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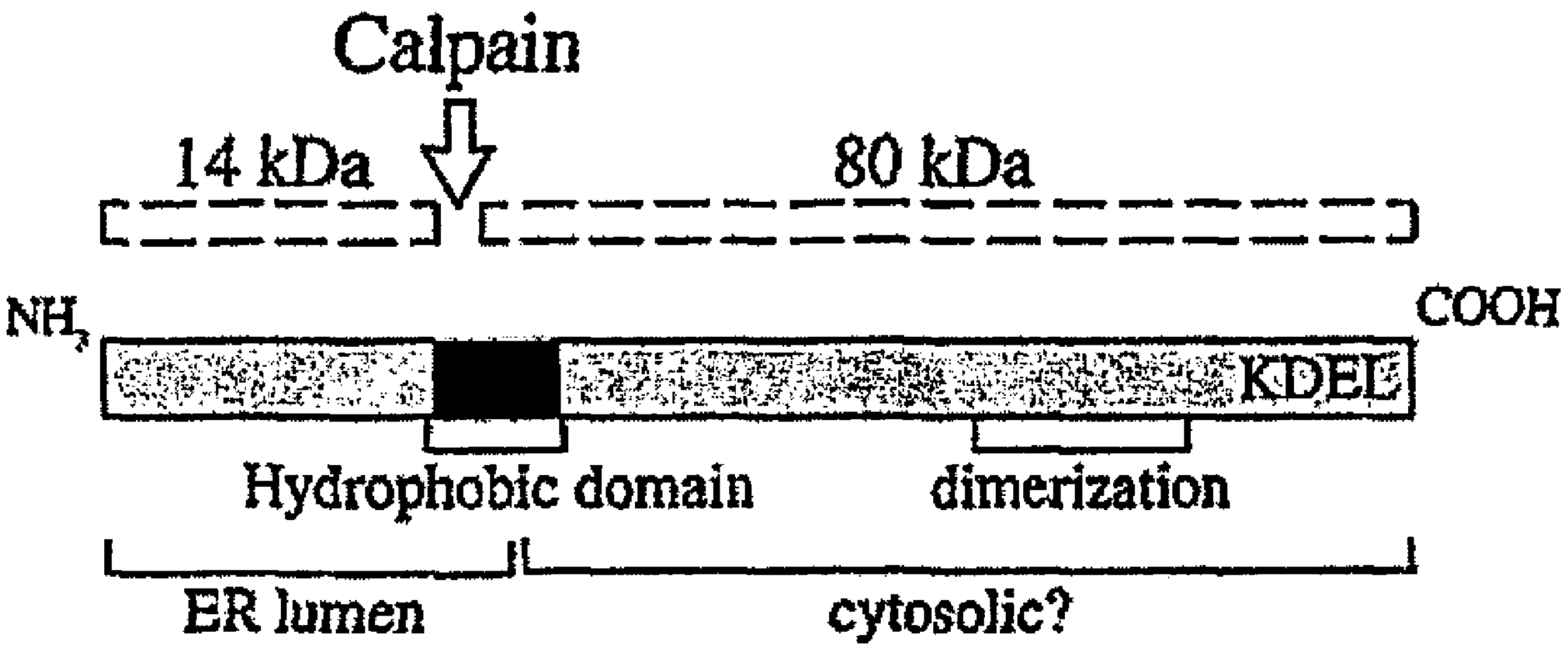
(72) Inventeurs/Inventors:
JUHL, HARTMUT, DE;
DAVID, KERSTIN, DE;
FENTZ, ANNE-KRISTIN, DE

(73) Propriétaire/Owner:
INDIVUMED GMBH, DE

(74) Agent: PIASETZKI NENNIGER KVAS LLP

(54) Titre : UTILISATION D'UN FRAGMENT D'ENDOPLASMINE ET DE DERIVES DE CELUI-CI EN TANT QUE BIOMARQUEUR POUR ADENOME ET/OU CARCINOME COLORECTAL, METHODE DE DETECTION ET SYSTEME D'ESSAI

(54) Title: USE OF AN ENDOPLASMIN FRAGMENT AND DERIVATIVES THEREOF AS A BIOMARKER FOR COLORECTAL ADENOMA AND/OR CARCINOMA; METHOD FOR DETECTION AND TEST SYSTEM



(57) Abrégé/Abstract:
The present invention is directed to a method for detecting colorectal adenoma and/or colorectal carcinoma comprising the steps: a) providing an isolated sample material which has been taken from an individual, b) determining the level of an endoplasmin fragment or a derivative thereof in said isolated sample material, c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values. The invention is further directed to a method for discriminating between colorectal adenoma and colorectal carcinoma as well as to a method for monitoring the development and/or course of colorectal adenoma and/or colorectal carcinoma and/or the treatment of colorectal adenoma and/or colorectal carcinoma. Moreover, the invention is directed to a test system and an array for use in these methods. Furthermore, the invention is directed to the use of an endoplasmin fragment as a biomarker for a detection of colorectal adenoma and/or colorectal carcinoma in an individual. Further, the invention is directed to a method for determining the effectiveness of a compound in the treatment colorectal adenoma and/or colorectal carcinoma.

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(71) Applicant *(for all designated States except US)*: **INDIVUMED GMBH** [DE/DE]; Orchideenstieg 17, 22297 Hamburg (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **JUHL, Hartmut** [DE/DE]; Kurt-Küchler-Strasse 5, 22609 Hamburg (DE). **DAVID, Kerstin** [DE/DE]; Chateauneufstrasse 10, 20535 Hamburg (DE). **FENTZ, Anne-Kristin** [DE/DE]; Heymannstrasse 10a, 20253 Hamburg (DE).

(74) Agent: **WALCHER, Armin**; Louis Pöhlau Lohrentz, P.O. Box 3055, 90014 Nürnberg (DE).

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(54) Title: USE OF AN ENDOPLASMIN FRAGMENT AND DERIVATIVES THEREOF AS A BIOMARKER FOR COLORECTAL ADENOMA AND/OR CARCINOMA; METHOD FOR DETECTION AND TEST SYSTEM

Fragments of endoplasmin (grp 94)

SEQ-ID NO.2
EEEAIQLDG LNASQIR

SEQ-ID NO.3
FAFQA EVNR

SEQ-ID NO.4
EEEAIQLDG LNASQIRELR EKSEKFAFQA EVNR

(57) Abstract: The present invention is directed to a method for detecting colorectal adenoma and/or colorectal carcinoma comprising the steps: a) providing an isolated sample material which has been taken from an individual, b) determining the level of an endoplasmin fragment or a derivative thereof in said isolated sample material, c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values. The invention is further directed to a method for discriminating between colorectal adenoma and colorectal carcinoma as well as to a method for monitoring the development and/or course of colorectal adenoma and/or colorectal carcinoma and/or the treatment of colorectal adenoma and/or colorectal carcinoma. Moreover, the invention is directed to a test system and an array for use in these methods. Furthermore, the invention is directed to the use of an endoplasmin fragment as a biomarker for a detection of colorectal adenoma and/or colorectal carcinoma in an individual. Further, the invention is directed to a method for determining the effectiveness of a compound in the treatment colorectal adenoma and/or colorectal carcinoma.

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USE OF AN ENDOPLASMIN FRAGMENT AND DERIVATIVES THEREOF AS A BIOMARKER FOR COLORECTAL ADENOMA AND/OR CARCINOMA; METHOD FOR DETECTION AND TEST SYSTEM

The present invention relates to the field of detection of colorectal adenoma and/or colorectal carcinoma.

Colorectal carcinoma is the third most frequently diagnosed carcinoma (9.4 %) worldwide. In 2003 nearly 945 000 new cases of colorectal carcinoma were diagnosed worldwide and approximately 492 000 people died of this disease. The incidence of colorectal carcinoma is increasing, while the mortality rate of colorectal carcinoma is decreasing. Incidence of colorectal carcinoma increases with age, beginning at around 40 years of age, and it is higher for men than for women (40.6 for men versus 30.6 for women, per 100 000 per year) (World cancer report, 2003, Ed. BW. Stewart and P. Kleihues. IARC Press, Lyon).

In most patients, development of colorectal carcinoma follows a multistep progression from premalignant adenoma to invasive malignancies that have the propensity for metastasis.

So far only unpleasant colorectal screening tests such as colonoscopy have been shown to achieve detection of early stage colorectal carcinoma and its precursors. However, high false negative rates have been observed for flat neoplastic lesions and polypoid lesions smaller than 10 mm diameter (Kudo S. (1997) Gastrointest. Endosc. Clin. N.Am. 7:87-98). Therefore, it would be desireable to have a test system allowing the early detection of colorectal adenoma and/or colorectal carcinoma as well as the specific discrimination between different tumor stages. This would also allow a specific adaptation of the therapy.

The currently known screening tests based on tumor marker detection in blood samples lack the required sensitivity and specificity. For instance, CEA-(Carcinoembryonic antigen)-levels in blood samples have been used to detect colon carcinoma. However, CEA levels are not specifically elevated in colon carcinoma and have been shown to be elevated also in patients with other malignant diseases (e.g., cancers of the stomach, pancreas, breast, and lung) and with various nonmalignant conditions (e.g., alcoholic liver disease, inflammatory bowel disease, heavy cigarette smoking, chronic bronchitis, and pancreatitis). (Posner MR, Mayer RJ: The use of serologic tumor markers in gastro intestinal malignancies. Hematol Oncol Clin North Am 8:533, 1994). Further, the CEA-levels are not elevated in colon adenomas. Furthermore, CEA-levels are not suitable to distinguish colorectal adenoma from colorectal carcinoma or different tumor stages.

An object of the invention is to provide means allowing an early detection of colon adenoma and/or colon carcinoma and/or allowing discrimination of different tumor stages.

It is a further object to provide a biomarker which can be used in the detection of colorectal adenoma and/or carcinoma and/or which can be used in the discrimination of different tumor stages.

Another object of the present invention is to provide a test system for detecting colorectal adenoma and/or carcinoma and/or for discrimination of different tumor stages which is cost effective and can be widely used.

Moreover, the test system should be easy to handle.

It is an further object of the present invention to provide a screening system for detecting the effectiveness of compounds which are specific for the treatment of adenoma and/or carcinoma depending on the specific stage of tumors.

The objects underlying the present invention are solved by the use of an endoplasmin fragment or a derivative thereof as a biomarker for the detection of

colorectal adenoma and/or colorectal carcinoma in an individual and/or for the discrimination of different tumor stages. The detection can be carried out in vivo and in vitro. Pursuant to a preferred embodiment, the detection is carried out in vitro.

The objects are further solved by a method for detecting colorectal adenoma and/or colorectal carcinoma comprising the steps:

a) providing an isolated sample material which has been taken from an individual,
b) determining the level of an endoplasmin fragment or a derivative thereof in said isolated sample material,
c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values.

The objects are further solved by a method for discriminating colorectal adenoma and colorectal carcinoma and/or for discriminating further tumor states comprising the steps:

a) providing an isolated sample material which has been taken from an individual,
b) determining the level of an endoplasmin fragment or a derivative thereof in said isolated sample material,
c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values.

The objects are also solved by a method for monitoring the development and/or the course and/or the treatment of colorectal adenoma and/or colorectal carcinoma comprising the steps:

a) providing an isolated sample material which has been taken from an individual,

b) determining the level of an endoplasmin fragment or a derivative thereof in said isolated sample material,
c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one ore more reference values.

In a preferred embodiment the effectiveness of a surgical or therapeutical procedure is controlled in order to decide as to whether the colorectal adenoma and/or colorectal carcinoma is completely removed. In another embodiment the therapy of a colorectal adenoma and/or colorectal cancer patient by treating the patient with one or more chemical substances, antibodies, proteins, peptides, small molecular drug, antisense-RNA, radiation, e.g. X-rays, or combinations thereof is controlled in order to control the effectiveness of the treatment.

The objects underlying the invention are solved by providing a test system for detecting colorectal adenoma and/or colorectal cancer and/or for the differentiation of different tumor stages in a sample of an individual comprising:

a) an antibody or a receptor which binds to an epitope of an endoplasmin fragment or a derivative thereof,
b) a solid support which supports said antibody or receptor,
c) a reagent for detecting the binding of said epitope of an endoplasmin fragment or a derivative thereof to said antibody or receptor.

The objects of the invention are furthermore solved by the provision of an array comprising detection molecules for detecting of colorectal adenoma and/or colorectal carcinoma cancer and/or for the differentiation of different tumor stages in an individual comprising as detection molecule:

a) a nucleic acid probe immobilized to a solid support for binding to and detecting mRNA encoding an endoplasmin fragment or a derivative thereof, or

b) an antibody immobilized to a solid support for binding to and detecting of an epitope of an endoplasmin fragment or a derivative thereof, or
c) a receptor immobilized to a solid support for binding to and detecting of an epitope of an endoplasmin fragment or a derivative thereof,

wherein preferably different amounts of detection molecules are immobilized to the solid support to increase the accuracy of the quantification.

