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Annexin for cancer risk assessment

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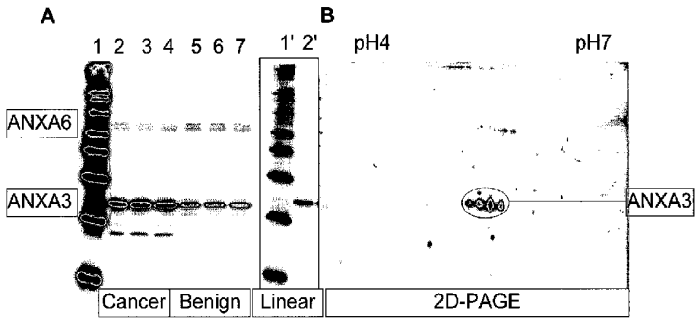

(57) Abstract: The invention relates to the use of at least one annexin protein for the treatment of cancer, in particular of the urogenital and/or intestinal tract, and to a method for diagnosing cancer, particularly of the urogenital and/or intestinal tract, and/or for discrimination between cancerous and non-cancerous tissue.

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Description

Annexin for cancer risk assessment

The present invention relates to the treatment and/or diagnosis of cancer, particularly of the urogenital and/or intestinal tract.

Cancer is one of the leading causes of human death in the western civilization and often linked with difficulties regarding its diagnosis.

For example, prostate cancer is one of the leading causes of cancer death in men but is a heterogeneous disease that is difficult to diagnose. Predicting the course that an individual tumor will take is almost impossible. The current state of diagnostic prostate cancer markers is essentially based on different isoforms of prostate specific antigen (PSA) and on the whole is not satisfactory in terms of false negatives and false positives. Recently, various alternative molecular markers have been suggested from body fluids or prostate tissue. At least three different subclasses of prostate cancer have been identified that seem related to tumor grade, incidence of recurrence, and metastases. Fatty acid synthase alone defines distinct molecular signatures for prostate cancer. Yet, there is urgent remaining need for more elaborate and reliable therapeutic and diagnostic parameters to characterize patients according to their risk of progression in order to develop novel appropriate multimodal therapy strategies for improved individual cancer control.

In view thereof, the problem addressed by the invention has been solved by the use of at least one annexin protein, preferably annexin A3, for the treatment of cancer, particularly of the urogenital and/or intestinal tract, preferably prostate cancer.

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In a first aspect the present invention provides a method for diagnosing prostate cancer by discrimination between cancerous and non-cancerous prostate tissue, the method comprising:

(a) determining the abundance of at least one intracellular annexin A3 protein in a urine sample or fraction thereof; and/or

(b) determining the abundance of at least one extracellular annexin A3 protein in a urine sample or fraction thereof;

wherein the levels of annexin A3 observed in the urine or fraction thereof from a subject with cancerous prostate tissue are lower than in urine or fraction thereof from a subject with non-cancerous prostate tissue and wherein the urine samples or fractions thereof are obtained from exprimate urine.

Besides, the present invention comprises the use of at least one annexin protein, preferably of annexin A3, for the manufacture of a medicament for treatment of cancer, particularly of the urogenital and/or intestinal tract, preferably of prostate cancer. In a preferred embodiment cancer treatment is done by the enhancement of the in vivo abundance of at least one annexin protein, in particular by the enhancement of the in vivo abundance of at least one extracellular annexin protein.

In view thereof, the problem addressed by the invention has been solved in a further aspect by a method for diagnosing cancer, particularly of the urogenital and/or intestinal tract, and/or for discrimination between cancerous and non-cancerous tissue comprising the separate steps of

- determining the intracellular abundance of at least one annexin protein and/or
- determining the extracellular abundance of at least one annexin protein,

in particular using urine samples or fractions thereof.

In a preferred embodiment the inventive method comprises the separate steps of

- determining the intracellular abundance of at least one annexin protein and
- determining the extracellular abundance of at least one annexin protein,

in particular using urine samples or fractions thereof.

