ng/ml. The mean value is 6.6±0.2 ng/ml for 112 healthy individuals (negative controls). This difference is statistically significant ($P<0.0001$, one-sided t-test with Welch's correction for unequal variances).

6. Serum Assay for the I-FABP Tumor Marker

We used an ELISA kit marketed by the company Hycult Biotechnology to assay the human I-FABP protein (Cat. No. HK406). This kit makes it possible to quantify the I-FABP protein in cell culture supernatants or in serum, plasma or urine, in order to determine the presence of ischemic lesions in the small intestine. We followed the procedure recommended by the manufacturer. The assaying of the I-FABP protein can be carried out by alternative techniques, well known to those skilled in the art.

FIG. **5** gives the results of this assay. In the serum panel that we tested, 15 patients out of 40 having colorectal cancer have a serum I-FABP concentration of greater than 40 pg/ml, whereas, in the control group, only 2 individuals out of 24 exceed this value. More clearly, 3 sera of patients having stage I colorectal cancer, 2 sera of patients having stage III colorectal cancer and 1 serum of a patient having stage IV colorectal cancer have a serum I-FABP concentration of greater than 100 pg/ml. No concentration above this value was found in the CRC-control group.

7. Serum Assay for the Apolipoprotein AI Tumor Marker

The assaying of serum Apolipoprotein AI was carried out by means of two different immunoassay techniques. Firstly, we used a microplate sandwich ELISA. The 96-well plates were coated with the anti-Apo AI monoclonal antibody, clone 1404 (Biodesign Cat. No. H45404) at 1 μg per well. After 3 washes with PBS-0.05% Tween 20 (PBS-T), the plates were saturated with 10% milk in PBS-T for 1 h at 37° C. The plates were washed a further 3 times in PBS-T, 100 μl of the dilutions of the standard range or 100 μl of the 1/100000 dilution of the test serum samples were deposited onto the plates, and the plates were incubated for 2 h at 37° C. The standard range was prepared by diluting the Apo AI protein (Biodesign Cat. No. A50620H) in PBS-T, BSA 1% (1.6 to 100 ng/ml). After 3 washes with PBS-T, the polyclonal detection antibody coupled to horseradish peroxidase (Biodesign Cat. No. K45452P) was added at 0.1 μg per well, and the plates were incubated for 2 h at 37° C. A further 3 washes with PBS-T were carried out, before adding the OptEIA substrate (BD), at 100 μl/well. After 20 min, when

the development of the color had taken place, the reaction was stopped with 2N sulfuric acid and the absorbence at 450 nm was measured.

FIG. **6**A gives the results of this assay. We demonstrated a decrease in serum concentration of Apo AI in individuals with colorectal cancer. The mean concentration in 38 individuals with stage I to IV CRC is 675±36 μg/ml, whereas it is much higher in 27 healthy individuals (controls): 1040±39 μg/ml. This difference is statistically very significant (P<0.0001, one-sided t-test). By way of comparison, in 13 individuals with liver cancer, the mean serum concentration of Apo AI is 1175±87 μg/ml with the sandwich ELISA technique used. The decrease in the serum concentration demonstrates that Apo AI is therefore a specific marker of colorectal cancer, it being possible for this decrease to be demonstrated by means of an immunoassay.

The second assaying technique that was used is a multiplex assay marketed by the company Linco, which makes it possible to assay several Apolipoproteins, including AI and AII, simultaneously, in the same sample (Cat. No. APO-62K). The procedure recommended by the manufacturer was applied.

FIG. **6**B gives the results of this assay. The decrease in the serum concentration of Apo AI in patients with CRC is confirmed with this second technique. The mean concentration of Apo AI in 34 individuals with stage I to IV CRC is 768±30 μg/ml, whereas it is much higher in 17 healthy individuals (controls): 1194±51 μg/ml. This difference is statistically very significant (P<0.0001, one-sided t-test).

8. Serum Assay for the Apolipoprotein AII Tumor Marker

The assaying of serum Apolipoprotein AII was carried out with the Linco multiplex kit. FIG. **7** gives the results of this assay. We demonstrated a decrease in the serum concentration of Apo AII in the individuals with colorectal cancer. The mean concentration of Apo AII in 34 individuals with stage I to IV CRC is 170±11 μg/ml, whereas it is much higher in 17 healthy individuals (controls): 277±16 μg/ml. This difference is statistically very significant (P<0.0001, one-sided t-test).

9. Serum Assay for the I-Plastin Tumor Marker

The I-Plastin protein was assayed using the antibodies described in Example 2 and an ELISA assay using the Vidas® automated device (bioMérieux). To do this, the ELISA assay was constructed using the reagents of the Vidas® HBs Ag Ultra kit (bioMérieux, Cat. No. 30315). The reagents were used as described in the corresponding information sheet (ref. 11728 D-FR-2005/05), with the following modifications:

1. The cones were sensitized with the capture antibody 3D11D10 at a concentration of 15 μg/ml.
2. The content of the second well of the HBs Ag Ultra cartridge was replaced with 300 μl of revealing antibody 8C8C5, coupled to biotin, diluted to 1 μg/ml in the buffer of the second well of the Vidas® HBs Ag Ultra kit (buffer with goat serum and sodium azide at 1 g/l).
3. The serum, plasma or stool samples (100 μl) were diluted directly in the second well of the HBs Ag Ultra cartridge.
4. The ELISA reaction was carried out using the Vidas® automated device and the HBs Ag Ultra protocol.
5. The results were obtained in the form of crude values after subtraction of the background noise (reading of the substrate before reaction).