The nucleic acid probe is for example selected from the group consisting of single-stranded or double-stranded DNA or RNA, aptamers and combinations thereof. Aptamers are single-stranded oligonucleotides that assume a specific, sequence-dependent shape and bind to protein targets with high specificity and affinity. Aptamers are identified using the SELEX process (Tuerk C. and Gold L. (1990) Science 249: 505-510; Ellington AD. and Szostak JW (1990) Nature 346, 818 –822).

The objects are furthermore solved by a method for determining whether a compound is effective in the treatment of colorectal adenoma and/or colorectal carcinoma cancer and/or for the differentiation of different tumor stages comprising the steps:

a) treating of a colorectal adenoma or colorectal carcinoma patient with a compound,
b) determining the level of an endoplasmin fragment or a derivative thereof in a sample material of said patient, and
c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values.

Preferred embodiments are specified in dependent claims.

According to the present invention the term “sample material” is also designated as “sample”.

Pursuant to the present invention the term "biomarker" is meant to designate a protein or protein fragment or a nucleic acid which is indicative for the incidence of the colorectal adenoma and/or colorectal carcinoma. That means the "biomarker" is used as a means for detecting colorectal adenoma and/or colorectal carcinoma.

The term "individual" or "individuals" is meant to designate a mammal. Preferably, the mammal is a human being such as a patient.

The term "healthy individual" or "healthy individuals" is meant to designate individual(s) not diseased of colorectal adenoma and/or colorectal carcinoma. That is to say, the term "healthy individual(s)" is used only in respect of the pathological condition of colorectal adenoma and/or colorectal carcinoma and does not exclude the individual to suffer from diseases other than colorectal adenoma and/or colorectal carcinoma.

The term "discrimination of different tumor stages" according to the present invention means the discrimination of colorectal adenoma versus colorectal carcinoma and/or the discrimination of different tumor stages, e.g. TNM I, II, III and IV.

The term "derivative thereof" is meant to describe any modification on DNA, mRNA or protein level comprising e.g. the truncated gene, fragments of said gene, a mutated gene, or modified gene or the respective gene products thereof. The term "derivatives thereof" includes nucleic acid sequences, such as DNA, RNA, mRNA or protein sequences or peptide sequences. The derivative can be a modification which is an result of a deletion, substitution or insertion in the gene. The gene modification can be a result of the naturally occurring gene variability. The term "naturally occurring gene variability" means modifications which is not a result of genetic engineering. The derivative can be a result of the processing of the gene or gene product within the body and/ or a degradation product. The modification on protein level can be due to enzymatic or chemical modification within the body. For example the modification can be a glycosylation or phosphorylation or farnesylation.

The term “endoplasmin fragment or a derivative thereof” as used in the present invention also comprises mutated endoplasmin fragments or modified endoplasmin fragments or fragments of modified endoplasmin. The modification of the “endoplasmin fragment” can be due to enzymatic or chemical modification.

The terms “tumor” and “cancer” are interchangeably used and have the same meaning.

In one embodiment the endoplasmin fragment or derivative thereof is a protein comprising an N-terminal fragment or a C-terminal fragment of endoplasmin. The term N-terminal fragment or C-terminal fragment is not to be understood in that these sequence comprise the complete N-terminus or C-terminus of endoplasmin, respectively, but that the fragment is generated from the N-terminal or C-terminal region of endoplasmin.

The fragment can also be generated from a middle part of the protein sequence of endoplasmin.

The sequence length of the fragment of endoplasmin (SEQ ID NO. 1) must be sufficiently long to be specific for endoplasmin. It has been shown that fragments of endoplasmin comprising at least 7 contiguous amino acids, preferably at least 8 contiguous, amino acids of the sequence of endoplasmin (SEQ ID NO. 1) are highly specific for endoplasmin. Pursuant to a preferred embodiment of the invention the fragment comprises at least 9 contiguous amino acids of endoplasmin (SEQ ID NO. 1).

A specific cleavage of the full-length endoplasmin (SEQ ID NO. 1) which is also designated as grp94 leads to two fragments of about 10-14 kDa and 80 kDa. It is assumed that the endoplasmin-cleavage is induced by Calpain (Fig. 4).

In one embodiment of the invention, the endoplasmin fragment comprises a C-terminal fragment having a molecular weight of about 80 kDa or the N-terminal

fragment having a molecular weight of about 10 to 14 kDa. According to another embodiment of the invention the fragments having a molecular weight of about 10 to 14 kDa or about 80 kDa are induced by cleavage by Calpain.

In an embodiment of the invention the endoplasmin fragment comprises a part of the sequence SEQ-ID-NO.1. In a preferred embodiment of the invention the endoplasmin fragment comprises a N-terminal fragment. Preferably the fragment comprises the peptide sequence SEQ ID-NO. 2 and/or the peptide sequence SEQ-ID-NO. 3. Pursuant to another embodiment of the invention the fragment comprises the peptide sequence SEQ-ID-NO. 4.

In another embodiment of the invention the endoplasmin fragment has a molecular weight of 10 to 14 kDa, more preferably of 10.3 kDa.

In a further embodiment of the invention the endoplasmin fragment is a peptide or protein which is or includes an amino acid sequence having at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4 and which is able to specifically detect colorectal adenoma and/or colorectal carcinoma.

Pursuant to another embodiment of the invention the endoplasmin fragment has an amino acid sequence having at least 80 %, preferably at least 90 %, more preferably at least 95% sequence identity with SEQ ID NO. 1 based on the sequence of the fragment.

Glucose-related proteins (grps) are a group of highly conserved proteins synthesized after stress induction. Grps act as molecular chaperones helping to transport, fold and process their respective target proteins. Immunohistochemical staining detects grp94 mainly in the cytoplasm. Grp94 is also designated as endoplasmin. Grp94 (endoplasmin) exhibits the dual properties of a luminal protein and an integral protein, suggesting that it exists in two different forms within the endoplasmic reticulum.

The term “epitope” is meant to designate any structural element of a protein or peptide or any proteinaceous structure allowing the specific binding of an antibody, an antibody fragment, a protein or peptide structure or a receptor.

The methods of the present invention are carried out with sample materials such as body fluids or tissue samples which already have been isolated from the human body. Preferably, the sample material is a tissue sample. Subsequently the sample material can be fractionated and/or purified. It is for example possible, to store the sample material to be tested in a freezer and to carry out the methods of the present invention at an appropriate point in time after thawing the respective sample material.

It has been surprisingly discovered by the present inventors that an endoplasmin fragment or a derivative thereof can be used as a biomarker, preferably as an early biomarker, for the detection of colorectal adenoma and/or carcinoma.

The inventors have now surprisingly found that the protein levels of endoplasmin fragments or derivatives thereof in a sample material such as a tissue sample or a body fluid are elevated in individuals having colorectal adenoma and/or carcinoma. Furthermore, the inventors have surprisingly found that the endoplasmin fragment protein level or a derivative thereof in a tissue sample or a body fluid can be used to distinguish healthy people from people with colorectal adenoma and/or carcinoma as well as colorectal adenoma from colorectal carcinoma. Furthermore, the level of the endoplasmin fragment can be used to discriminate between different tumor stages, e.g. TNM I, II, III and/or IV. The endoplasmin fragment is a specific biomarker which can be used to discriminate colorectal adenoma and colorectal carcinoma and/or different tumor stages. The different tumor stages can be for example TNM I, II, III and/or IV. The endoplasmin fragment is a selective and specific marker which can be used to confirm the diagnosis obtained by histopathology.

The inventors have, however, also found that an elevated level of complete endoplasmin in the sample material such as tissue or biological fluids ,e.g. serum or plasma, is specific for colorectal adenoma. Therefore, an elevated level of the

complete endoplasmin is also indicative for colorectal adenoma. In view of that, all explanations given above or below apply for complete endoplasmin respectively.

However, the inventors have also surprisingly found, that an elevated level of fragments of endoplasmin in a sample material such as tissue or a body fluid is a much more specific and sensitive biomarker for colorectal adenoma and/or carcinoma.

Pursuant to the present invention, sample material can be tissue, cells or a body fluid. The sample material can be a body fluid such as blood, blood plasma, blood serum, bone marrow, stool, synovial fluid, lymphatic fluid, cerebrospinal fluid, sputum, urine, mother milk, sperm, exudate and mixtures thereof. In a embodiment of the invention the body fluids are fractionated by chromatography.

Preferably, the body fluid has been isolated before carrying out the methods of the present invention. The methods of the invention are preferably carried out in vitro by a technician in a laboratory.

According to a preferred embodiment of the invention, the level of endoplasmin fragment is measured in blood plasma, blood serum or urine. Blood serum can be easily obtained by taking blood from an individual to be medically examined and separating the supernatant from the clotted blood.

The level of the endoplasmin fragment or a derivative thereof is higher with progressive formation of colorectal adenoma. The colorectal adenoma is a benign neoplasma which may become malign. When developing colorectal cancer from benign colorectal adenoma, the level of the endoplasmin fragment or a derivative thereof in body fluids, preferably blood serum, further increases.

After transformation of colorectal adenoma into colorectal cancer, the pathological condition of the afflicted individual can be further exacerbated by formation of metastasis. The higher levels of the endoplasmin fragment are correlated with the incidence of colorectal adenoma and/or carcinoma and/or metastasis.

The present invention provides an early stage biomarker which allows to detect the neoplastic disease at an early and/or still benign stage and/or an early tumor stage. Early detection enables the physician to timely remove the colorectal adenoma and dramatically increases the chance of the individual to survive.

Moreover, the present invention allows to monitor the level of an endoplasmin fragment or a derivative thereof in a body fluid such as blood serum over an extended period of time, such as years.

The long term monitoring allows to differentiate between colorectal adenoma and colorectal carcinoma. The level of an endoplasmin fragment or a derivative thereof can be routinely checked in body fluid, for example, once or twice a year. If an increase of the level of an endoplasmin fragment or a derivative thereof is detected this can be indicative for colorectal adenoma. A further increase of the level of an endoplasmin fragment or a derivative thereof can then be indicative for the transformation into malignant colorectal carcinoma.

Moreover, the course of the disease and/or the treatment can be monitored. If the level of an endoplasmin fragment or a derivative thereof further increases, for example after removal of the colorectal adenoma, this can be indicative for exacerbation of the pathological condition.

That means, the level of an endoplasmin fragment or a derivative thereof is a valuable clinical parameter for detecting and/or monitoring of colorectal adenoma and/or colorectal carcinoma. The level of an endoplasmin fragment or a derivative thereof in body fluids increases after incidence of colorectal adenoma. Therefore, the level of an endoplasmin fragment or a derivative thereof is an important clinical parameter to allow an early diagnosis and, consequently, an early treatment of the disease. Furthermore, the level of an endoplasmin fragment can be used to check the efficiency of surgery and/or other therapy.

The method of the invention for detection of colorectal adenoma and/or colorectal carcinoma and/or for discriminating between different tumor stages comprises the step of providing an isolated sample material which has been taken from an individual, then determining the level of an endoplasmin fragment or a derivative thereof in the isolated sample material, and finally comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values. In one embodiment, one or more further biomarker(s) is/are additionally detected in an isolated sample material which has been taken from an individual, the level of the biomarker(s) is/are determined and compared with one or more reference value(s).

The reference value can be calculated as the average level of an endoplasmin fragment or a derivative thereof determined in a plurality of isolated samples of healthy individuals or individuals suffering from colorectal adenoma and/or colorectal carcinoma. This reference value can either be established from healthy persons covering a range to be considered as normal, or a range which is considered to be elevated from patients who suffer from colorectal adenoma and/or colorectal carcinoma. Usually, the reference values are given as a range of reference values. Therefore, the term reference value and range of reference values as used in the present invention are defined to have the same meaning.

A specific value within a range of reference values can then be indicative for healthy condition or the pathological condition of colorectal adenoma and/or colorectal carcinoma. This range of reference values can be established by taking a statistically relevant number of tissue samples or body fluid samples, such as serum samples, of healthy individuals and of individuals suffering from colon adenoma and/or colon carcinoma as it is done for any other medical parameter range such as, e.g., blood sugar. The reference value for a healthy condition can also be obtained from healthy tissue of a colorectal adenoma and/or colorectal tumor patient.