In a further preferred embodiment the inventive method comprises the separate steps of

- determining the intracellular abundance of at least one annexin protein and
- determining the extracellular abundance of at least one said annexin protein,

in particular using urine samples or fractions thereof.

After determination of the intra- and extracellular abundance of at least one annexin protein ratios of the extracellular abundance over the intracellular abundance or the other way around may be determined. Preferably, ratios of the extracellular abundance over the intracellular abundance are determined. The obtained ratios are advantageous diagnostic parameters for cancer and/or for discrimination between cancerous and non-cancerous tissue.

According to the present invention the term „extracellular" is understood as the extracellular space including the outer surface of plasma membranes of cells.

According to the present invention, the term "non-cancerous tissue" comprises healthy tissue and pathogenic tissue, in particular benign prostatic hyperplasia, chronic prostatitis, Crohn's disease, colitis ulcerosa, inflammable tissue and fibroses, in particular secondary fibroses.

According to the present invention the term „abundance" is understood as the intracellular and/or extracellular level and concentration respectively of a protein.

According to the present invention, the term "annexin protein" and "protein" in general comprise isoforms, mutants, truncated versions and post-translational modified forms thereof. Post-translational modified forms can in particular include proteinaceous forms obtainable by proteolytic processing.

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According to the present invention, the term "treatment" is equivalent to "therapy", thus comprising the treatment of troubles associated with cancer.

In the case of a preferred embodiment the annexin protein is at least a member of the group consisting of annexin A1, annexin A2, annexin A3, annexin A4, annexin A5, annexin A6, annexin A7, annexin A8 and annexin A10 and wherein preferably the abundance of the at least one annexin protein is determined together with the abundance of at least a further protein. With respect to said further protein it is referred to the following description.

In another embodiment, the abundance of at least one annexin protein is determined together with the abundance of a small molecule or nucleic acid marker.

Annexins are calcium-binding proteins thought to influence various intra- and extracellular functions, including membrane trafficking, lymphocyte migration, cell motility, calcium flux, and signal transduction. They are highly abundant, and the calcium-dependent bulk masking of negatively charged membrane lipids may be important for annexin function.

In a previous proteomics study comparing the differential abundance of proteins between benign and tumorous tissue from 31 prostate cancer patients, the inventors identified annexin A3 as more being variously differentially abundant in tumors, and potentially represented a diagnostic marker for various sub-types of prostate cancer. Annexin A3 is a relatively infrequent annexin family member that was upregulated an average of 2.4 fold across all 31 patients (between 1.1 and 5.4 fold with 95% confidence; P = 0.045). In a tentative sub-cluster of 22 patients that was suggested by cluster analysis, Annexin A3 was upregulated an average of 4.4 fold (between 2.2 and 9.1 fold with 95 % confidence; P = 0.0008),

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suggesting that in certain types of tumor Annexin A3 abundance may be involved in the cancerous phenotype. For further details it is referred to the patent application PCT/EP2005/001567, which is incorporated herein in its entirety.

Several annexins are reported to be down-regulated in association with prostate cancer, including annexin A1, annexin A2, annexin A4, annexin A7 and annexin A10. It has been reported that "some differential (annexin A3) value between malignant and benign" prostate tissue. Recently, annexin A3 has been shown to be necessary for DNA replication in cultured hepatocytes and seems to be expressed higher in small hepatocytes which have higher growth potential and proliferation rates than parenchymal hepatocytes. The results of the inventors suggest that annexin A3, typically a rare member of this family, may therefore provide a biomarker or target or therapeutic principle for cancer treatment of certain patients.