The concentration of the tumor marker present in the body fluid to be assayed (blood, serum, plasma, stool) was calculated according to the procedure described in paragraph 2 regarding the assaying of LEI.

The amounts obtained for the patients analyzed are reported in FIG. **8**. The 2 sera of patients having colorectal cancer who were tested show a clear increase in their amount of serum I-Plastin.

10. Serum Assay for the Group-B Tumor Markers

The Beta2-Microglobulin, CEA, CA19-9 and Testosterone tumor markers were assayed using the assay kits of the applicant, respectively Vidas® β2-Microglobulin, Vidas® CEA, Vidas® CA19-9™ and Vidas® Testosterone, according to the procedure specific to each kit.

The E-Cadherin protein was assayed using the E-Cadherin EIA kit (Takara Biochemicals, Tokyo, Japan) according to the procedure of the kit.

The Regenerating Islet-Derived Protein 3 Alpha protein, otherwise known as pancreatitis associated protein (PAP1), was assayed using the PANCREPAP ELISA kit (DynaBio, Marseille, France) according to the procedure of the kit.

The Galectin-3 and LDH proteins were assayed using the antibodies described in Example 2. The Proteasome 20 S was assayed using the antibodies described in patent EP 0434670. To do this, the ELISA assays were constructed using the Vidas® automated device (bioMérieux) and the reagents of the Vidas® HBs Ag Ultra kit (bioMérieux, Cat. No. 30315). The reagents were used as described in the corresponding information sheet (ref. 11728 D-FR-2005/05), with the following modifications:

1. The cones were sensitized with the capture antibody at a concentration of between 5 and 30 μg/ml.
2. The content of the second well of the HBs Ag Ultra cartridge was replaced with 300 μl of revealing antibody, coupled to biotin, diluted to 1 μg/ml in buffer with goat serum and sodium azide at 1 g/l.
3. The serum, plasma or stool samples were diluted directly in the second well of the HBs Ag Ultra cartridge after, if necessary, a dilution in buffer of the second well.
4. The ELISA reaction was carried out using the Vidas® automated device and the HBs Ag Ultra protocol. The step of incubating the sample with the capture and revealing antibodies was between 14 and 100 cycles.
5. The results were obtained in the form of crude values after subtraction of the background noise (reading of the substrate before reaction).

The concentration of the tumor marker present in the body fluid to be assayed (blood, serum, plasma, stool) was calculated according to the procedure described in paragraph 2 regarding the assaying of LEI. The assay conditions for various tumor markers have been reproduced in Table 7.

TABLE 7

	Protein		
	Galectin-3	LDH-B	Proteasome 20 S
Capture antibody	12F8A12 at 15 μg/mL	3F11E11 at 10 μg/mL	GD6 at 30 μg/mL
Revealing antibody	14A5G1	12F10G8	7A11
Goat serum in dilution buffer	with	with	without
Stool volume	50 μL	50 μL	200 μL
Serum volume	50 μL	50 μL	100 μL

TABLE 7-continued

	Protein		
	Galectin-3	LDH-B	Proteasome 20 S
Sample deposit	2nd well	2nd well	1st well
Incubation time	100 cycles	14 cycles	14 cycles

The amounts obtained for the patients analyzed with the beta2-Microglobulin, CEA, CA19-9, Testosterone, E-Cadherin, Regenerating Islet-Derived Protein 3 Alpha, Galectin-3, LDH and Proteasome 20S tumor markers have been reported respectively in FIGS. **9** to **17**.

Three sera of patients having colorectal cancer show an increase in their amount of serum β2-Microglobulin.

Ten sera of patients having colorectal cancer show an increase in their amount of serum CEA. More clearly, 1 serum of a patient having stage III colorectal cancer and 7 sera of patients having stage IV colorectal cancer show a considerable increase in their amount of serum CEA.

Nine sera of patients having colorectal cancer show an increase in their amount of serum CA 19-9. More clearly, 1 serum of a patient having stage III colorectal cancer and 7 sera of patients having stage IV colorectal cancer show a considerable increase in their amount of serum CA 19-9.

Ten sera of patients having colorectal cancer show a decrease in their amount of serum Testosterone. More clearly, 1 serum of a patient having stage II colorectal cancer, 1 serum of a patient having stage III colorectal cancer and 2 sera of patients having stage IV colorectal cancer show a fall in their amount of serum Testosterone.

Two sera of patients having colorectal cancer show an increase in their amount of serum Regenerating Islet-Derived Protein 3 Alpha.

Four sera of patients having stage IV colorectal cancer, 2 sera of patients having stage III colorectal cancer and 1 serum of a patient having stage II colorectal cancer show a clear increase in their amount of serum Galectin-3.

EXAMPLE 4: USE OF THE SERUM ASSAYS FOR TUMOR MARKERS IN COMBINATION

The applicant showed in Example 3 that abnormally elevated or abnormally reduced amounts of tumor markers could be observed in the bloodstream of certain patients having colorectal cancer. Surprisingly, the increase or the decrease in the amount, in the blood, of two given markers is not systematically observed in the same patients. As a result, the combination of several tumor markers makes it possible to increase the number of patients identified as having colorectal cancer. Thus, a patient A may present an increase or a decrease in one or more tumor markers (group X), it being possible for said markers of group X to be normal in a patient B; in this same patient B, one or more other tumor markers (group Y) may be elevated or reduced, it being possible for said markers of group Y to be normal in patient A.