Preferably, two reference values are calculated which are designated as negative control and positive control 1. The reference value of the negative control is calculated from healthy individuals or healthy tissue of colorectal adenoma and/or

colorectal tumor patients and the positive control is calculated from individuals suffering from colorectal adenoma or colorectal carcinoma. More preferably, three reference values are calculated which are designated as negative control and positive control 1 and positive control 2. Positive control 1 can be calculated from individuals suffering from colorectal carcinoma and positive control 2 can be calculated from colorectal adenoma.

In an another embodiment of the present invention, the reference values can be individual reference values calculated as the average level of an endoplasmin fragment or a derivative thereof determined in a plurality of isolated samples taken from the individual over a period of time.

When monitoring the level of an endoplasmin fragment or a derivative thereof over an extended period of time, such as months or years, it is possible to establish an individual average level. The level of an endoplasmin fragment or a derivative thereof can be measured, for example, from the same blood serum sample when measuring blood sugar and can be used to establish an individual calibration curve allowing to specifically detect any individual increase of the level of an endoplasmin fragment or a derivative thereof.

The reference value for further biomarkers can also be calculated in the same way as described for endoplasmin. The average levels of the endoplasmin fragment or further biomarkers may be the mean or median level.

In another aspect the present invention further provides a test system for detecting colorectal adenoma and/or colorectal carcinoma in an isolated sample material of an individual. The test system is based either on the specificity of an antibody or a receptor to specifically bind to an epitope or a suitable structural element of an endoplasmin fragment or a derivative thereof. A receptor can be any structure able to bind specifically to an endoplasmin fragment or a derivative thereof. The receptor can be, for example, an antibody fragment such as an Fab or an $F(ab')_2$ fragment or any other protein or peptide structure being able to specifically bind to an endoplasmin

fragment or a derivative thereof. The receptor can also be an aptamer specifically binding to an endoplasmin fragment or a derivative thereof.

The antibody, antibody fragment or receptor is bound to a solid support such as, e.g., a plastic surface or beads to allow binding and detection of an endoplasmin fragment or a derivative thereof. For example, a conventional microtiter plate can be used as a plastic surface. The detection of the binding of an endoplasmin fragment or a derivative thereof can be effected, for example, by using a secondary antibody labelled with a detectable group. The detectable group can be, for example, a radioactive isotop or an enzyme like horseradish peroxidase or alkaline phosphatase detectable by adding a suitable substrate to produce, for example, a colour or a fluorescence signal.

The test system can be an immunoassay such as an enzyme-linked immunosorbentassay (ELISA) or an radio immunoassay (RIA) or luminescence immunossay (LIA). However, any other immunological test system using the specificity of antibodies or fragments of antibodies can be used such as Western blotting or immuno precipitation.

The present invention also provides an array comprising detection molecules for detecting colorectal adenoma and/or colorectal carcinoma in an individual, wherein the detection molecule can be a nucleic acid probe immobilized on a solid support for binding to and detecting of mRNA encoding endoplasmin fragments, mutations, variants or derivatives thereof, or an antibody immobilized on a solid support for binding to and detecting of an epitope of an endoplasmin fragment or a derivative thereof, or a receptor immobilized on a solid support for binding to and detecting an epitope of an endoplasmin fragment or a derivative thereof. Preferably, the array comprises further detection molecules for biomarkers for detecting colorectal adenoma and colorectal carcinoma. Preferably, the nucleic acid probe comprises nucleic acid sequences selected from the group consisting of the nucleic acid sequences corresponding to SEQ-ID-NO.2 and/or SEQ-ID-NO.3 or SEQ-ID-NO.4.

Alternatively, the present invention also comprises an inverse array comprising patient samples immobilized on a solid support which can be detected by the above defined detection molecules.

Preferably the array comprises detection molecules which are immobilized to a solid surface at identifiable positions.

The term “array” as used in the present invention refers to a grouping or an arrangement, without being necessarily a regular arrangement. An array comprises preferably at least 2, more preferably 5, most preferably 10 different sets of detection molecules. Preferably, the array of the present invention comprises at least 50 sets of detection molecules, further preferred at least 100 sets of detection molecules. Pursuant to another embodiment of the invention the array of the present invention comprises at least 500 sets of detection molecules. The detection molecule can be for example a nucleic acid probe or an antibody or a receptor as already described above.

The nucleic acid probe can be any natural occurring or synthetic oligonucleotide, aptamers as well as cDNA, cRNA and the like.

The described array can be used in a test system according to the invention. The term “array” as used in the present invention relates to both macroarrays and microarrays.

Pursuant to another embodiment of the invention, the level of an endoplasmin fragment or a derivative thereof is determined by mass spectroscopy.

Mass spectroscopy allows to specifically detect an endoplasmin fragment or a derivative thereof via its molecular weight and to quantify the amount of an endoplasmin fragment or a derivative thereof very easily.

Any suitable ionization method in the field of mass spectroscopy known in the art can be employed to ionize the endoplasmin fragment or a derivative thereof molecule, fragments, mutations, variants or derivatives thereof. The ionization methods comprise electron impact (EI), chemical ionization (CI), field ionization (FDI), electrospray ionization (ESI), laser desorption ionization (LDI), matrix assisted laser desorption ionization (MALDI) and surface enhanced laser desorption ionization (SELDI).

Any suitable detection method in the field of mass spectroscopy known in the art can be employed to determine the molecular mass of an endoplasmin fragment or a derivative thereof. The detection methods comprise quadrupol mass spectroscopy (QMS), fourier transform mass spectroscopy (FT-MS) and time-of-flight mass spectroscopy (TOF-MS).

Preferably, the mass spectroscopy is a surface enhanced laser desorption ionization-time of flight-mass spectroscopy (SELDI-TOF-MS). Before carrying out a SELDI-TOF-MS, the endoplasmin fragment or a derivative thereof in the isolated sample is preferably immobilized on a chip or solid support with an activated surface. The activated surface comprises preferably immobilized antibodies against an endoplasmin fragment or a derivative thereof such as, for example, rabbit polyclonal-antibodies. After binding of the endoplasmin fragment or a derivative thereof to the antibodies, a time-of-flight analysis in a SELDI-TOF mass spectrometer is carried out, which delivers intensity signals for determination of the endoplasmin fragment or a derivative thereof level.

Moreover, mass spectroscopy allows to simultaneously detect other proteins which can have a relevance with respect to the detection of colorectal adenoma and/or colorectal cancer.

In an embodiment of the present invention the sensitivity and/or specificity of the detection of colorectal adenoma and/or colorectal carcinoma is enhanced by the additional detection of one or more further biomarkers.

Preferably, the sensitivity and specifity of the methods, arrays, test systems and uses according to the present invention are increased by the combination of detecting an endoplasmin fragment and derivatives thereof as well as alpha-defensin 1, 2 or 3 or derivatives thereof.

The term "alpha-defensin 1, 2, 3 or a derivative thereof" as used in the present invention also comprises truncated alpha-defensin 1, 2 or 3, fragments of alpha-defensin1, 2 or 3, mutated alpha-defensin 1, 2 or 3, or modified alpha-defensin 1, 2 or 3. The modification of "alpha-defensin 1, 2 or 3" can be due to enzymatic or chemical modification. alpha-defensins 1, 2 and 3 are also designated as human neutrophil peptides (HNP) 1, 2 and 3, respectively. The three peptides have mass/charge ratios (m/z) of 3445±10, 3374± 10 and 3489 ±10.

HNP 1-3 peptides are part of the defensin family of peptides which are fundamental components of the immune system and have the capacitiy to kill/inactivate a broad range of pathogens. Defensins are also known to function as regulators of both the innate and the adaptive immune system.

Previous studies indicate that HNP 1-3 expression in tumors primarily originates from tumor invading eosinophils and neutrophils. However, it can also be produced by cancer cells (e.g., bladder cancer cells).
(*Albrethsen J, Bogebo R, Gammeltoft S, Olsen J, Winther B, Raskov H. Upregulated expression of human neutrophil peptides 1, 2 and 3 (HNP 1-3) in colon cancer serum and tumours: a biomarker study. BMC Cancer. 2005, 5:8.*)

In a further embodiment of the present invention the sensitivity and/or specificity of the detection of colorectal adenoma and/or colorectal carcinoma and/or of the discrimination of different tumor stages can be enhanced by detection of an endoplasmin fragment in combination with one or more further biomarker(s) selected from the group consisting of alpha-defensin 1, 2 or 3, transthyretin, p53, C3a, CEA (carcinoembryonic antigen), CA 19-9, CA 15-3, CA-125, Kras, β-Catenin, Her-2/neu, C-reactive protein plasma and derivatives thereof and
mutations in E-cadherin, MSH2, MSH3, MLH1, PMS1, PMS2, MSH6 genes and

microsatellite instability of MHL1 or MSH2 and
SNPs (single nucleotide polymorphysm) and combinations thereof.

Another biomarker for colorectal adenoma and/or carcinoma may comprise a protein or polypeptide having a molecular weight of 4,838 ± 25 Da, preferably 4,838 ± 10 Da. In a preferred embodiment the endoplasmin fragment and the protein or polypeptide having a molecular weight of 4,838 ± 25 Da, preferably 4,838 ± 10 Da are used both to detect colorectal adenoma and/or carcinoma.

In one embodiment a fragment of endoplasmin or a derivative thereof in combination with C3a or a derivative and/or transthyretin or a derivative thereof are used as biomarkers to detect colorectal adenoma and/or carcinoma and /or to discriminate different tumor stages.

The term "transthyretin or a derivative thereof" as used in the present invention also comprises truncated transthyretin, fragments of transthyretin, mutated transthyretin, or modified transthyretin. The modification of "transthyretin" can be due to enzymatic or chemical modification. Moreover, the term "transthyretin" is also used to designate monomeric or multimeric forms of transthyretin. For example, the term "transthyretin" especially covers the monomeric protein chain usually being part of the homotetrameric protein transthyretin.

Transthyretin is also designated as prealbumin. Transthyretin is a tetrameric protein having a molecular weight of about 54,000 Da that is synthesized mainly in the liver Transthyretin is normally a homotetramer comprising four protein chains having each a molecular weight of about 14,000 Da. Using mass spectroscopy the inventors have detected several variants of the transthyretin protein chains having a molecular weight of inter alia 13,776 Da, 13,884 Da or 14,103 Da. The inventors have found out that especially the level of molecular variants of transthyretin having a molecular weight of 13,776 Da and 13,884 Da is decreased in a body fluid such as serum in case of incidence of colorectal adenoma and/or colorectal carcinoma.

The term "C3a or a derivative thereof" as used in the present invention also comprises truncated C3a, fragments of C3a, mutated C3a, modified C3a or the precursor C3 (SEQ ID NO. 5) or fragments of C3. In one embodiment the derivative has or comprises a protein sequence having an identity of at least 80% preferably of at least 90%, more preferably of at least 98% with the sequence SEQ-ID-NO. 6.

The modification of "C3a" can be due to enzymatic or chemical modification. In particular, the term C3a or a derivative thereof especially comprises a truncated C3a-protein preferably having a molecular weight in the range of 8,950 ± 25 Da; more preferably in the range of 8,950 ± 20 Da. In a preferred embodiment the truncated C3a-protein has a molecular weight of 8,939 Da. Preferably, the C3a-protein has no C-terminal Arginin and optionally a molecular weight in the range of 8,950 ± 20 Da. In one embodiment the C3a derivative is C3a-desArg (SEQ ID NO. 7). In one embodiment the C3a derivative is obtained by cleavage of C3a by mastcell-chymase. In another embodiment the C3a is obtained by cleavage of C3 by C3-convertase. The present invention also includes combination of the aforementioned embodiments.

C3a belongs to the group of anaphylatoxins. C3a, C4a and C5a are proteolytic products of serine proteases of the complement system. C3a (SEQ-ID-NO. 6) is derived from the third component (C3) (SEQ-ID-NO. 5) of the blood complement system during complement activation. C3a is a hormone with local effectiveness. Approximately 40% of the amino acid residues in C3a are involved in a helical conformation. Serum anaphylatoxins are involved in a variety of cellular immune responses, as well as being potent proinflammatory agents. C3a produces powerful effects on blood vessel walls, contraction of smooth muscle and an increase in vascular permeability. The C-terminal arginine in C3a is of fundamental importance for its biological activity. Anaphylatoxins are regulated by carboxypeptidase N (anaphylatoxin inactivator), which removes within seconds the carboxyterminal arginine. This mechanism converts the intact anaphylatoxin into a less active C3a-desArg form (SEQ ID NO. 7).

This allows the detection of colorectal adenoma and/or colorectal carcinoma with an increased sensitivity and/or specificity and/or the discrimination of different tumor states. Further, the methods of the present invention will be well accepted by the patients since these methods are not as unpleasant as a colonoscopy. Pursuant to the present invention sample material isolated from the individual , which can be a tissue specimen or a biological fluid, e.g. plasma or serum, is screened with the methods of the invention. The sample material can be obtained, for example, by taking blood or by a biopsy.