Annexins are cytoplasmic, but are also found extracellularly, although they lack secretory leader sequences. For instance, annexin A3 has been identified as an antigen for anti-sperm antibodies involved in male infertility. It has been found that annexin A1 was exposed on epithelial surfaces in the vicinity of solid lung tumors, and that administration of a radiolabelled antibody against the protein caused tumor regression in animal experiments. Indeed, annexin A5 translocation to cell surfaces is associated with apoptosis and annexin A1, also known as Lipocortin 1 is released to the extracellular space in large abundances from neutrophils and monocytes/macrophages as an anti-inflammatory agent. In fact, annexin A1 may be the primary mediator of the anti-inflammatory effect of glucocorticoids.

There is no mechanism for annexin secretion reported combining secretion, especially the cellular expulsion of annexin A3, the exosome

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pathway, and altered regulation of immune surveillance of the prostate. Exosomes are membrane vesicles of 30 to 100 nm in diameter, which are produced and secreted in vitro by living cells of diverse origin, and are thought to be involved in the transfer of tumor antigens to antigen presenting cells, as well as in the stimulation of specific immune responses. Annexin family members, including annexin A3 and annexin A8, are commonly found in exosomes.

It has been postulated that exosomes are involved in the release of heat shock proteins to the extracellular environment in the absence of cellular necrosis. The luminal exosome environment could permit the low pH values necessary for the proposed annexin calcium ion channel function in vivo, that has been controversially discussed because of incompatibility with cell viability. Indeed, the reported instances of physiological annexin ion channels occur in the matrix vesicles involved in osteoblast bone formation, and in the terminal differentiation and death of chondrocytes, which are both circumstances atypical of normal cellular viability. According to the inventors annexin ion channels could be involved in the osmotic rupture of exosome vesicles (either within multivesicular vesicles prior to secretory fusion with the cytoplasmic membrane, or extracellularly) and thereby modulate extracellular milieu of tumors or other tissues, such as bone in the case of osteoporosis.

It has recently been reported that irradiated tumor cells are efficiently phagocytised by macrophages, but when cell surface phosphatidylserine is masked by annexin A5, the macrophage pathway is reduced and a strong CD8+ dendritic cell-dependent immune response is elicited. As referred to above, annexin A1 is an anti-inflammatory modulator that reduces neutrophil recruitment, and thereby reduces tissue inflammation. It binds to specific extracellular ALX (lipoxin A) receptors on neutrophils and macrophages, and can thereby modulate macrophage phago-

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cytosis. At the site of action within the tissues, annexin A1 and its N-terminal peptide (Ac2-26) promotes phagocytosis of apoptotic neutrophils, thereby reducing the level of inflammation and the immune response through anti-inflammatory cytokines such as TGF (Transforming Growth Factor)-β1, and, accordingly, the antigen-induced T cell proliferation of Th1 (T-helper 1) and Th2 (T-helper 2) T-cells is also inhibted by the peptide Ac2-26. Changes of annexin A3 in tumors may influence immune surveillance of prostate tissue by altering the properties and/or concentration of the extracellular annexin pool, and by thereby modulating the interplay between a macrophage/granulocyte dominated response, and/or a humoral one.

According to one embodiment of the present invention the abundance of at least one annexin protein is determined together with the abundance of at least another annexin protein, preferably of the group consisting of annexin A1, annexin A2, annexin A3, annexin A4, annexin A5, annexin A6, annexin A7, annexin A8 and annexin A10.

In the case of a further embodiment of the present invention the abundance of at least one annexin protein is determined together with the abundance of at least a further protein of the group consisting of serum amyloid P, isopeptidase T, muscle-type fatty acid binding protein, galectin 1 , heat shock protein 90, BiP (Human protein: P11021 - 78 kDa glucose-regulated protein precursor, GRP 78, Immunoglobulin heavy chain binding protein, Endoplasmic reticulum lumenal Ca2+ binding protein grp78), protein disulfide isomerase, epidermal-type fatty acid binding protein, enoyl coenzyme A hydratase and nucleophosmin.