The various tumor markers assayed by the applicant may thus be combined by means of various mathematical algorithms well known to those skilled in the art. By way of illustration, and without this example being exhaustive in nature, the following method was carried out:

1. A threshold value was set for each tumor marker.
2. When the amount of the tumor marker in the blood was increased in the case of colorectal cancer, the amount in the blood, obtained for a given patient, was divided by its threshold value. When the amount of the tumor marker in the blood was decreased in the case of colorectal cancer, the amount in the blood, obtained for a given patient, was inverted and then multiplied by its threshold value.
3. When the "amount in the blood divided by threshold value" ratio was greater than 1, the ratio was multiplied by a coefficient, for example 10. The value thus obtained was named the "score", for the patient studied, for the tumor marker under consideration.
4. The scores obtained for various tumor markers were added, with them being weighted by a factor specific to each marker. In the case of the example below, all the weighting factors were set at 1.
5. The sum of the scores was divided by the total number of scores added, and the value thus obtained was named the "total score".
6. The patient is diagnosed as having colorectal cancer when his or her total score is increased relative to a threshold score.

The total scores for a selection of 2, 4 and 8 markers comprising LEI are given in Table 8.

The combination of the LEI and CEA tumor markers thus makes it possible to obtain, for the same group of 24 patients, increased total scores "2" in 12 patients having colorectal cancer, whereas assaying LEI or CEA alone showed an increase, respectively, in 4 and 10 patients only.

The combination of the LEI, Beta2-Microglobulin, Aminoacylase 1 and CEA tumor markers thus makes it possible to obtain, for the same group of 24 patients, increased total scores "4" in 16 patients having colorectal cancer, whereas assaying LEI, Beta2-Microglobulin, Aminoacylase 1 or CEA alone showed an increase, respectively, in 4, 4, 4 and 10 patients only.

The combination of the LEI, Beta2-Microglobulin, Aminoacylase 1, Proteasome 20S, L-FABP, PAP, E-Cadherin and CEA tumor markers thus makes it possible to obtain, for the same group of 24 patients having colorectal cancer, increased total scores "8" in 19 patients, whereas assaying LEI, Beta2-Microglobulin, Aminoacylase 1, Proteasome 20S, L-FABP, PAP, E-Cadherin or CEA alone showed an increase, respectively, in 4, 4, 4, 8, 6, 2, 2 and 10 patients only.

TABLE 8

Pathological condition	Sample identifier	Stage	TNM	LEI ng/ml	Total score 2^a	Total score 4^b	Total score 8^c
CRC+	CLSP047/F0	I	T1N0M0	211.96	0.29	0.44	0.43
CRC+	CLSP080/F0	I	T2N0N0	133.49	0.15	0.17	0.27
CRC+	CLSP076/F0	II	T3N0M0	132.93	0.25	0.40	1.90
CRC+	GHBD020/F0	II	T3N0M0	232.71	0.20	0.44	1.75
CRC+	GHBD021/F0	II	T3N0M0	160.39	0.14	0.29	0.40
CRC+	GHBD023/F0	II	T3N0M0	153.67	0.54	0.51	0.44
CRC+	GHBD025/F0	II	T3N0M0	131.25	0.17	3.31	2.15
CRC+	GHBD029/F0	II	T3N0M0	157.59	0.13	4.88	3.06

TABLE 8-continued

Pathological condition	Sample identifier	Stage	TNM	LEI ng/ml	Total score 2[a]	Total score 4[b]	Total score 8[c]
CRC+	CBSE005/F0	III	T3N1M0	4224.68	25.47	97.52	76.65
CRC+	CLSP050/F0	III	T2N1M0	120.60	0.28	4.02	4.64
CRC+	CLSP072/F0	III	T3N1M0	169.92	6.70	9.80	6.04
CRC+	CLSP078/F0	III	T3N1M0	535.09	0.38	0.44	5.14
CRC+	CLSP089/F0	III	T4N2M0	130.69	30.30	19.73	10.16
CRC+	CLSP090/F0	III	T1N1M0	317.94	0.23	0.28	0.32
CRC+	GHBD019/F0	III	T3N1M0	156.47	8.19	4.36	2.42
CRC+	CBSE008/F0	IV	T4N3M1	178.33	0.25	18.41	11.18
CRC+	CBSE021/F0	IV	T4N2M1	549.68	290.43	193.70	85.52
CRC+	CBSE026/F0	IV	T4N1M1	1356.05	240.50	160.42	74.05
CRC+	CLSP068/F0	IV	TxNxM1	1571.87	409.46	273.15	129.00
CRC+	CLSP156/F0	IV	T4N0M1	160.95	54.10	36.17	17.54
CRC+	CSEM005/F0	IV	T4N2M1	348.79	11.41	7.80	6.62
CRC+	CSEM005/F1	IV	T4N2M1	2664.06	16.40	16.40	18.31
CRC+	CLSP153/F0	III/IV	T3N2Mx	193.46	23.72	15.88	10.41
CRC+	CBSE003/F0	IV	T2N0M1	139.66	146.08	106.47	56.61
CRC−	N017197/F0			209.72	0.40	0.40	0.39
CRC−	N017250/F0			229.34	0.24	0.24	0.46
CRC−	N017349/F0			615.37	0.74	0.74	0.50
CRC−	N017357/F0			794.01	0.61	0.61	0.61
CRC−	N017373/F0			320.75	0.39	0.39	0.39
CRC−	N018640/F0			163.76	0.20	0.20	0.42
CRC−	N018667/F0			282.61	0.46	0.36	0.38
CRC−	N030068/F0			417.23	0.61	0.47	0.46
CRC−	N143574/F0			203.55	0.57	0.50	0.49
CRC−	N146628/F0			335.89	0.35	0.47	0.51
CRC−	N146978/F0			234.39	0.27	0.36	0.51
CRC−	N148324/F0			290.46	0.33	0.41	0.46
CRC−	N314199/F0			101.55	0.32	0.27	0.35
CRC−	N314324/F0			160.39	0.20	0.22	0.49
CRC−	N370510/F0			212.52	0.52	0.41	0.44
CRC−	N417852/F0			115.00	0.27	0.25	0.42
CRC−	N469695/F0			214.77	0.23	0.22	0.41
CRC−	N491079/F0			185.06	0.21	0.33	0.45
CRC−	N491124/F0			231.59	0.34	0.34	0.31
CRC−	N491722/F0			830.54	0.60	0.73	0.61
CRC−	N518569/F0			87.54	0.19	0.19	0.46
CRC−	N688038/F0			238.31	0.64	0.64	0.57
CRC−	N688118/F0			251.21	0.28	0.28	0.36
CRC−	N835827/F0			363.37	0.66	0.50	0.48
CRC−	N836221/F0			216.45	0.44	0.40	0.40
	Threshold			830.54	0.74	0.74	0.61