The sensitivity and specificity are defined as follow:

The sensitivity is the number of true positive detected patients (%) with regard to the number of all patients (100 %). The patients can be individuals having colorectal adenoma and/or colorectal carcinoma.

The specificity is the number of true negative detected individuals (%) with regard to the number of all healthy individuals (100%).

The sensitivity and specificity can be alternatively defined by the following formulas:

		diagnosis	
		+	-
test	+	TP	FP
	-	FN	TN

TP: True positive (test positiv, diagnosis correct);
FP: False positive (test positiv, diagnosis incorrect);
TN: True negative (test negative, diagnosis correct);
FN: False negative (test negative, diagnosis incorrect);

The sensitivity is calculated by the following formula:

TP/(TP + FN)

and the specificity is calculated by the following formula:

TN/ (TN + FP)

The result of each analysis group, which is selected from TP, FP, TN, FN, is calculated for a plurality of isolated samples selected from the group consisting of healthy individuals, colorectal adenoma patients and/or colorectal carcinoma patients. TP, FP, TN, FN relates to the number of individuals that are correlated with the status true positive, false positive, true negative, false negative, respectively.

The methods of the present invention can be carried out in combination with other diagnostic methods for detection of colorectal adenoma and/or colorectal carcinoma to increase the overall sensitivity and/or specificity. The detection of an endoplasmin fragment allows a very early detection of colorectal adenoma and can therefore be used as an very early biomarker. Furthermore, the detection of an endoplasmin fragment allows the discrimination of different tumor stages.

Preferably, the methods of the present invention are carried out as an early detection and/or monitoring method. If the results of the methods of the present invention should indicate the incidence of colorectal adenoma and/or colorectal adenoma in samples of body fluid, further examinations such as colonoscopy could be carried out. If the results obtained by histochemistry with tissue samples indicate the incidence of colorectal adenoma and/or colorectal adenoma the level of an endoplasmin fragment optionally in combination with a further biomarker should be analysed, in order to discriminate the different tumor stages.

The present invention further provides a method for determining whether a compound is effective in the treatment colorectal adenoma and/or colorectal carcinoma. Furthermore, the present invention provides a method for selecting a specific compound which is useful for treating a specific tumor stage.

Preferably, the method for determining whether a compound is effective in the treatment of colorectal adenoma and/or colorectal carcinoma and/or for the tretament of specific tumor stages comprises the steps of:

a) treating of a colorectal adenoma or colorectal carcinoma patient with a compound
b) determining the level of an endoplasmin fragment or a derivative thereof in a sample material of said patient
c) comparing the determined level of an endoplasmin fragment or a derivative thereof with one or more reference values.

The term "patient" as used in the present application covers humans as well as non-human beings such as animals. The animals are preferably selected from the group consisting of rodents, e.g. mouse, rat, hamster, and other animals, e.g. guinea-pig, rabbit, hare, dog and pig.

These animals can be used to specifically induce certain disease states, like colorectal adenoma and colorectal carcinoma, for research purposes. The induction of said disease states can, for example, be effected by treatment of the animals, for example, with radioactive or chemical substances known to induce colorectal cancer or colorectal adenoma disease state. The disease states can also be induced using viral transfection systems. It is also possible to use genetically modified animals, in which one or more specific gene function(s) has/have been altered, or knock-out animals such as knock-out mice in which a specific gene function has been deleted.

The compound for treatment of colorectal adenoma and/or colorectal carcinoma can be one or more chemical substances, antibody(ies), protein, peptide(s), small

molecular drugs of antisense mRNA(s). Alternatively, instead of one or more compounds irradiation can be used alone or in combination with one or more compounds.

The level of an endoplasmin fragment or a derivative thereof in a sample material of said patient can be determined by the above described detection techniques.

The following figures and example are given for illustrative purposes only. The invention is not to be construed to be limited to the following examples.

Figures

Fig. 1 shows the putative cleavage site of endoplasmin by calpain to generate an about 80 kDa and an about 14 kDa fragment.

Fig. 2 shows SELDI-TOF-MS protein profiles of lysates from normal colon, adenoma and tumor tissue. The intensity of p10.3 (N-terminal part of endoplasmin, 10,300 Da) increases significantly in tissue from healthy to adenoma to cancer patients.

Fig. 3 shows the analysis of endoplasmin fragment p10.3 by SELDI-TOF-MS. 30 samples from colon cancer and 29 samples from adenoma tissue as well as corresponding normal healthy colon tissue were analysed. The mean concentrations of the endoplasmin fragment p10.3 are significantly higher in

the adenoma group (A) and/or carcinoma group (T) compared to the healthy group (N).

Fig. 4 shows the detection of p10.3 in different cancer types by SELDI TOF MS. The expression level of p10.3 was significant lower in analysed samples of stomach, esophageal, lung, prostate and breast cancer compared to different colon carcinoma stages. The lysates were analysed on a SAX Protein Chip®Array. The difference in expression levels of p10.3 between normal and tumor tissues are statistically significant. N = normal tissue, T = cancer tissue.

Fig. 5 shows SELDI-TOF-MS protein profiles of lysates from normal colon, adenoma and cancer tissue. The peak intensities of p3489 (a member of the alpha-defensin family, p3.4) increase significantly in tissue from colorectal adenoma to cancer patients. N = normal tissue, A = adenoma tissue, T = cancer tissue.

Fig. 6 shows SELDI TOF MS protein profiles of lysates from normal colon, adenoma and cancer tissue. The peak intensity of p4848 (p4.8) is significantly elevated in tissues of colorectal adenoma patients compared to healthy control and colorectal cancer tissues. N = normal tissue, A = adenoma tissue, T = cancer tissue.

Fig. 7 shows that the combination of two markers (p10.3 and p4.8 or p10.3 and p3.4) improved in a heterogenous patient population both, the sensitivity and specificity for adenoma and cancer tissues. A) A sensitivity of 90% for cancer tissue (79 % for adenoma) and a specificity of 90% were achieved, if the detection was based on a combination of p10.3 and p4.8 B) Using the combination of p10.3 and the alpha defensin family member p3.4 the according numbers were 70% for cancer samples (82% for adenoma) with a specificity of 90%. N = normal colon mucosa, T = carcinoma tissue, A =

adenoma tissue. The numbers in front of the letter designate the number of separated individuals. The other numbers refer to the Cut-off values.

Examples

Unless otherwise stated all methods were carried out following the protocol of the manufacturer of the analytical systems.

Example 1:

Sample Preparation

Tissue Samples Tumor tissue from adenoma and colorectal cancer patients (TNM stage III) as well as matched normal colon tissue from each patient were analyzed. *Patients:* Ethical guidelines and patient confidentiality have been strictly assured and all patients gave written consent to participate in this study. All patients had comparable preoperative preparations such as fasting time and medication at the time of surgery.

Preparation of tissue extracts Cryostat sections (5µm) from fresh frozen tumor and normal tissues were stained with hematoxilin/eosin to control the histopathology and the amount of tumor/stroma. Tissue blocks with a tumor content or adenoma content over 50% as well as normal tissues were cut into sections (8x20µm) and immediately transferred into 500 µl Dulbeccos phosphate buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 6,5 mM $Na_2HPO_4 \cdot 12H_2O$, 1,5 mM KH_2PO_4, pH 7.4). The sections were centrifuged at 13,200 rpm for 10 min at 4°C. The pellets were extracted by a 150 µl lysis buffer (20mM Tris, 50mM NaCl, 0,5% Tween pH 7.2, 0.1% complete® (Roche, Mannheim, Germany) for 10 min on ice. After extensive vortexing the lysates were centrifuged for 10 min at 13,200 rpm. The protein concentrations of the supernatants were measured by BCA assay (BCA Assay, Pierce, Rockford, IL, USA) and were adjusted for each sample to a concentration of 5µg/50µl with lysis-buffer.

Example 2

Ciphergen ProteinChip® array preparation. The protein lysates were analyzed on a strong anionic exchanger array (SAX; Ciphergen Biosystems, Femont, CA, USA). SAX protein arrays (strong anionic exchanger) were processed in a bioprocessor (Ciphergen Biosystems, Inc). Chips were equilibrated with binding buffer (0.1M Tris/HCl, pH 7.5) for 2x5 min and subsequently incubated with 50µl protein lysate on each spot. After 45 min the unbound material was removed and the chips were washed 3 times with buffer and 2 times with water. After drying, 2 applications of sinapinic acid (1.0 µl) were added and the chips were analyzed with the Ciphergen Protein ChipReader (model PBSII). To minimize data variability, measurement was performed within two days using samples from all patient groups randomly distributed on the chips. As a standard control for normalization, one tissue extract from a cancer patient was used in parallel for all measurements.

SELDI-TOF-MS analysis. The mass spectra of proteins were generated by using an average of 195 laser shots at a laser intensity of 180. The detector was run at a sensitivity of 6. For data aquisition, the detection size range was between 2,000 and 40,000 Da. The laser was focused at 10,000 Da. The data were analyzed with the ProteinChip Data Analysis Program (version 3.1, Ciphergen Biosystems) and with the Biomarker Wizard Program (version 3.1, Ciphergen Biosystems).The peak intensities were normalized to the total ion current.

Example 3

Statistical evaluation of the data

For the three patient groups cut-off values are calculated by the C&RT(CART) algorithmus on the basis of decision-tree analysis (Breiman, L., Friedman, J. H., Olshen, R. A., & Stone, C. J. (1984). *Classification and regression trees.* Monterey, CA: Wadsworth & Brooks/Cole Advanced Books & Software). The Cutoff-values have been calculated in order to select and specify the limiting values between the different analysis groups. The evaluation has been performed with STATISTICA

Software Vs 7.1 from STATSOFT INC, the decision-tree analysis is performed with Data-Miner Modul subprogram Standard Classification Trees (CAndRT) (StatSoft, Inc. (2005). STATISTICA (data analysis software system), version 7.1. www.statsoft.com.)

The statistical data are evaluated on the basis on the mean value and standard deviation. Further, the Figures 3, 5 and 6 show a confidence interval of mean ± 0,95, indicating to find the true mean values of the prospect patient groups with 95% probability within this interval. The statistical evaluation is performed by the T-Test (Table 1). The tests were considered to be significant with p-levels < 0,05. The whiskers of the box plots show the standard deviation.

Example 4

Protein samples of tumor tissue from one adenoma and one colorectal cancer patient (TNM stage II) as well as normal colon tissue has been prepared and analysed according to Examples 1 and 2. Figure 2 shows SELDI-TOF protein profiles of lysates from normal colon, adenoma and tumor tissue. The intensity of p10.3 increases significantly in tissue from healthy to cancer patients.

Figure 3 shows the mean intensities of p10.3 (endoplasmin fragment) in normal, adenoma and colon cancer tissue. 30 colon cancer and 29 adenoma tissue samples as well as corresponding normal colon tissue were analysed according to examples 1 to 3. Differences in mean intensities of p10.3 in normal and colon tumor tissue as well as in normal and adenoma tissue were statistically significant. For the discrimination of tumor samples a specificity of 97% and sensitivity of 93% was achieved. The specificity for adenoma samples was 90% with a sensitivity of 86%.

Figure 5 shows the mean intensities of one of the identified alpha-defensin family members (peak 3,489) in the same patient population. The mean intensities in the adenoma and colon cancer groups were significantly elevated compared to normal colon tissue levels. A discrimination of adenoma samples was achieved with a

sensitivity of 52% and a specificity of 90%. The respective values for discrimination of tumor samples were 90% and 89.6%.

Figure 6 shows the specifically elevated level of peak 4838 in the adenoma group samples. The adenoma samples could be discriminated from the tumor and normal patient samples with a sensitivity of 76% and a specificity of 100%.

Table 1 summarizes the detected peak intensities in the patient group for peaks 10,324, 4,838 and the defensin family member peaks 3,375, 3,445 and 3,489.