Furthermore, the abundance of at least one annexin protein can be determined together with the abundance of at least a further protein of the group consisting of 14-3-3 family, proteasome, particularly prosome and/or macropain, activator subunit 2, cytokeratin family, KNP-I alpha

protein (NCBI ACCESSION BAA95554.1 GI:7768772) and KNP-I beta protein (NCBI ACCESSION BAA21139.1 GI:2250701).

In some cases the diagnostic value of conventional tumor markers for diagnosis is limited. For instance, high or extremely low serum prostate antigen (PSA) values provide a reasonable reliable diagnostic index for prostate cancer. However, preoperative PSA values ranging between 2 and 10 ng/ml, especially between 4 and 10 ng/ml, particularly between 2 and 6 ng/ml, are extremely poor regarding diagnostic reliability, in particular with respect to prediction of postoperative cure rates in radical prostatectomies.

Thus, in a particular preferred embodiment of the invention the abundance of at least one annexin protein is determined together with the abundance of at least one blood or serum marker, in particular of at least one member of the Kallikrein protease family, preferably of prostate specific antigen (PSA). The abundance of various forms of PSA, in particular total PSA (tPSA) abundances, relative or absolute abundances of free PSA (fPSA) and relative or absolute abundances of complexed PSA (cPSA), may be determined together with the abundance of annexin A3. It is further within the scope of the embodiment that other members of the Kallikrein protease family may be used in this respect. The abundances of these proteins to one another may also be used in combination with one or more measured or calculated annexin parameters for diagnostic purposes according to the invention. The annexin parameters that can be useful are obviously not restricted to those used by way of demonstration in this present disclosure.

In the case of a further preferred embodiment the abundance of at least one annexin protein is determined together with the abundance of at least an epithelial cell marker, particularly prostate specific membrane antigen (PSMA).

According to a especially preferred embodiment of the invention annexin A3 and/or annexin A8, preferably annexin A3, are used.

According to the present invention the cancer to be treated and/or diagnosed can be derived from the urogenital and/or intestinal tract. Preferably, cancer is chosen from the group consisting of prostate cancer, kidney cancer, bladder cancer, urethra cancer, ovarian cancer, uterine cancer or colon cancer. Preferably, the cancer to be diagnosed is prostate cancer and/or colon cancer. With respect to prostate cancer the inventive method preferably allows for the discrimination between prostate cancer tissue samples, benign prostatic hyperplasia (BPH) tissue samples, chronic prostatitis tissue samples, fibrosis afflicted tissue samples and healthy tissue samples.

Concerning cancer of the intestinal tract, particularly colon cancer, the inventive method preferably allows for the discrimination between cancer tissue sample and samples of tissue which are affected by inflammatory bowl diseases, particularly Crohns's disease and/or colitis ulcerosa.

According to a further embodiment of the invention it is possible to treat and/or to diagnose subgroups of cancers. Furthermore, different cancer stages may be treated and/or diagnosed by the invention. It is further possible to monitor the transition of non-cancerous tissue into cancerous tissue by means of the present invention.

In the case of a further preferred embodiment of the invention excrement samples or fractions thereof, especially of urine, in particular of exprimate urine, are subjected to a separation process prior to determining the abundance of at least one annexin protein to yield cell pellets and supernatants. Preferably, the separation process is done by centrifugation, especially by low speed centrifugation of cells out of a liquid me-

dium (e.g., 200 x g for 5 minutes at 4 °C), as obvious to experts in the art. Obviously any suitable centrifugation protocol, including successive centrifugations under different conditions, or combinations of centrifugations with other methods, could be conceivably employed to separate soluble or exosome-bound annexin from intracellular annexin for measurement. Other means of separation of soluble or exosome-bound annexin from intracellular annexin can also be employed, or combinations thereof, as obvious to experts in the art (e.g., magnetic beads, filtration, chromatography, etc).

According to a further embodiment of the present invention, the cell pellets are used for determining the intracellular abundance of at least one annexin protein, preferably of annexin A3.