[a]combination of LEI and CEA

[b]combination of LEI, Beta2-Microglobulin, Aminoacylase 1 and CEA

[c]combination of LEI, Beta2-Microglobulin, Aminoacylase 1, Proteasome 20S, L-FABP, PAP, E-Cadherin and CEA

EXAMPLE 5: FECAL TUMOR MARKER ASSAYS

The stools are extracted using a piece weighing approximately 1 g, to which 10 ml of 100 mM sodium phosphate buffer, pH 7.2, containing 1 g/L of azide are added. The mixture is homogenized on a vortex for 1 min. The sample is then subjected to 4 cycles of ultrasound for 7 s on ice. The unsolubilized fraction is removed by centrifugation at 2000 g, for 10 min at 4° C. The supernant is stored at −30° C. until it is assayed.

The ELISA assays described in Example 3 were used to search for the tumor markers in the stools after, if necessary, an appropriate dilution of the stools in the buffer of the first well of the HBs Ag Ultra cartridge.

The assay determinations with the tests, Aminoacylase 1, Galectin-3 and Proteasome 20S, have been represented, respectively, in FIGS. **18** to **20**. An increase in the amount of Aminoacylase 1, of Galectin-3 and of Proteasome 20S is observed, respectively, for 10, 14 and 8 stools of patients having colorectal cancer.

EXAMPLE 6: DETECTION OF THE TUMOR MARKERS BY THE ELISPOT TECHNIQUE

1. Cell Culture

The LnCAP prostate cancer line is cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% FCS (all Gibco). The cells are used as a negative control.

The Caco-2 colorectal cancer line is cultured in DMEM medium containing 2 mM L-glutamine, without FCS (all Gibco).

The HT-29 colorectal cancer line is cultured in MEM medium containing 2 mM L-glutamine and 10% FCS (all Gibco).

The HT-29/B6 colorectal cancer line is cultured in DMEM medium containing 4 mM L-glutamine, without FCS (all Gibco).

The cells are maintained at 37° C., in an incubator with 5% CO_2.

2. The ELISPOT Technique

This procedure makes it possible to determine the number of cells secreting the protein. The 96-well ELISPOT plates

with PVDF membranes (Multiscreen IP, Millipore) are coated with the mouse anti-tumor marker monoclonal antibody at 10 μg/ml (capture antibody, see Table 9 below, which gives the antibodies used in ELISPOT), 100 μl per well, in sterile PBS, overnight at +4° C. The plates are then washed with PBS and saturated with culture medium containing 10% FCS. In parallel, the cells are trypsinized, counted, and then diluted to 10^5 cells/ml. 200 μl of this cell suspension are distributed per well, as are cascade dilutions of this stock solution. The plates are then incubated for 20 h at 37° C. in a humid atmosphere at 5% CO_2, and then washed with PBS containing 0.05% Tween-20. The remaining cells are then lyzed by treatment with ice-cold water for 10 minutes, and then the plates are again washed. The revealing antibody, the biotinylated monoclonal directed against the tumor marker to be assayed (Table 9), is then added at 0.1 μg/well (incubation for 2 h at ambient temperature). The spots are revealed by adding extravidin-alkaline phosphatase (Sigma) and the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Biorad). The background noise corresponds to the number of spots measured in the LnCap wells and varies between 0 and 8 spots under the reading conditions used. The average number of nonspecific spots was subtracted from the specific signal.

TABLE 9

Marker	Capture Ab	Detection Ab
LEI	10E1H1	21B10A5
Ezrin	4A9H5	4A7A6C1
Galectin-3	12F8A12	14A5G1

3. Results

The number of Caco-2, HT-29 and HT-29/B6 cells secreting the tumor marker of interest, per million incubated cells, is shown in FIG. **21**. The ELISPOT technique makes it possible to confirm the release or the secretion of the tumor markers by the colon cancer lines. It will be possible to carry out a search for circulating tumor cells in patients using this technique, according to the method of patent application WO 03/076942 filed by the applicant.