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Table 1:

Variable (m/z)	Valid n N	Valid n A	Valid n T	Valid n A+T	Mean ± Std.Dev. N	Mean ± Std.Dev. A	Mean ± Std.Dev. T	Mean ± Std.Dev. A+T	p-Value N vs. A	p-Value N vs. T	p-Value N vs. A+T	p-Value A vs. T
3375	29	29	30	59	3.155 ± 3.476	6.54 ± 4.73	20.98 ± 12.89	13.88 ± 12.4	0.0029	0.0000001	0.000011	0.0000001
3445	29	29	30	59	2.66 ± 3.28	5.86 ± 5.1	19.79 ± 13.26	12.85 ± 12.3	0.01	0.0000001	0.000034	0.000002
3489	29	29	30	59	1.14 ± 1.06	2.39 ± 2.32	14.96 ± 13.39	8.78 ± 11.5	0.01	0.000001	0.00061	0.000006
4838	29	29	30	59	0.619 ± 0.166	1.68 ± 1.026	0.58 ± 0.21	1.12 ± 0.91	0.000001	0.49	0.0040	0.0000001
10324	29	29	30	59	0.95 ± 0.38	2.37 ± 1.24	3.44 ± 1.57	2.91 ± 1.5	0.0000001	0.0000001	0.0000001	0.00531

n: number of patients

N: normal tissue

A: adenoma tissue

T: cancer tissue

All data in Table 1 were obtained by SELDI-TOF-MS

Example 5

Expression of p10.3 in different tissues

In order to identify p10.3 as colon-specific biomarker cell extracts were prepared and analysed by mass spectroscopy according to Examples 1 and 2 for different tumor tissue namely breast, stomach, esophagus, lung and prostate. As shown in Fig. 4 and Table 2 the highest intensity of p10.3 can be found in colorectal carcinoma (stadium I and III). However, the endoplasmin fragment is expressed at a lower level in esophagus, lung and prostata cancer and normal tissue.

Table 2: Intensities of p10.3 in different cancer types. The lysates were analysed on a SAX Protein Chip®Array.

Tissue type		Intensity p10.3
Breast	normal tumor	1.002 0.672
Lung	normal tumor	1.431 1.907
Stomach	normal tumor	1.849 1.629
Esophagus	normal tumor	0.506 0.944
Prostate	normal tumor	0.933 0.852
Colon (stadium I)	normal tumor	0.948 2.830
Colon (stadium III)	normal tumor	1.035 3.283

It can be seen from Fig. 4 that endoplasmin fragments, especially the protein fragment having an apparent molecular weight of 10,300 Da (p10.3), are

highly specific for colon carcinoma. In contrast to tumor of breast, lung, stomach, esophagus or prostate, there is a significant difference in the level of p10.3 between healthy and diseased patients. Moreover, the level of p10.3 increases with the cancer stage (stadium I versus stadium III).

Example 6

30 colon cancer and 29 adenoma tissue samples as well as corresponding normal colon tissue were isolated and analysed as described in Examples 1 to 3 except that a second marker, a protein either having a molecular weight of 4,838 Da (p4.8) or a molecular weight of 3,489 Da (p3.4), has been used in combination with the endoplasmin fragment peak. In combination with a second marker protein, e.g. 4,838 Da (p4.8) or 3,489 Da (p.3.4), simultaneous separation of normal tissue from adenoma and/or cancer tissues was achieved. Using p10.3 as first and p4.8 as second marker, 26 out of 29 (specificity 90%) normal tissue samples, 23 of 29 adenoma samples (sensitivity 79%) as well as 27 of 30 cancer samples (sensitivity 90%) were correctly separated (Figure 7A). The combination with peak 3489 achieved a specificity of 90% and a sensitivity of 82% for adenoma samples and 70 % for tumor samples (Figure 7B).

Results

As shown in Figure 3 the amount of the endoplasmin fragment differs significantly between the three groups (N= normal A=adenoma T=cancer). The endoplasmin fragment level increases from healthy individuals over colorectal adenoma patients to colorectal carcinoma patients.

These data show that an endoplasmin fragment, optionally in combination with other biomarkers such as a protein having a molecular weight of 4,838 Da (p4.8) or of 3,489 Da (p3.4), is (are) an excellent biomarker(s) for the detection of colorectal adenoma and/or colorectal carcinoma.

In contrast to already known biomarkers CEA and CA 19-9 it is possible to discriminate between healthy individuals and adenoma patients. Furthermore, it is possible to discriminate adenoma patients and colorectal carcinoma patients. The sensitivity and specificity of the endoplasmin fragment test is high and allows an early specific detection of adenomas as well as the discrimination between different tumor stages.

Sequence Listing

SEQ-ID NO. 1

Sequence of Endoplasmin (grp 94)

```
  1   MRALWVLGLC CVLLTFGSVR ADDEVDVDGT VEEDLGKSRE GSRTDDEVVQ REEEAIQLDG
 61   LNASQIRELR EKSEKFAFQA EVNRMMKLII NSLYKNKEIF LRELISNASD ALDKIRLISL
121   TDENALSGNE ELTVKIKCDK EKNLLHVTDT GVGMTREELV KNLGTIAKSG TSEFLNKMTE
181   AQEDGQSTSE LIGQFGVGFY SAFLVADKVI VTSKHNNDTQ HIWESDSNEF SVIADPRGNT
241   LGRGTTITLV LKEEASDYLE LDTIKNLVKK YSQFINFPIY VWSSKTETVE EPMEEEEAAK
301   EEKEESDDEA AVEEEEEEKK PKTKKVEKTV WDWELMNDIK PIWQRPSKEV EEDEYKAFYK
361   SFSKESDDPM AYIHFTAEGE VTFKSILFVP TSAPRGLFDE YGSKKSDYIK LYVRRVFITD
421   DFHDMMPKYL NFVKGVVDSD DLPLNVSRET LQQHKLLKVI RKKLVRKTLD MIKKIADDKY
481   NDTFWKEFGT NIKLGVIEDH SNRTRLAKLL RFQSSHHPTD ITSLDQYVER MKEKQDKIYF
541   MAGSSRKEAE SSPFVERLLK KGYEVIYLTE PVDEYCIQAL PEFDGKRFQN VAKEGVKFDE
601   SEKTKESREA VEKEFEPLLN WMKDKALKDK IEKAVVSQRL TESPCALVAS QYGWSGNMER
661   IMKAQAYQTG KDISTNYYAS QKKTFEINPR HPLIRDMLRR IKEDEDDKTV LDLAVVLFET
721   ATLRSGYLLP DTKAYGDRIE RMLRLSLNID PDAKVEEEPE EEPEETAEDT TEDTEQDEDE
781   EMDVGTDEEE ETAKESTAEK DEL
```

SEQ-ID NO. 2

Fragment of endoplasmin (grp 94)

```
EEEAIQLDG LNASQIR
```

SEQ-ID NO. 3

Fragment of endoplasmin (grp 94)

```
FAFQA EVNR
```

SEQ-ID NO. 4

Fragment of endoplasmin (grp 94)

```
EEEAIQLDG LNASQIRELR EKSEKFAFQA EVNR
```

SEQ-ID NO. 5

C3 Protein

```
ID   CO3_HUMAN_2;   parent: CO3 HUMAN
FT   CHAIN     23   1663    Complement C3.
SQ   Sequence   1641 AA;
SPMYSIITPN ILRLESEETM VLEAHDAQGD VPVTVTVHDF PGKKLVLSSE KTVLTPATNH
MGNVTFTIPA NREFKSEKGR NKFVTVQATF GTQVVEKVVL VSLQSGYLFI QTDKTIYTPG
STVLYRIFTV NHKLLPVGRT VMVNIENPEG IPVKQDSLSS QNQLGVLPLS WDIPELVNMG
QWKIRAYYEN SPQQVFSTEF EVKEYVLPSF EVIVEPTEKF YYIYNEKGLE VTITARFLYG
KKVEGTAFVI FGIQDGEQRI SLPESLKRIP IEDGSGEVVL SRKVLLDGVQ NLRAEDLVGK
SLYVSATVIL HSGSDMVQAE RSGIPIVTSP YQIHFTKTPK YFKPGMPFDL MVFVTNPDGS
PAYRVPVAVQ GEDTVQSLTQ GDGVAKLSIN THPSQKPLSI TVRTKKQELS EAEQATRTMQ
ALPYSTVGNS NNYLHLSVLR TELRPGETLN VNFLLRMDRA HEAKIRYYTY LIMNKGRLLK
AGRQVREPGQ DLVVLPLSIT TDFIPSFRLV AYYTLIGASG QREVVADSVW VDVKDSCVGS
LVVKSGQSED RQPVPGQQMT LKIEGDHGAR VVLVAVDKGV FVLNKKNKLT QSKIWDVVEK
ADIGCTPGSG KDYAGVFSDA GLTFTSSSGQ QTAQRAELQC PQPAARRRRS VQLTEKRMDK
VGKYPKELRK CCEDGMRENP MRFSCQRRTR FISLGEACKK VFLDCCNYIT ELRRQHARAS
HLGLARSNLD EDIIAEENIV SRSEFPESWL WNVEDLKEPP KNGISTKLMN IFLKDSITTW
EILAVSMSDK KGICVADPFE VTVMQDFFID LRLPYSVVRN EQVEIRAVLY NYRQNQELKV
RVELLHNPAF CSLATTKRRH QQTVTIPPKS SLSVPYVIVP LKTGLQEVEV KAAVYHHFIS
DGVRKSLKVV PEGIRMNKTV AVRTLDPERL GREGVQKEDI PPADLSDQVP DTESETRILL
QGTPVAQMTE DAVDAERLKH LIVTPSGCCE QNMIGMTPTV IAVHYLDETE QWEKFGLEKR
QGALELIKKG YTQQLAFRQP SSAFAAFVKR APSTWLTAYV VKVFSLAVNL IAIDSQVLCG
AVKWLILEKQ KPDGVFQEDA PVIHQEMIGG LRNNNEKDMA LTAFVLISLQ EAKDICEEQV
NSLPGSITKA GDFLEANYMN LQRSYTVAIA GYALAQMGRL KGPLLNKFLT TAKDKNRWED
PGKQLYNVEA TSYALLALLQ LKDFDFVPPV VRWLNEQRYY GGGYGSTQAT FMVFQALAQY
QKDAPDHQEL NLDVSLQLPS RSSKITHRIH WESASLLRSE ETKENEGFTV TAEGKGQGTL
SVVTMYHAKA KDQLTCNKFD LKVTIKPAPE TEKRPQDAKN TMILEICTRY RGDQDATMSI
LDISMMTGFA PDTDDLKQLA NGVDRYISKY ELDKAFSDRN TLIIYLDKVS HSEDDCLAFK
VHQYFNVELI QPGAVKVYAY YNLEESCTRF YHPEKEDGKL NKLCRDELCR CAEENCFIQK
SDDKVTLEER LDKACEPGVD YVYKTRLVKV QLSNDFDEYI MAIEQTIKSG SDEVQVGQQR
TFISPIKCRE ALKLEEKKHY LMWGLSSDFW GEKPNLSYII GKDTWVEHWP EEDECQDEEN
QKQCQDLGAF TESMVVFGCP N
```

SEQ-ID NO. 6

C3A: 77 amino acids, 9,094 Da , pl 9.7

ID CO3_HUMAN_5; parent: CO3 HUMAN
FT PEPTIDE 672 748 C3a anaphylatoxin.
SQ Sequence ;
SVQLTEKRMD KVGKYPKELR KCCEDGMREN PMRFSCQRRT RFISLGEACK
KVFLDCCNYITELRRQHARA SHLGLAR

SEQ-ID NO. 7

C3A-desArg: 76 amino acids, pl 9.54

SVQLTEKRMD KVGKYPKELR KCCEDGMREN PMRFSCQRRT RFISLGEACK
KVFLDCCNYITELRRQHARA SHLGLA

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for detecting colorectal adenoma or colorectal carcinoma, said method comprising the steps of:
   a) providing a sample material isolated from a test individual,
   b) determining a level of an endoplasmin fragment in said isolated sample material, and
   c) comparing the determined level of the endoplasmin fragment with at least one reference value, wherein the endoplasmin fragment has a molecular weight of 10 to 14 kDa and comprises peptide sequence SEQ-ID No. 2, SEQ-ID No. 3, or SEQ-ID No. 4.

2. A method for discriminating between colorectal adenoma, colorectal carcinoma, and further tumor stages, said method comprising the steps of:
   a) providing a sample material isolated from a test individual,
   b) determining a level of an endoplasmin fragment in said isolated sample material, and
   c) comparing the determined level of the endoplasmin fragment with at least one reference value, wherein the endoplasmin fragment has a molecular weight of 10 to 14 kDa and comprises peptide sequence SEQ-ID No. 2, SEQ-ID No. 3, or SEQ-ID No. 4.

3. A method for i) monitoring the development, course, or treatment of colorectal adenoma or colorectal carcinoma, or ii) discriminating between different tumor stages, said method comprising the steps of:
   a) providing a sample material isolated from a test individual,
   b) determining a level of an endoplasmin fragment in said isolated sample material, and
   c) comparing the determined level of the endoplasmin fragment with at least one reference value, wherein the endoplasmin fragment

has a molecular weight of 10 to 14 kDa and comprises peptide sequence SEQ-ID No. 2, SEQ-ID No. 3, or SEQ-ID No. 4.