As already mentioned in the above description, annexins are intricately involved in processes of osteoblastosis and osteolysis. Annexins, are implicated in the process of bone mineralization. This is noteworthy because prostate cancer metastases are unusual among cancers in exhibiting a high frequency of osteoblastic bone lesions. Most cancer metastases are characterised by osteoclast osteolytic (bone dissolving) activity, whereas prostate metastases exhibit both osteoclastic and mineral depositing osteoblastic activity.

Physiological mineralization is a highly complex and regulated process. Bone mineralization is initiated by small vesicles, called matrix vesicles, that are released from the plasma membrane of mineralizing skeletal cells. The first mineral phase forms inside the matrix vesicles. Since these are membrane-enclosed, channel proteins are required for the mineral ions to enter. Annexins form channels into the matrix vesicles by which Ca^{2+} enters, leading to the initiation of calcium phosphate mineralization. Once the intravesicular crystals reach a certain size they rupture the membrane. This is in turn related to inflammation, a feature common

to cancer and annexin biology, and involves an interplay between bone and the immune system. Therefore, according to a particular preferred embodiment the inventive method can be used to diagnose and/or treat osteoporosis. In a particular embodiment of the inventive method annexin abundances, preferably the abundance of annexin A3 and/or annexin A8, are determined in body fluids, body secretions, tissue samples, groups of cells or cells, especially by methods known to those skilled in the art, in order to diagnose and/or treat osteoporosis. Such treatment may involve the application of substances that influence the abundance, subcellular/extracellular localisation, post-translational modification or activity of annexin proteins. Activity in this respect especially includes ion-channel activity, which may be appropriately increased or decreased. Substances that may be used for the treatment of osteoporosis explicitly include annexin A3, truncated or mutant versions thereof, or antibodies or other affinity reagents, in particular known in the state of the art. Said substances can further include nucleic acids, or chemically related substances, such as peptide nucleic acids (pNA), which may be also used a small interfering RNAs (siRNAs), in particular known in the state of the art.

Examples of protein analysis of exprimate massaged prostate urine cell pellets from patients diagnosed with cancer, benign prostatic hyperplasia (BPH) or control patients with conditions diagnosed as unrelated to cancer are shown in fig. 3, fig. 4, and fig. 5, respectively. The top panels in each of fig. 3 to fig. 5 show annexin A3 enhanced chemiluminescence (ECL) signal from western blot, and the bottom panels show the entire loaded protein signal as stained with Ponceau S ('Protein'). Each gel contains a molecular weight ladder (M) as well as duplicates of 7.5 µg of total cell protein lysate from a prostate tumour containing annexin A3 as a positive control (+C). Annexin A3 signals from samples on different gels can be compared by normalisation to the average value of the replicate respective positive controls. It was found by the inventors in a pre-

liminary study that the pellets of exprimate urine samples of cancer patiens have much less annexin A3 than either benign prostatic hyperplasia (BPH) patients or healthy control patients (Fig. 6). For instance, regarding the reference value of 0.2-fold the amount of annexin A3 signal (abundance) as in PR_26CA, only 5/30 (5:25) exprimate urine samples of cancer patients have more than 0.2 fold the reference value, while 23/30 (23:7) of the exprimate urine samples of BPH patients and 18/30 (18:12) of the exprimate urine samples of healthy control patients exceeded this reference value. These results show that on average, those cell pellets from samples of exprimate urine of cancer patients contain less annexin A3 than samples of exprimate urine from BPH patients or healthy control patients.

According to a further embodiment of the invention the supernatants resulting from the separation process of the urine samples, in particular of exprimate urine samples, and fractions thereof are used for determining the extracellular abundance of at least one annexin protein, preferably of annexin A3.

It is particularly preferred to use the supernatants for diagnosing cancer, in particular of the urogenital and/or intestinal tract, and/or for discrimination between cancerous and non-cancerous tissue.