EXAMPLE 7: IMMUNOHISTOCHEMICAL DETECTION OF THE TUMOR MARKERS USING COLONIC TISSUES

1. Methodology

Firstly, the tissue-microarray slides are deparaffinized. For this, they are incubated successively in the following baths for 10 minutes: methylcyclohexane (twice), 100% ethanol, 95% ethanol, 70% ethanol and water. The slides are then rinsed with TBS containing 0.1% Tween 20 (TBS-T), for 10 min, with stirring. The antigens are reactivated in 10 mM citrate buffer, pH 6, by heating to 90° C. for 40 min, and then by allowing to cool to ambient temperature for 30 min. The endogenous peroxidases are inhibited by incubation in TBS-T containing 3% H_2O_2, for 5 min. The slides are then saturated with 3% BSA in TBS-T, for 1 h at 37° C., in a humid chamber.

The slides are then incubated for 2 h with the anti-Leukocyte Elastase Inhibitor (clone 3D9C2), anti-Ezrin (clone 5G2D12), anti-Aminoacylase 1 (clone 8A8A10) or anti-I-Plastin (clone 8D6A3) primary antibody diluted to 10 μg/ml in TBS-T containing 3% BSA (incubation at 37° C. in a humid chamber). After 3 washes of 10 min in TBS-T, the slides are incubated for 2 h at 37° C., in a humid chamber, with the horseradish peroxidase-coupled anti-mouse secondary antibody (Cat. No. 115-035-003 Jackson Immunoresearch) diluted to 1/400 in the saturating solution. The slides are washed 3 times for 10 minutes in TBS-T, and then 3 times for 10 min in PBS. The slides are developed with the Sigma Fast substrate (Cat. No. D-4168, Sigma-Aldrich) for 5 min. The staining is stopped by washing in PBS. Counterstaining with Harris hematoxylin (Cat. No. MHS16, Sigma-Aldrich) is carried out for 30 sec. After washing with water and with PBS, the slides are mounted for observation under a microscope.

The antibodies used for the immunohistochemical labeling were selected specifically for this application, independently of their reactivity in ELISA or in Western blotting.

2. Immunohistochemical Detection of Leukocyte Elastase Inhibitor

Tissue-microarray slides were used to screen a large number of samples. These samples are colonic tissues spotted onto slides. The characteristics of the patients (characteristics of the colonic tissue spots present on the colorectal cancer tissue-microarray), and also the results of the immunolabelings with the anti-Leukocyte Elastase Inhibitor antibody, are reproduced in Table 10.

TABLE 10

Diagnosis	Histology and genetic characterization	Labeling in epithelial cells	Labeling in the stroma
Malignant tumor	Conserved adenocarcinoma	Positive	Negative
Malignant tumor	Conserved adenocarcinoma	Positive	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Benign tumor	Adenoma	Negative	Negative
Malignant tumor	Conserved adenocarcinoma	Positive	Negative
Malignant tumor	LOH adenocarcinoma	Positive	Negative
Malignant tumor	LOH adenocarcinoma	Positive	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Malignant tumor	LOH adenocarcinoma	Negative	Negative
Malignant tumor	LOH adenocarcinoma	Positive	Negative
Malignant tumor	MSI-high adenocarcinoma	Positive	Negative
Malignant tumor	MSI-high adenocarcinoma	Positive	Negative
Malignant tumor	Colloid adenocarcinoma	Negative	Negative
Malignant tumor	Colloid adenocarcinoma	Negative	Negative
Normal	Normal mucosa	Negative	Negative
Normal	Normal mucosa	Negative	Negative

The results in the table demonstrate that, in the healthy colonic mucosa biopsies, there is no labeling (10 negatives). The labeling is also negative in the adenoma (1/1). The labeling is positive in the epithelial cells of the colonic adenocarcinomas (+ in 8/11 patients). There is no labeling in the stroma.

3. Immunohistochemical Detection of Ezrin

Tissue-microarray slides were used to screen a large number of samples. These samples are colonic tissues spotted onto slides. For each patient with a colonic adenocarcinoma, 3 samples were taken at the center of the tumor, 3 samples were taken at the invasion front and 3 samples were

taken in the healthy tissue. Table 11 shows the results of the immunolabelings with the anti-Ezrin antibody; the level of labeling indicated is the maximum intensity over the 3 samples analyzed.

TABLE 11

Patient identifier	Tumor center	Tumor invasion front	Healthy tissue
55	+	++	0
127	+	+	0
329	+	++	+
475	+	++	+
544	+	++	+
726	+	+	+
1203	++	++	+
1310	++	+++	+
2003	+	0	+
2296	++	++	0
2301	+	++	+
2377	+	+	0
3095	+	+	0
3430	+	+	0
3636	+	+	0
3748	+	+	0
3839	+	++	0
3891	0	0	0
4054	+	+	0
4322	+	++	0
445	0	++	+
4474	++	++	0
4792	+	+	+
4958	++	++	+
5101	+	++	+
5318	++	+++	0
5374	+	+	0
5472	+	0	+
6340	++	+	0
6353	++	+	0

In a sampling of 30 patients, 25 exhibit overexpression of Ezrin in the tumor (tumor center or invasion front) compared with the adjacent healthy tissue.

4. Immunohistochemical Detection of Aminoacylase 1

Tissue-microarray slides were used to screen a large number of samples. These samples are colonic tissues spotted onto slides. For each patient with a colonic adenocarcinoma, 3 samples were taken at the center of the tumor, 3 samples were taken at the invasion front and 3 samples were taken in the healthy tissue. Table 12 shows the results of the immunolabelings with the anti-Aminoacylase antibody; the level of labeling indicated is the maximum intensity over the 3 samples analyzed.