4. The method according to any one of claims 1 to 3, wherein the endoplasmin fragment has a molecular weight of 10.3 kDa.

5. The method according to any one of claims 1 to 4, wherein a level of the endoplasmin fragment in a sample material isolated from a colorectal adenoma patient or a colorectal carcinoma patient is increased as compared to a level of the endoplasmin fragment in a sample material isolated from a healthy individual.

6. The method according to claim 5, wherein a first increase in the level of the endoplasmin fragment in a first sample material is indicative for colorectal adenoma and wherein a second increase in the level of the endoplasmin fragment in a second sample material, isolated from said test individual at a later point in time than said first sample material, is indicative for colorectal carcinoma, with the proviso that said second increase is greater than said first increase.

7. The method according to any one of claims 1 to 6, wherein in step (b) at least one further biomarker for detecting said colorectal adenoma or colorectal carcinoma is determined in said isolated sample material; and wherein in step (c) the determined level of said at least one further biomarker is compared to at least one further reference value.

8. The method according to claim 7, wherein said at least one further biomarker is selected from the group consisting of:
a protein having a molecular weight of 4,838 ± 25 Da as determined by surface enhanced laser desorption ionization-time of flight mass spectroscopy (SELDI-TOF-MS),
alpha-defensin 1, alpha-defensin 2, alpha-defensin 3, transthyretin, p53,

C3a, CEA (carcinoembryonic antigen), CA 19-9, CA 15-3, CA-125, Kras, β-Catenin, Her-2/neu, C-reactive protein,
mutations in E-cadherin, MSH2, MSH3, MLH1, PMS1, PMS2, and MSH6 genes,
microsatellite instability of MHL1, microsatellite instability of MSH2, SNPs, and combinations thereof.

9. The method according to any one of claims 1 to 8, wherein the at least one reference value is calculated as the average level of the endoplasmin fragment determined in a plurality of sample materials isolated from a group of healthy individuals, colorectal adenoma patients or colorectal carcinoma patients.

10. The method according to any one of claims 1 to 8, wherein the at least one reference value is an individual reference value calculated as the average level of the endoplasmin fragment determined in a plurality of sample materials isolated from said test individual over a period of time.

11. The method according to any one of claims 1 to 10, wherein the isolated sample material is a body fluid or tissue.

12. The method according to claim 11, wherein the body fluid is selected from the group consisting of: blood, blood plasma, serum, bone marrow, stool, synovial fluid, lymphatic fluid, cerebro spinal fluid, sputum, urine, mother milk, sperm, exudate and mixtures thereof.

13. The method according to any one of claims 1 to 12, wherein the level of the endoplasmin fragment in said isolated sample material is determined on DNA, mRNA, or protein level.

14. The method according to any one of claims 1 to 13, wherein the level of the endoplasmin fragment in said isolated sample material is determined

by a nucleic acid hybridisation technique, immunological methods, proteomics technique, or mass spectroscopy.

15. The method according to claim 7, wherein the at least one further reference value is calculated as the average level of the further biomarker determined in a plurality of sample materials isolated from a group of healthy individuals, colorectal adenoma patients or colorectal carcinoma patients.

16. The method according to claim 7, wherein the at least one further reference value is an individual reference value calculated as the average level of the at least one further biomarker determined in a plurality of sample materials isolated from said test individual over a period of time.

17. The method according to claim 7, wherein the level of the at least one further biomarker in said isolated sample material is determined on DNA, mRNA, or protein level.

18. The method according to claim 7, wherein the level of the at least one further biomarker in said isolated sample material is determined by a nucleic acid hybridisation technique, immunological methods, proteomics technique, or mass spectroscopy.

19. The method according to any one of claims 1 to 18, wherein the method is carried out in combination with other diagnostic methods for colorectal adenoma or colorectal carcinoma to increase sensitivity or specificity.

20. A use of an endoplasmin fragment as a biomarked for (i) detection of colorectal adenoma or colorectal carcinoma, or (ii) discrimination of different tumor stages, in a test individual or a sample material isolated from said test individual, wherein said endoplasmin fragment has a

molecular weight of 10 to 14 kDa and comprises peptide sequence SEQ-ID No. 2, SEQ-ID No. 3, or SEQ-ID No. 4.

21. The use according to claim 20, for (i) an early detection of colorectal or colorectal carcinoma, or (ii) an early discrimination of different tumor stages in said isolated sample material.

22. The use according to claim 20 or 21, in combination with at least one further biomarker for colorectal adenoma or colorectal carcinoma to increase sensitivity or specificity.

23. The use according to claim 22, wherein said at least one further biomarker is selected from the group consisting of:
a protein having a molecular weight of 4,838 ± 25 Da as determined by surface enhanced laser desorption ionization-time of flight mass spectroscopy (SELDI-TOF-MS),
alpha-defensin 1, alpha-defensin 2, alpha-defensin 3, transthyretin, p53, C3a, CEA (carcinoembryonic antigen), CA 19-9, CA 15-3, CA-125, Kras, β-Catenin, Her-2/neu, C-reactive protein,
mutations in E-cadherin, MSH2, MSH3, MLH1, PMS1, PMS2, and MSH6 genes,
microsatellite instability of MHL1, microsatellite instability of MSH2, SNPs, and combinations thereof.

24. The use according to claim 20, wherein the endoplasmin fragment has a molecular weight of 10.3 kDa.

25. A method for determining whether a treatment of a patient having colorectal adenoma or colorectal carcinoma with a compound is effective, said method comprising the steps of:

a) determining a level of an endoplasmin fragment in a sample material isolated from said patient, and

b) comparing the determined level of the endoplasmin fragment with one or more reference values, wherein said endoplasmin fragment has a molecular weight of 10 to 14 kDa and comprises peptide sequence SEQ-ID No. 2, SEQ-ID No. 3, or SEQ-ID No. 4.

26. The method according to claim 25, wherein in step (b) at least one further biomarker for (i) detecting colorectal adenoma or colorectal carcinoma, or (ii) discriminating different tumor stages is determined, in said isolated sample material and wherein in step (c) the determined level of said at least one further biomarker is compared to at least one further reference value.

27. The method according to claim 26, wherein said at least one further biomarker is selected from the group consisting of:
a protein having a molecular weight of 4,838 ± 25 Da as determined by surface enhanced laser desorption ionization-time of flight mass spectroscopy (SELDI-TOF-MS),
alpha-defensin 1, alpha-defensin 2, alpha-defensin 3, transthyretin, p53, C3a, CEA (carcinoembryonic antigen), CA 19-9, CA 15-3, CA-125, Kras, β-Catenin, Her-2/neu, C-reactive protein,
mutations in E-cadherin, MSH2, MSH3, MLH1, PMS1, PMS2, and MSH6 genes,
microsatellite instability of MHL1, microsatellite instability of MSH2, SNPs, and combinations thereof.

28. The use according to claim 25, wherein the endoplasmin fragment has a molecular weight of 10.3 kDa.

Figure 1

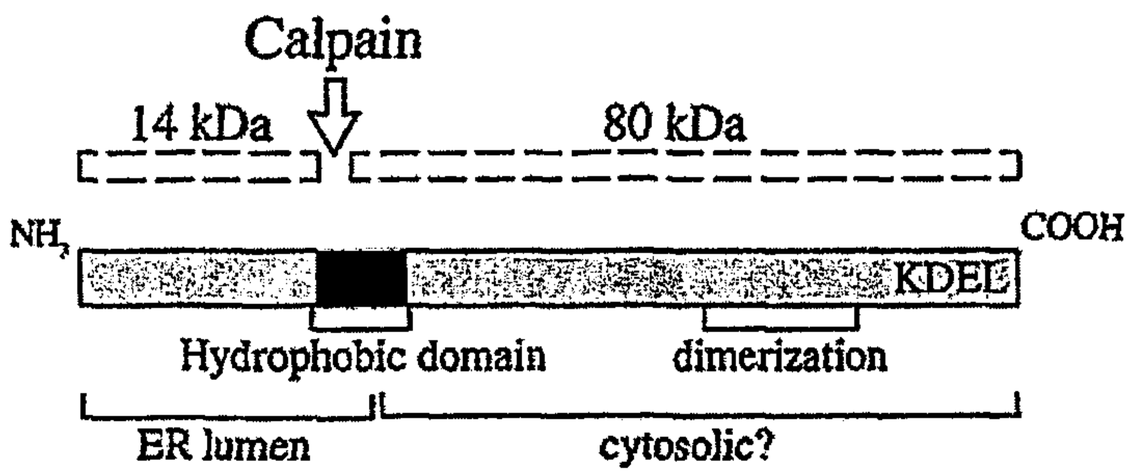

Calpain
14 kDa
80 kDa
NH2
COOH
KDEL
Hydrophobic domain
dimerization
ER lumen
cytosolic?

Figure 2

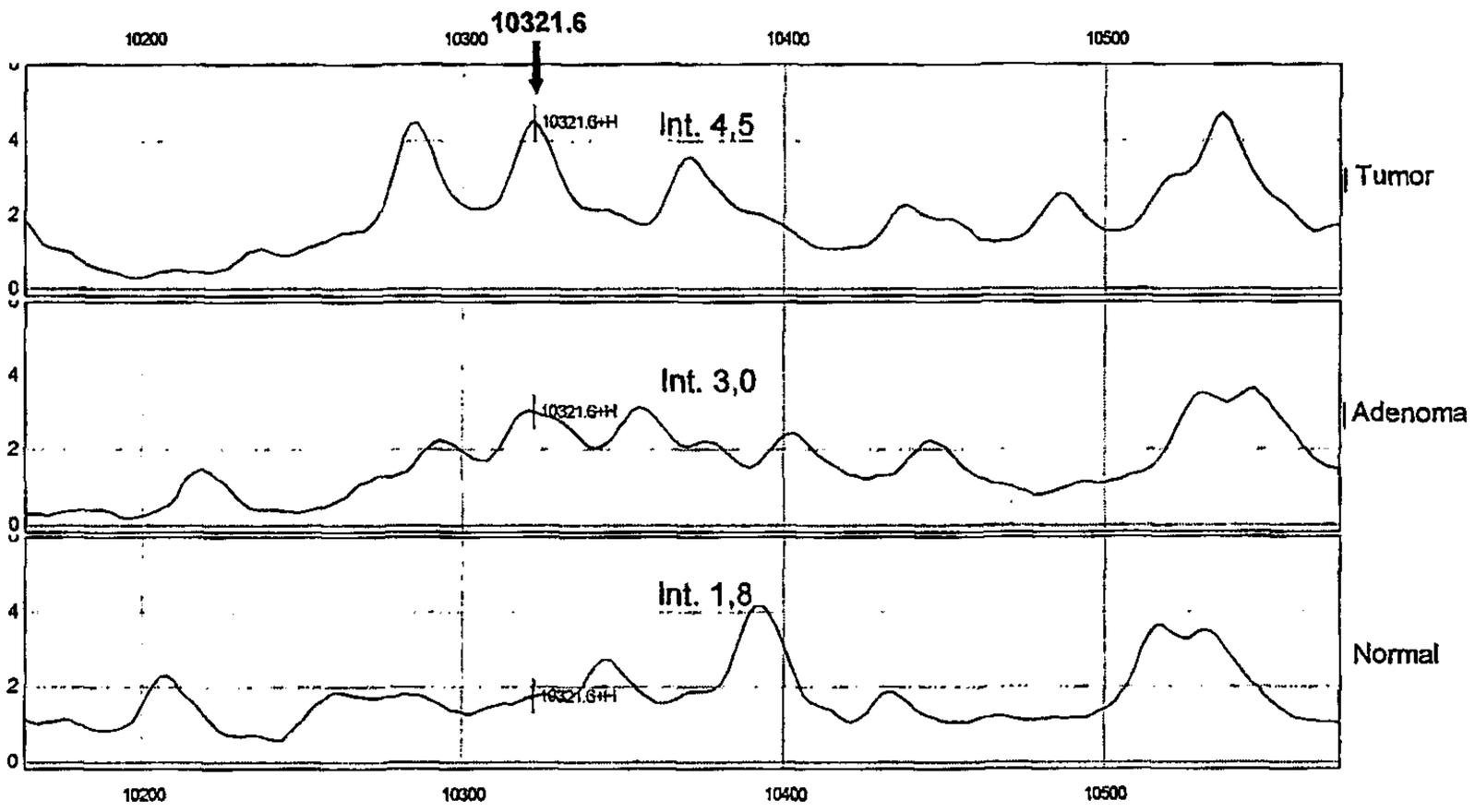

10200
10300
10321.6
10400
10500
10321.6+H
Int. 4,5
Tumor
Int. 3,0
Adenoma
Int. 1,8
Normal
0
2
4