In the case of a further preferred embodiment of the present invention a cation chelator, especially a Ca^{2+}-chelator, particularly EDTA and/or EGTA, is added to the urine sample or fraction thereof prior to determining the abundance of at least one annexin protein, preferably of annexin A3. In a preferred embodiment the addition of the cation chelator occurs prior to subjection of the samples or fractions thereof to the separating process.

Preferably the determination of the abundance of at least one extracellular annexin protein, particularly annexin of A3, is performed in an cation chelator treated, particularly EDTA and/or EGTA treated, supernatant and for comparison in a supernatant lacking a cation chelator, particularly EDTA and/or EGTA. The supernatant is preferably derived from the same urine sample, in particular exprimate urine sample, or fraction thereof.

Based on the reasoning that in particular annexin A3 translocation from the interior to the exterior of cells is differentially affected during the development of prostate cancer, the present invention includes the determination of whether there is any difference in the intracellular/extracellular localisation of annexin A3 in assocation with cancer. The extracellular environment as already mentioned in the above specification is understood as the extracellular space including the outer surface of plasma membranes of cells. Exprimate urine obtained subsequent to clinical prostate massage contains cells exuded from the prostate. As well as the possibility of extracellular annexin A3 in exosomes, free annexin A3 could bind to negatively charged groups such as phospholipids on the surface of cells in a calcium-dependent manner. The latter annexin A3-fraction could be released from the surface of cells into the supernatant by addition of EDTA/EGTA to the medium to chelate calcium.

A further investigation in a double-blinded, four-center study demonstrated that the ratio of total annexin A3 of pellet over supernatant was able to diagnose cases labelled fibrosis in the group of non-cancer patients. Fibrosis is associated with benign processes and indicative of non-cancer. The AUROC was 0.7072 for 'pu.anx.tot.ratio' for a total of 103 non-cancer cases. The correlation for the ratio was negative, thus increased total annexin A3 amounts in supernatants were crucial for assorting into this group. This is logic as for cancer cases a decreased annexin A3 value in supernatants was observed (see below).

The further profiling of non-cancer patients (BPH, chronic prostatitis, fibrosis, PIN1-3) by ratios of annexin A3 in pellets/supernatants is an important aspect of the invention for subsequent sequential and/or multiparameter steps of data analysis beyond diagnostic decision cancer vs. non-cancer.

Additionally, the inventors determined annexin 3 abundances in supernatants and cell pellets of a separate independent series of patients, to compare the relative annexin A3 abundance in both cell pellets and supernatants of exprimate urine samples. Again, it was found for this different patient cohort that abundances of annexin A3 in samples of exprimate urine pellets from cancer patients are lower than abundances of annexin A3 in samples of exprimate urine pellets from BPH patients or healthy patients. Concerning the supernatants, the annexin A3 abundances from these same patients are higher in the EDTA-treated supernatants of exprimate urine samples of cancer patients than in EDTA-treated supernatants of exprimate urine samples of BPH patients or healthy patients. From individual ratios of extracellular (EDTA-treated supernatants) and intracellular (1000 x g pellets) fractions of exprimate urine an even clearer image emerged, as shown in fig. 7. Taken together, these data indicate false positive rates around or below 10 % and moreover the ratios of annexin A3 expression in supernatants (annexin A3-S) vs. pellets (annexin A3-P) allows a discrimination of cancer vs. BPH vs. controls as shown in table 1: essentially annexin A3-S is high in cancer and BPH, and low in controls, whereas annexin A3-P is high in BPH and controls and low in cancer; thus having high S (or S/P), low P for cancer; high S (or S/P) and high P for BPH; and low S (or S/P) and high P for controls; individual ratios (S/P) give clearest picture as compared to pellets alone (fig. 7). Additional calibration for protein abundances further improves the picture.

According to a further embodiment of the invention the abundance of at least one annexin protein, preferably annexin A3, is determined by immunohistochemical methods, in particular using tissue samples, such as tissue sections.