TABLE 12

Patient identifier	Tumor center	Tumor invasion front	Healthy tissue
55	++	++	0
127	0	0	0
329	++	++	0
475	++	++	+
544	+	0	0
726	0	0	0
1203	0	+	0
1310	0	+	+
2003	++	0	0
2296	+	+	0
2301	+	+	+
2377	+	+	+
3095	+	+	0
3430	+	+	+
3636	++	+	+
3748	++	++	0
3839	++	++	+
3891	++	++	0
4054	+	++	0
4322	+++	+++	+
445	+	++	+
4474	+	++	+
4792	++	++	+
4958	+	+	+
5101	+	+	++
5318	+++	++	0
5374	+	+	0
5472	++	++	+
6340	++	++	+
6353	++	++	++

In a sampling of 30 patients, 21 exhibited overexpression of Aminoacylase in the tumor (tumor center or invasion front) compared with the adjacent healthy tissue.

5. Immunohistochemical Detection of I-Plastin

Tissue-microarray slides were used to screen a large number of samples. These samples are colonic and rectal tissues spotted onto slides. The characteristics of the patients (characteristics of the colonic tissue spots present on the colorectal cancer tissue-microarray), and also the results of the immunolabelings with the anti-1-Plastin antibody, are reproduced in Table 13.

TABLE 13

Diagnosis	Histology and genetic characterization	Labeling in the epithelial cells	Labeling in the stroma
Malignant colon tumor	Conserved adenocarcinoma	++	Negative
Malignant colon tumor	Conserved adenocarcinoma	++	Negative
Normal colon	Normal mucosa	+	Negative
Normal colon	Normal mucosa	+	Negative
Normal colon	Normal mucosa	+	Negative
Benign colon tumor	Adenoma	+	Negative
Malignant colon tumor	Conserved adenocarcinoma	+	Negative
Malignant colon tumor	LOH adenocarcinoma	++	Negative
Malignant colon tumor	LOH adenocarcinoma	++	Negative
Normal colon	Normal mucosa	+	Negative
Normal colon	Normal mucosa	+	Negative
Normal colon	Normal mucosa	+	Negative
Malignant colon tumor	LOH adenocarcinoma	++	Negative

TABLE 13-continued

Diagnosis	Histology and genetic characterization	Labeling in the epithelial cells	Labeling in the stroma
Malignant colon tumor	LOH adenocarcinoma	++	Negative
Malignant colon tumor	MSI-high adenocarcinoma	+	Negative
Normal colon	Normal mucosa	+	Detached
Normal colon	Normal mucosa	+	Detached
Malignant colon tumor	Colloid adenocarcinoma	+	Negative
Normal colon	Normal mucosa	++	Negative
Normal colon	Normal mucosa	++	Negative
Normal rectum	Normal rectal mucosa	++	Negative
Normal rectum	Normal rectal mucosa	Nonspecific	Negative
Normal rectum	Normal rectal mucosa	Nonspecific	Negative
Normal rectum	Normal rectal mucosa	Nonspecific	Negative
Malignant rectal tumor	LOH adenocarcinoma	+	Negative
Malignant rectal tumor	LOH adenocarcinoma	++	Negative
Malignant rectal tumor	LOH adenocarcinoma	++	Negative
Malignant rectal tumor	LOH adenocarcinoma	++	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	++	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	++	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	+	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	+	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	+	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	+	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	++	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	++	Negative
Benign rectal tumor	Adenoma with low-grade dysplasia	++	Negative

The results in the table demonstrate that:

in the healthy colonic mucosa biopsies, the labeling is weak in 8 samples (+) and 2 samples are ++. The labeling is also weak (+) in the colonic adenoma (1/1). The labeling is strongly positive ++ in the epithelial cells of the colonic adenocarcinomas (++ in 6/9 patients and 3 weak +, including the colonic colloid adenocarcinomas). There is no labeling in the stroma;

in the healthy rectal mucosa biopsies, labeling is present in the surface epithelium in a nonspecific manner (3/4) and at ++ level in one sample. The labeling is strongly positive ++ in the rectal adenomas (5/9) or discreet + (4/9). The labeling is also strong ++ in the epithelial cells of the rectal adenocarcinomas (++ in 3/4 patients, 1 weak +). There is no labeling in the stroma.

EXAMPLE 8: DETECTION OF THE TUMOR MARKERS BY MEANS OF THE LC-MRM-MS TECHNIQUE

1. Methodology

In order to be able to decrease the detection limit to a few ng/ml, an improved MRM-MS method was used. The successive steps of this method are: 1) immunodepletion of the abundant proteins, 2) trypsin digestion, 3) SPE (solid-phase extraction) fractionation of the peptides, 4) liquid chromatography (LC) coupled to MRM-MS.

The setting up was carried out on spike samples by adding the ACY, Ezrin, L-FABP, PDI or I-Plastin recombinant proteins to a control serum pool, at a concentration of 10-250 ng/ml. Apolipoproteins A1 and A2 are naturally present in serum.

Immunodepletion.

The depletion of the abundant proteins in the serum was carried out using the commercial Vivapure anti-HSA kit from Vivascience. Alternatively, the Proteoextract Albumin/IgG kit from Calbiochem and the Aurum™ serum Protein Minikit from Bio-Rad were also used. It is also possible to produce the specific resins in the laboratory, by coupling a monoclonal antibody directed against the protein to be depleted, to a CNBr-activated Sepharose 4B resin (Amersham Bioscience), according to the manufacturer's instructions.

Enzymatic Digestion.