Figure 3

Endoplasmin fragment

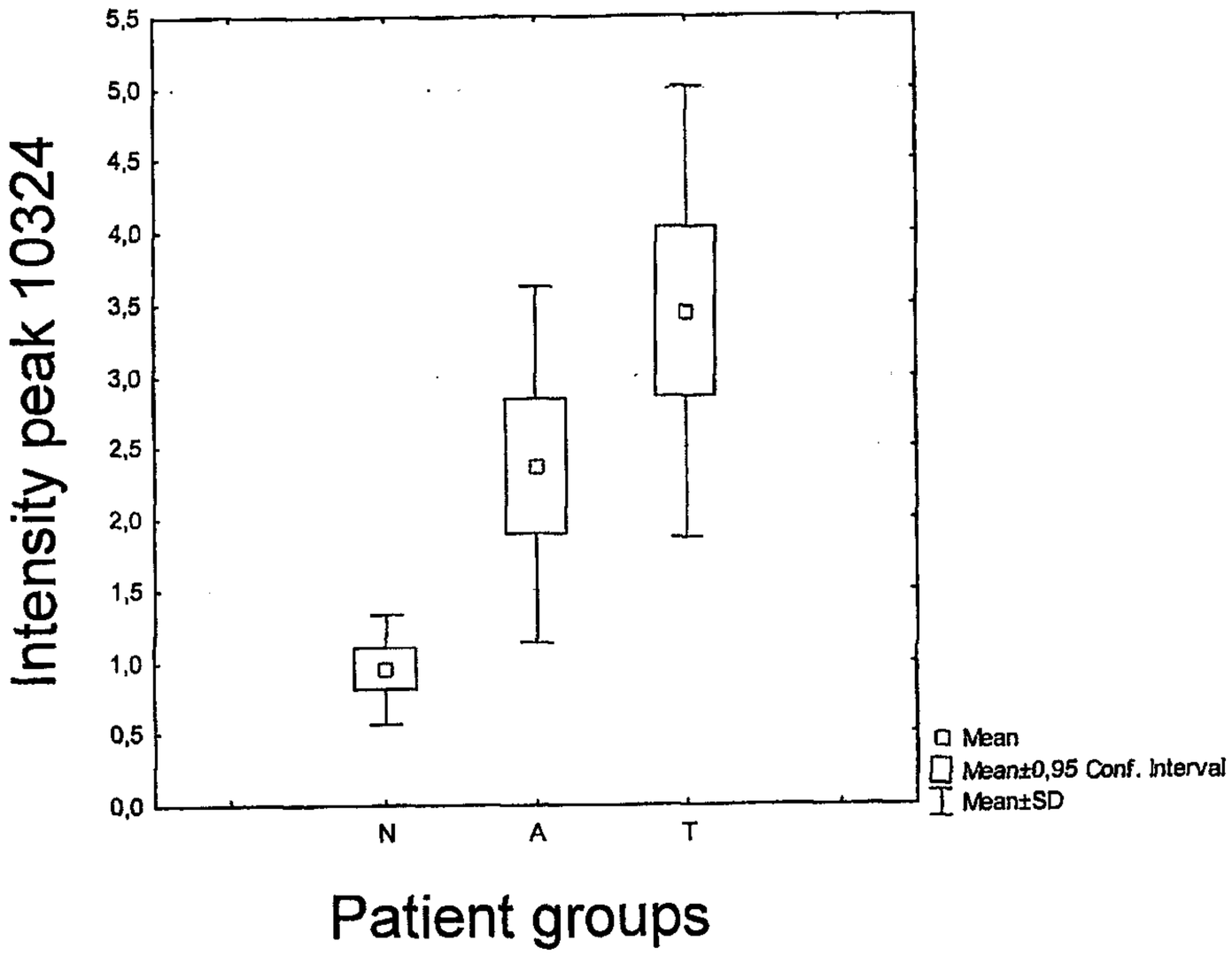


Figure 4

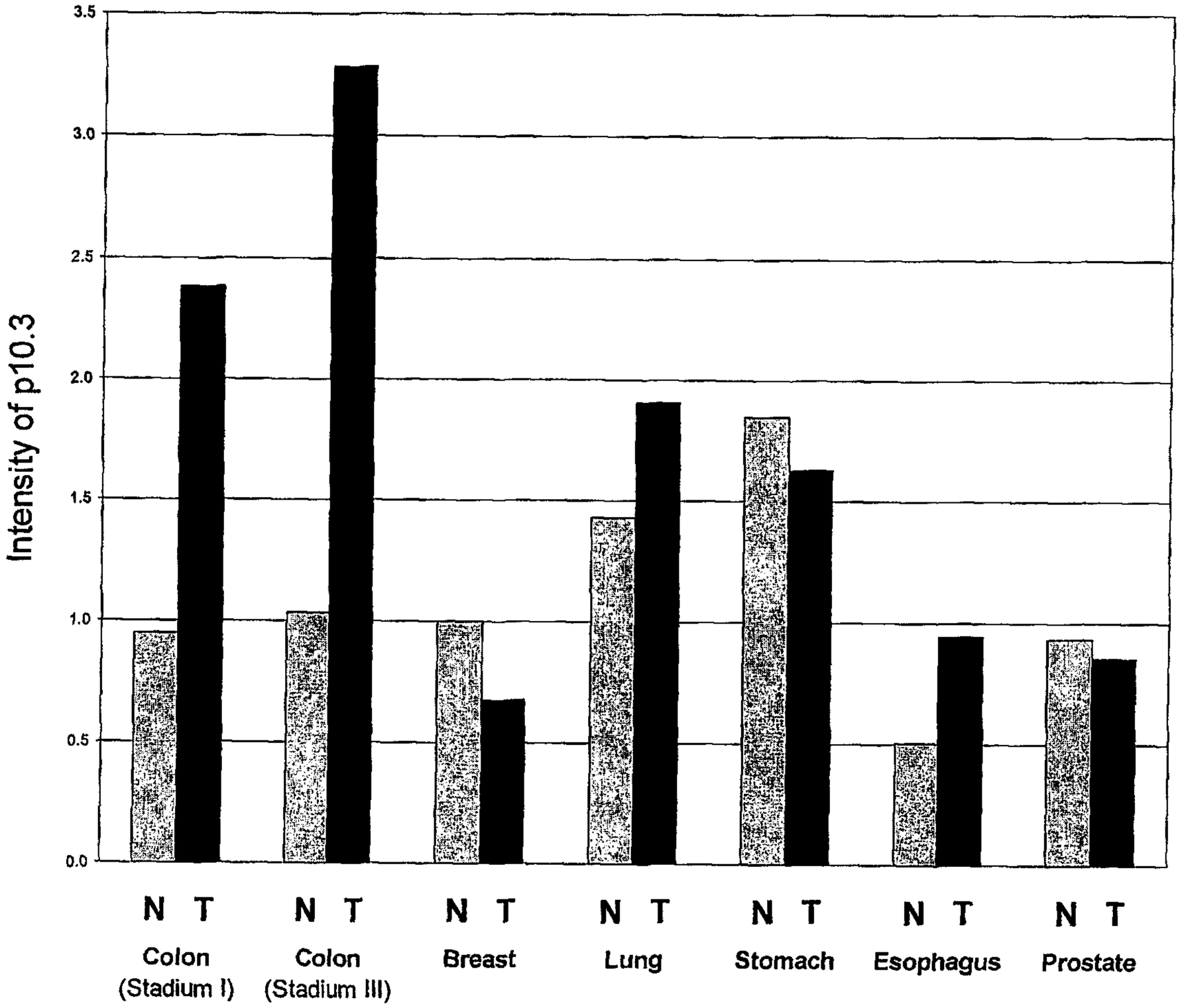

Intensity of p10.3
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N T
Colon (Stadium I)
N T
Colon (Stadium III)
N T
Breast
N T
Lung
N T
Stomach
N T
Esophagus
N T
Prostate