The present invention additionally comprises the use of at least one anti-annexin antibody, in particular of anti-annexin A3 antibody, for diagnosis of cancer, in particular of the urogenital and/or intestinal tract, and/or for discrimination between cancerous and non-cancerous tissue. In a preferred embodiment the anti-annexin antibody is used for pathohistological-diagnostically staining of tissue samples, in particular tissue sections. The samples may be obtained by biopsies or complete tissue excision. In particular, the tissue samples to be stained by the anti-annexin antibody are derived from prostate biopsies or prostate tissue after protectomy.

A polyclonal rabbit serum containing antibodies against annexin A3 was obtained and used to localise annexin A3 in prostate tissues by immune histochemistry. Because of the large number of annexin family members, the inventors characterised the specificity of the anti-annexin A3 polyclonal antibody by Western blot prior to immune histochemistry. The vast majority of the signal obtained by Western blot of prostate benign and cancer tissue cell lysates comes from annexin A3 (fig. 1). A marginal abundance of signal was observed for higher molecular weight protein, which is presumably annexin A6. This antibody produced a strong clean band using approximately 120 ng recombinant 60 kDa GST(Glutathione-S-Transferase)-annexin A3 under the same conditions. A thorough quantification, based on radioactive values from 2D gels from biopsies, protein stains of 1D and 2D gels and 1D and 2D Western blots from biopsies and exprimate urines, lead to determination of detection limits of protein concentrations in exprimate urine samples ranging from 0.02 to > 15 ng/ml. The limit of detection was somewhere below but

near to 0.01 ng/ml. In terms of protein content the range is from 0.001 to more than 0.3 ng/µg total protein.

It is therefore probable that this antibody recognises predominantly annexin A3 in immune histochemistry, as shown in fig. 2, where the corresponding annexin A3 signal is restricted to epithelial cells in healthy prostate, and additionally to cancerous cells in tumours. Stromal cells exhibited enhanced staining in early cancer. The rationale of mechanism for differences of annexin A3 in prostate cancer and BPH tissue tentatively indicates a transition from intracellular, more localized and in total lower expression to extracellular and in total higher expression when compared to controls.

The distribution of annexin A3 staining was suggestive of cytoplasmic and membrane localisation (fig. 2), although the overall level of staining in individual cells seemed lower in cancer than benign tissue (fig. 2), the overall level of annexin A3 in the same cancer tissue was higher (e.g. fig. 1), which may be explained by more annexin A3-containing cells in cancerous tissue, and/or more extracellular annexin A3 in cancerous tissue.

In a comprehensive study (four centers, double-blinded) taking into account and measuring for the first time total annexin amounts in supernatants and pellets of exprimate urine of 250 patients and moreover quantifying the potential contribution of neutrophils to the annexin A3 signal (by parallel quantification of neutrophil marker NGAL), it was observed that annexin A3 levels were reduced in the supernatants of exprimate urine of patients with cancer. In general terms, this result indicates, that higher levels of annexin A3 are observed in exprimate urine of non-cancer patients with fibrosis/BPH, than in cancer patients, however, these levels were much reduced to negligible in non-exprimate conventional urine. Therefore it is concluded that the annexin A3 meas-

ured in exprimate urine originates predominantly in the prostate, and is released into the urine as a consequence of the prostatic massage process. It has been shown above that annexin A3 is expressed primarily in the ductal epithelial cells of the healthy prostate. For discrimination of cancer vs. non-cancer alone, the present data indicate that amount of annexin A3 in supernatants has the biggest diagnostic value (AUROC-values for a combined readout of annexin A3 per µg protein and total annexin A3 in supernatants of exprimate urine of prostate cancer patients with initial PSA-values between 4-10 were 0.78-0.82). Here it was observed a disturbance of pellet-annexin A3 in case of higher NGAL-values; so corresponding AUROC's for pellet annexin A3 were in the range of 0.55-0.65).