The depleted serum samples are denatured in a 6M urea solution buffered with 10 mM of Tris, pH 8, and containing 30 mM of dithiothreitol, for 40 minutes at 40° C., and then alkylated with 50 mM iodoacetamide, at ambient temperature, for 40 minutes, in the dark. They are diluted 6-fold in water, and then the trypsin digestion is carried out at 37° C., overnight, using an enzyme/substrate ratio of 1:30 (Promega). The digestion is stopped by adding formic acid at a final concentration of 0.5%. The digested samples are desalified by solid-phase extraction (SPE) using the Oasis HLB 3 cc reverse-phase cartridges (60 mg) (Waters). After application of the sample, the cartridges are washed with 1 ml of formic acid at 0.1%, and the elution is then carried out with a methanol/water mixture (80/20 v/v) containing 0.1% of formic acid. The eluates are dried under vacuum.

SPE Fractionation.

The dry samples are taken up in 1 ml of acetate buffer and loaded onto Oasis MCX (mixed cation exchange) 60 mg mixed cartridges (hydrophobic and cation exchange) (Waters) pre-equilibrated in acetate buffer and methanol. The cartridges are washed with 1 ml of acetate buffer and 1 ml of methanol. The peptides of interest (Table 14) are eluted with 1 ml of a methanol/acetate buffer mixture (50/50 v/v). The pH of the acetate buffer is chosen according to the isoelectric point of the peptide of interest. The eluates are dried under vacuum, and dissolved in 200 μl of a solution of acetonitrile/water (3/97 v/v) containing 0.1% of formic acid. A 50 μl aliquot was injected into the LC coupled to an MS-MS system.

Liquid Chromatography and Mass Spectrometry.

The LC-MS analysis was carried out on an HP 1100 series high pressure chromatographic system (HPLC) with a binary pump and injector (Agilent Technologies), coupled to a mass spectrometer, either a Sciex API 2000 triple quadripole, or a Sciex API 4000 Qtrap (hybrid triple quadripole-ion trap MS) (MDS Sciex) for better sensitivity. The LC separation was carried out on a C_{18} Symmetry column

(Waters), at an elution flow rate of 300 μl/min. (Eluent A=0.1% formic acid in water, eluent B=0.1% formic acid in acetonitrile, linear gradient of 5% B to 50% B in 25 min, then of 50% B to 100% B in 3 min). The MS analysis is carried out in the positive ionization mode at a voltage of 5500 V, applied as a needle voltage, enabling ionization in the source. The instrument verification and data acquisition are carried out with the Analyst 1.4.1 software. The nebulizing gas (air) and curtain gas (nitrogen) flows are 30 and 20 psi, respectively. The Turbo V™ ion source is adjusted to 400° C., the auxiliary nitrogen flow to 40 psi. The MRM transitions recorded for each peptide are reproduced in Table 14. The collision energy (CE), the declustering potential (DP) and the collision cell exit potential (CXP) are optimized for each of the MRM transitions selected.

2. Results

For each tumor marker (proteins of Table 14), the list of theoretical MRM transitions was generated using the MIDAS (MRM-initiated detection and sequencing) software. This list comprises all the double-charged or triple-charged parent ions of the theoretical tryptic peptides in a mass range of from 800 to 3000 Da and all the possible fragment ions of y or b type. For each protein, each possible transition was tested in order to determine the most sensitive and most specific transitions. The result of this selection is reproduced in Table 14. Using a heavy peptide of AQUA type (Sigma) or alternatively a heavy recombinant protein that will serve as an assay standard, it is possible to quantify, in an absolute manner, the tumor marker of interest in a complex biological medium.

TABLE 14

Sequence (SEQ ID No.)	pI	Q1	Q3	DP	CE	CXP
Apolipoprotein A1						
AKPALEDLR	6.38	338.2	288.2	14	20	5
(SEQ ID No. 16)			403.2	14	15	10
			532.3	14	15	25
ATEHLSTLSEK	5.53	405.9	173.1	20	20	10
(SEQ ID No. 17)			363.2	20	25	20
LSPLGEEMR	4.54	516.3	201.1	20	20	5
(SEQ ID No. 18)			831.4	20	20	40
			621.3	30	30	15
QGLLPVLESFK	6.11	615.9	299.2	20	30	15
(SEQ ID No. 19)			819.5	20	35	40
			186.1	20	35	5
THLAPYSDELR	5.39	651.3	239.1	20	30	5
(SEQ ID No. 20)			352.2	20	35	15
			879.4	20	35	25
VQPYLDDFQK	3.71	626.8	228.1	15	25	5
(SEQ ID No. 21)			1025.5	15	20	30
			513.2	15	25	25
DYVSQFEGSALGK	4.13	700.8	279.1	15	35	5
(SEQ ID No. 22)			378.2	15	20	10
			1023.5	15	20	25
			204.1	15	25	5
Apolipoprotein A2						
EQLTPLIK	6.4	471.3	260.2	20	30	15
(SEQ ID No. 23)			147.1	20	30	25
			684.5	20	20	35
Aminoacylase						
AVGVPALGFSPMNR	10.35	708.4	517.3	20	30	23
(SEQ ID No. 24)			808.4	20	35	28
			228.1	20	32	7
			1089.6	20	32	32