Figure 5

Analysis of peak 3,489 [Da] by SELDI-TOF-MS

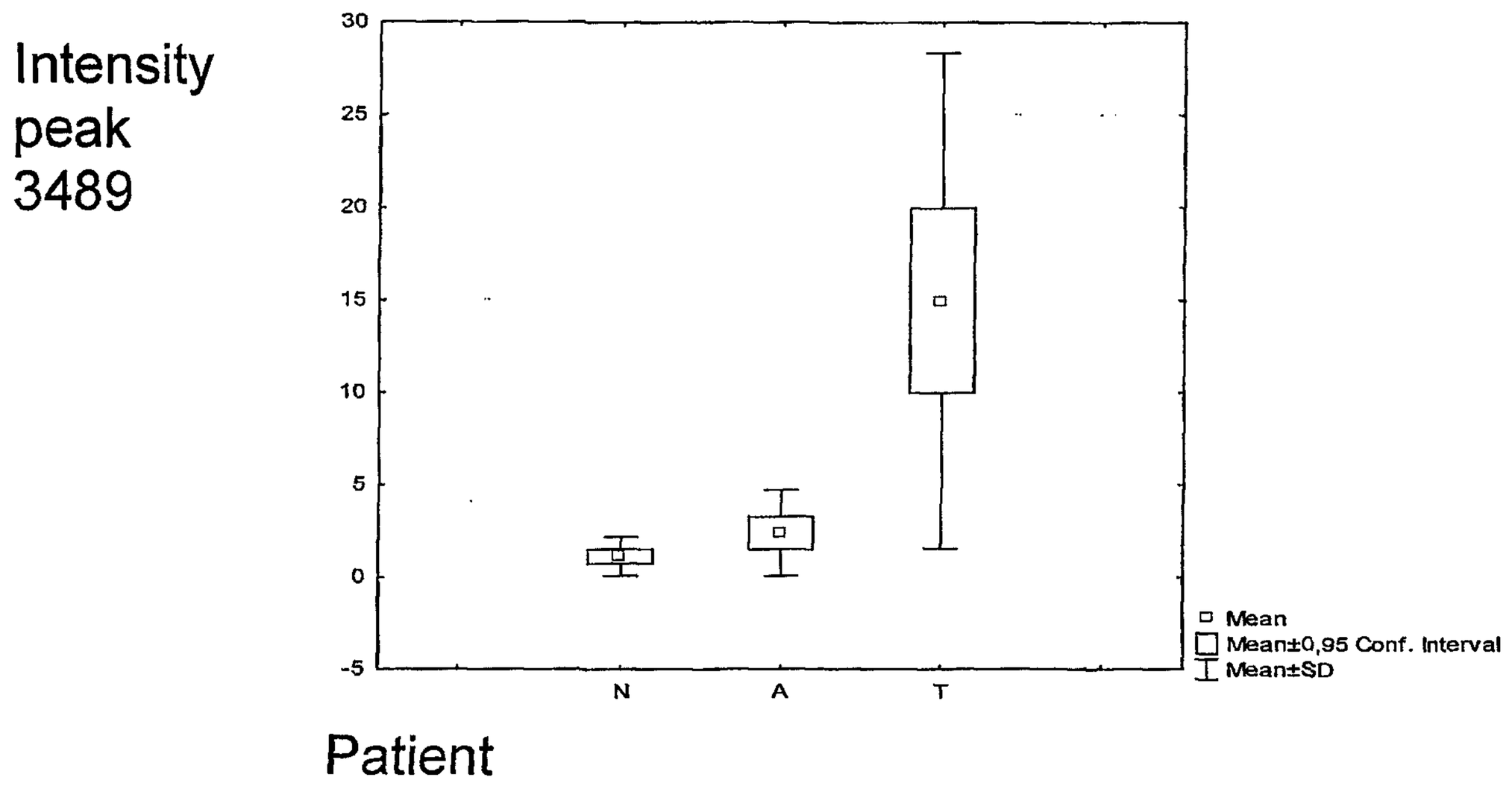


Figure 6

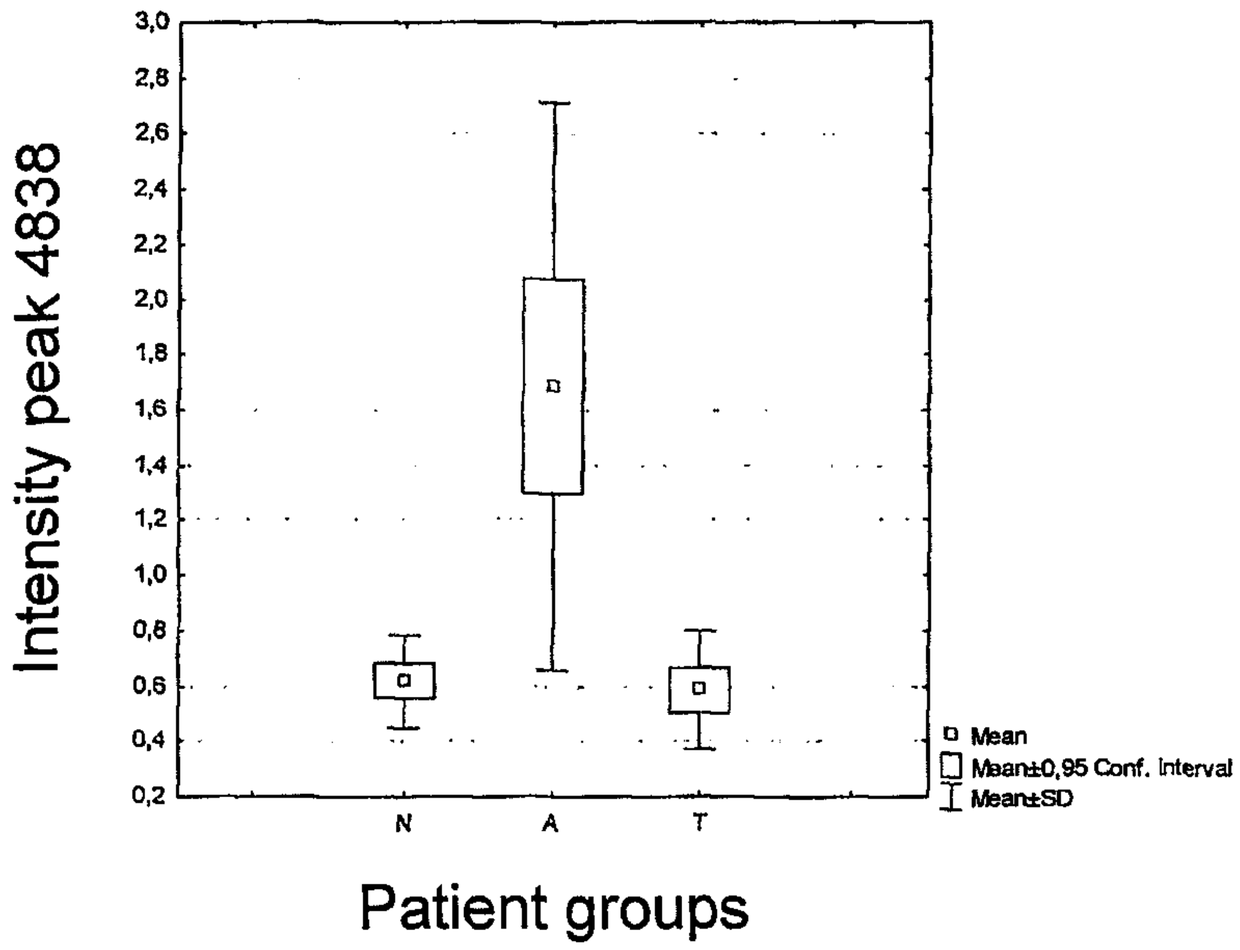

Analysis of peak 4838 [Da] by SELDI-TOF-MS
Intensity peak 4838
3,0
2,8
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
N
A
T
Mean
Mean±0,95 Conf. Interval
Mean±SD
Patient groups

Figure 7A

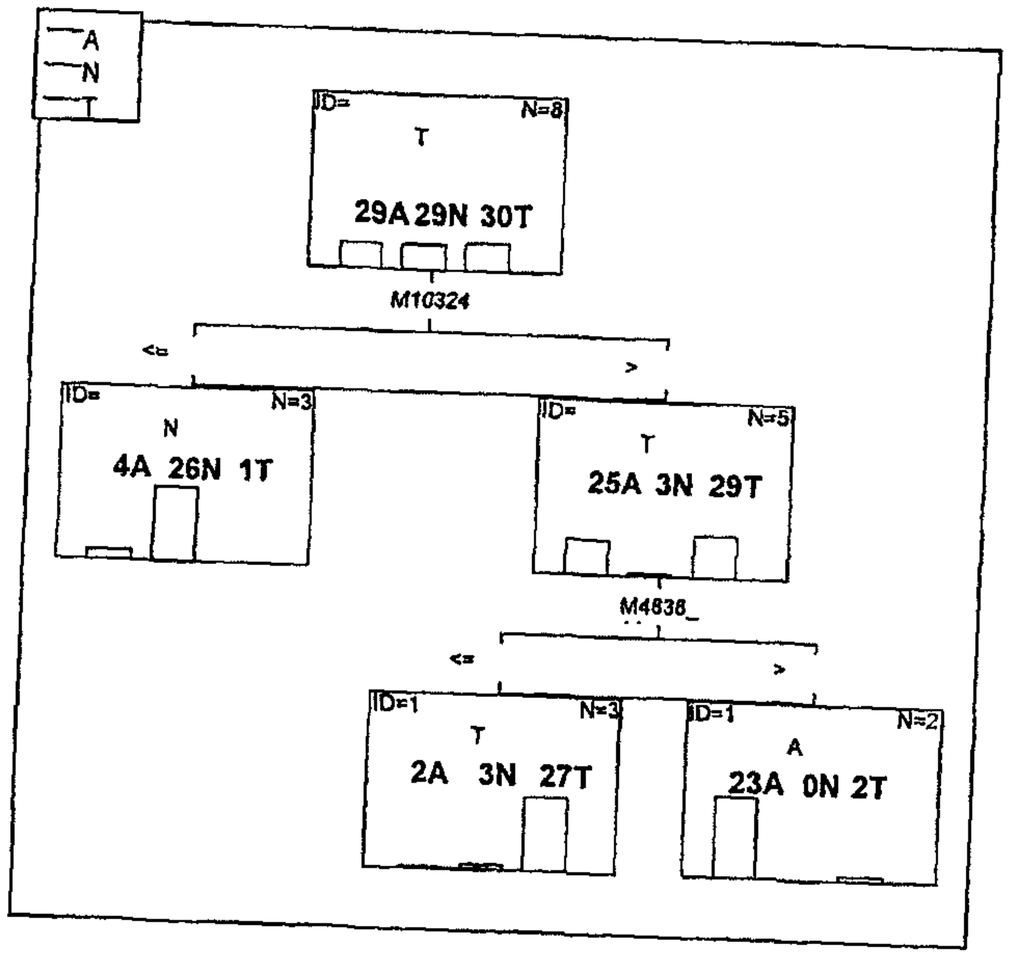
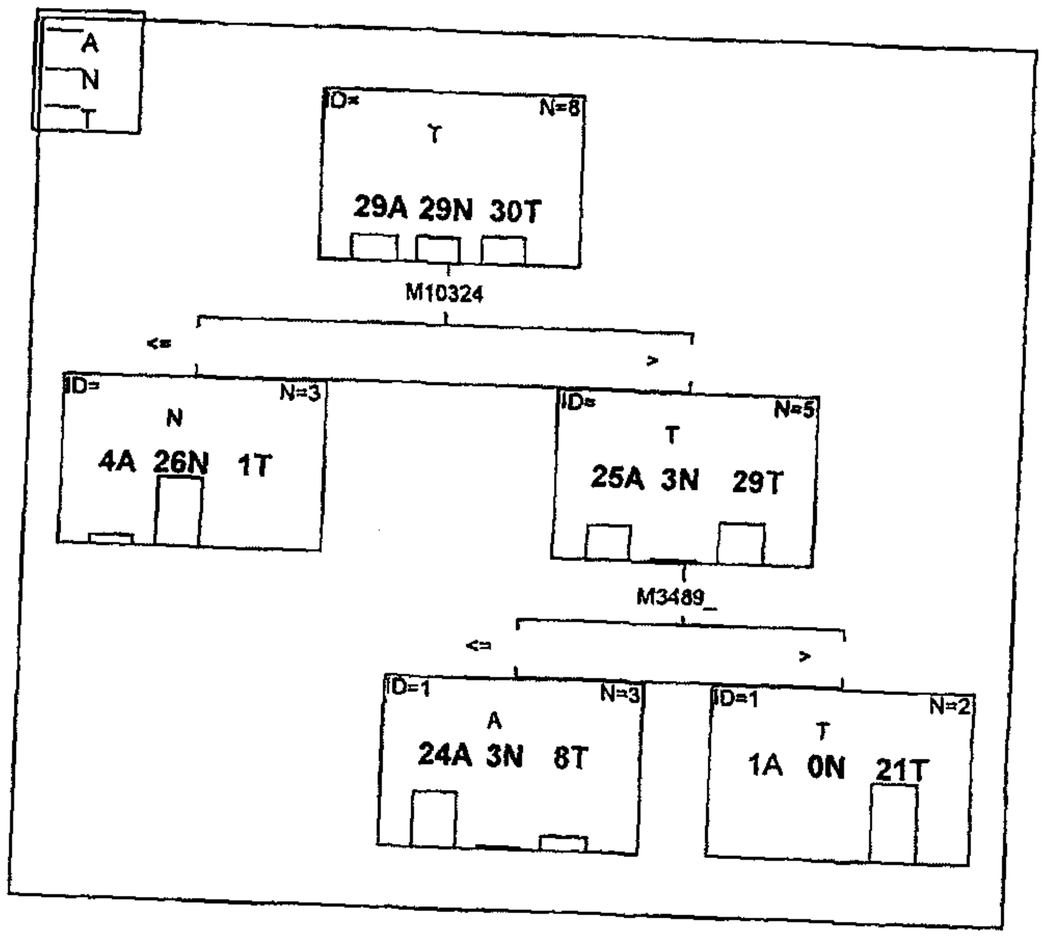
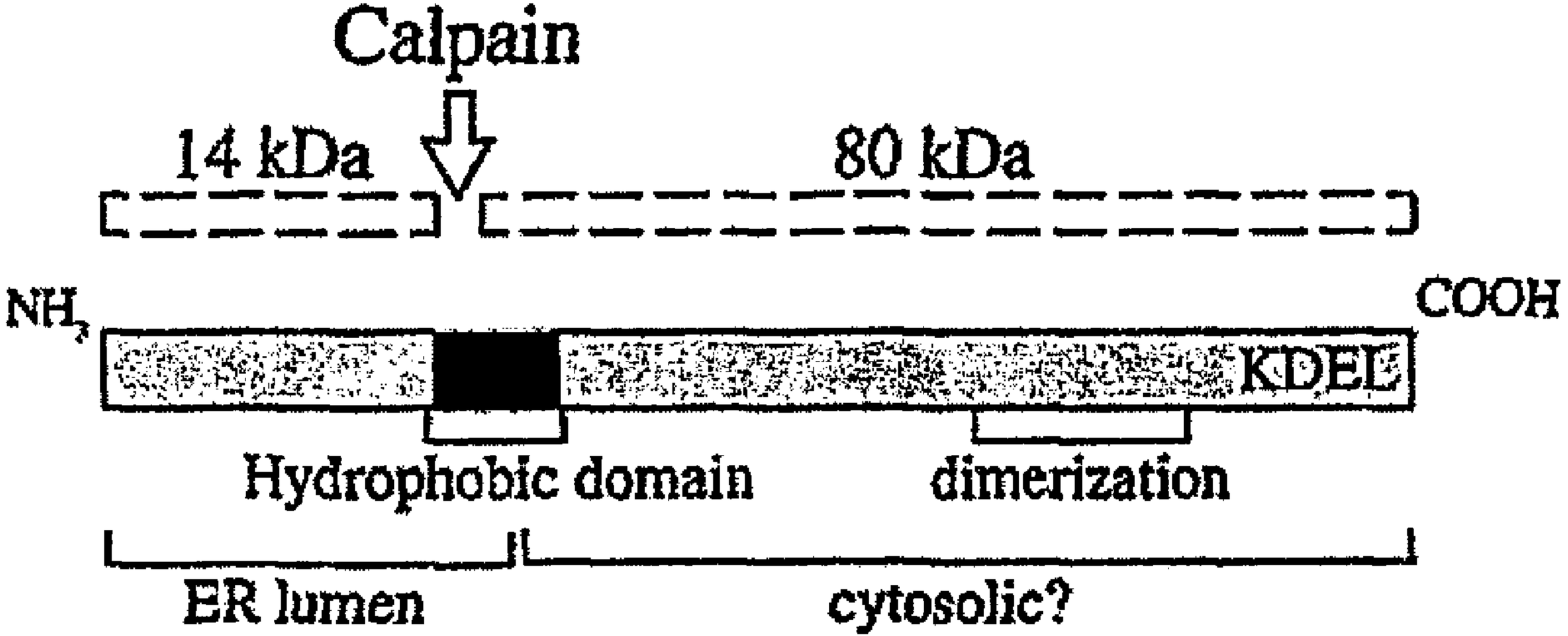


Figure 7B




Calpain
14 kDa
80 kDa
NH2
COOH
KDEL
Hydrophobic domain
dimerization
ER lumen
cytosolic?