TABLE 14-continued

Sequence (SEQ ID No.)	pI	Q1	Q3	DP	CE	CXP
VVNSILAFR	10.35	509.8	199.1	15	25	5
(SEQ ID No. 25)			820.5	15	25	28
			506.3	15	25	20
			393.2	15	25	15
EGSVTSVNLTK	6.99	567.8	248.2	15	28	7
(SEQ ID No. 26)			661.4	15	28	25
			762.4	15	28	32
GPEEEHPSVTLFR	4.58	749.3	819.5	20	38	35
(SEQ ID No. 27)			956.5	20	38	30
Ezrin						
IGFPWSEIR	7.04	552.8	787.2	20	27	25
(SEQ ID No. 28)			288	20	27	10
			690.1	20	27	22
			504.1	20	27	20
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			760.2	20	25	25
SGYLSSER	10.1	449.7	478.2	20	25	20
(SEQ ID No. 30)			591.4	20	22	20
			391.0	20	22	15
APDFVFYAPR	6.76	591.8	272.2	20	28	10
(SEQ ID No. 31)			1111.6	20	28	35
L-FABP						
AIGLPEELIQK	4.54	605.8	856.5	32	29	22
(SEQ ID No. 32)			1026.58	32	30	27
			185.1	32	30	5
			242.2	32	33	5
GVSEIVQNGK	6.36	515.8	658.4	28	30	35
(SEQ ID No. 33)			545.3	28	30	30
			157.1	28	30	10
			446.2	28	30	25
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			201.1	35	28	10
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			249.1	23	27	10
PDI						
EADDIVNWLK	3.74	601.8	560.3	20	27	18
(SEQ ID No. 36)			659.4	20	27	22
DHENIVIAK	5.24	519.8	253.1	20	25	10
(SEQ ID No. 37)			331.2	20	25	13
			786.4	20	25	30
			657.4	20	25	25
LITLEEEMTK	4.01	603.8	328.2	20	30	11
(SEQ ID No. 38)			766.3	20	30	25
			980.5	20	30	32
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			635.4	20	25	26
I-Plastin						
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The invention claimed is:

1. A method comprising:

assaying the expression levels of Leukocyte Elastase Inhibitor and Liver Fatty Acid-Binding Protein in a biological fluid sample from a person suspected of having colorectal cancer, wherein:

the biological fluid sample is selected from the group consisting of samples of whole blood, serum, plasma, urine, saliva, effusions, bone marrow, and stool extract, and

the assaying is performed by passing the biological fluid sample through a mass spectrometer.

2. The method as claimed in claim **1**, wherein the biological fluid sample is selected from the group consisting of samples of whole blood, serum, plasma, urine, effusions, bone marrow, and stool extract.

3. The method as claimed in claim **1**, further comprising assaying for at least one member selected from the group consisting of Ezrin, Aminoacylase 1, Intestinal Fatty Acid-Binding Protein, Apolipoprotein AI, and Apolipoprotein AII.

4. The method as claimed in claim **1**, further comprising assaying for at least one member selected from the group consisting of Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Keratin type II Cytoskeletal 8, Keratin type I Cytoskeletal 18, Keratin type I Cytoskeletal 19, Epithelial-Cadherin, CEA, Villin, CA19-9, CA 242, CA 50, CA 72-2, Testosterone, TIMP-1, Cripto-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, methylated DNA in the blood, specific alterations in fecal DNA fragments, and fecal human hemoglobin.

5. The method as claimed in claim **1**, further comprising assaying for at least one member selected from the group consisting of Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Epithelial-Cadherin, CEA, CA19-9, Testosterone, TIMP-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, and fecal human hemoglobin.

6. The method as claimed in claim 1, further comprising assaying for CEA.

7. The method as claimed in claim 1, further comprising assaying for Beta 2-Microglobulin, Aminoacylase 1, and CEA.

8. The method as claimed in claim 1, further comprising assaying for Beta 2-Microglobulin, Aminoacylase 1, Proteasome 20S, PAP, E-Cadherin, and CEA.

9. A method comprising:

assaying the expression levels of Leukocyte Elastase Inhibitor and Liver Fatty Acid-Binding Protein in a biological sample from a person suspected of having colorectal cancer, wherein:

the biological sample is selected from the group consisting of samples of whole blood, serum, plasma, urine, saliva, effusions, bone marrow, and stool, and

the assaying is performed on the biological sample via immunoassay using an automated device.

10. The method as claimed in claim 9, further comprising assaying for at least one member selected from the group consisting of Ezrin, Aminoacylase 1, Intestinal Fatty Acid-Binding Protein, Apolipoprotein AI, and Apolipoprotein AII.

11. The method as claimed in claim 9, further comprising assaying for at least one member selected from the group consisting of Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Keratin type II Cytoskeletal 8, Keratin type I Cytoskeletal 18, Keratin type I Cytoskeletal 19, Epithelial-Cadherin, CEA, Villin, CA19-9, CA 242, CA 50, CA 72-2, Testosterone, TIMP-1, Cripto-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, methylated DNA in the blood, specific alterations in fecal DNA fragments, and fecal human hemoglobin.

12. The method as claimed in claim 9, further comprising assaying for at least one member selected from the group consisting of Beta2-Microglobulin, Proteasome 20S, Galectin-3, L-Lactate Dehydrogenase Chain B, Calreticulin, Regenerating Islet-Derived Protein 3 Alpha, Tumor-Associated Calcium Signal Transducer 1, Epithelial-Cadherin, CEA, CA19-9, Testosterone, TIMP-1, Intelectin-1, Protein Disulfide Isomerase, Cytokeratin 20, Translationally-Controlled Tumor Protein, (Pro)defensin-A5, and fecal human hemoglobin.

13. The method as claimed in claim 9 further comprising assaying for CEA.

14. The method as claimed in claim 9, further comprising assaying for Beta 2-Microglobulin, Aminoacylase 1, and CEA.

15. The method as claimed in claim 9, further comprising assaying for Beta 2-Microglobulin, Aminoacylase 1, Proteasome 20S, PAP, E-Cadherin, and CEA.

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