It is also known that in a prostate with cancer, only a small percentage of the epithelial ductal cells are cancerous, therefore the measured differences in extra-cellular annexin A3 abundance should be non-significant according to logical reasoning. Nevertheless, a considerable and significant reduction of average annexin A3 abundance in the exprimate urine of cancer patients relative to non-cancerous patients was observed. It is not possible to rationalise why this should be the case because the non-cancerous epithelial cells should continue to secrete annexin A3 according to intuitive conventional wisdom. Possibly, the presence of cancer causes the secretion of a trans-acting substance, such as cytokine, that affects the annexin A3 secretion from the bulk of epithelial cell in a prostate with a cancerous lesion. It is unclear whether this trans-acting factor would originate in the cancerous cells themselves, or in other cells. It is well documented that trans-acting factors influence the relationship between cancerous cell and their mesenchymal/stromal environment, and vice versa. Irrespective of the mechanim(s) responsible, the present empirical observations are unambiguous, and clearly but surprisingly demonstrate that lower levels of annexin A3 in the exprimate urine provide a predictive measure of the probability that the patient has tumor-

ous cells in the prostate. This diagnostic use of annexin A3 levels in exprimate urine can be combined with other diagnostic indexes, such as the level of prostate specific antigen (PSA), as demonstrated by way of example. These results also suggest that the presence of the annexin A3 protein is associated with the healthy phenotype. Therefore, annexin A3 protein can be applied in a therapeutic manner to treat cancer, by enhancing the levels of extracellular annexin A3.

The mechanisms underlying the observed results are under investigation, and they potentially reflect a transition of some kind of completely healthy prostate epithelium proceeding to a non-cancerous stage (fibrosis/BPH) which is associated with elevated levels of annexin A3 in both pellets and supernatants of exprimate urine, with the above ratio of total annexin A3 (p/s) with highest diagnostic value. In cancer, there is an obvious and surprising correlation to decreased annexin A3 amounts in supernatants of exprimate urine of cancer patients; pellet annexin A3 amounts appear to have a contaminating contribution by NGAL-positive leukocytes/neutrophils.
It is thus highly desirable to measure annexin A3 levels in both pellet and supernatant. This information on the protein level is not accessible by genomic methods e.g., as superficially insinuated but not substantiated by USA patent application US2003/0108963A1.

In summary, annexin A3 exhibited predominantly intracellular staining in healthy tissue, and extra-epithelial location in early cancerous tissue: with advanced cancer exhibiting markedly reduced annexin A3 staining within cancer cells.

According to a further embodiment of the invention the urine samples or fractions thereof are obtained from urine, in particular from exprimate

urine, which is recovered subsequent to prostate massage, particularly by rectal finger insertion.

In a further embodiment of the invention the urine samples or fractions thereof are purified, particularly are freed from neutrophils, monocytes or peripheral blood mononuclear cells (PMBCs), especially by means of magneto beads. Preferably, samples or fractions thereof of morning urine are used.

According to one embodiment of the invention annexin levels are measured in faeces or epithelial cells of the intestinal tract. Further, annexin levels can be used to treat and/or to diagnose epithelial cancers of gastrointestinal tract in any fraction or preparation of faeces (any potentially exosome-producing epithelial surface). Annexin levels are used further to treat and/or to diagnose colorectal cancer. According to a preferred embodiment the methods of the invention can be combined with a determination of neutrophils, in particular of calprotectin and/or neutrophil gelatinase-associated lipocalin (NGAL), to discriminate inflammatory conditions (Crohn's disease or colitis ulcerosa from cancer).

It is further within the scope of the present invention that at least one annexin protein, preferably annexin A3 and/or annexin A8, in particular annexin A3, can be used as diagnostic marker and/or therapeutic target for diseases disclosed in the description, in particular for prostate cancer, colorectal cancer and/or osteoporosis, preferably for subgroups thereof.

According to the present invention, it is possible to treat cancer, in particular of the urogenital and/or intestinal tract. This is preferably achieved by the enhancement of the in vivo abundance of at least one annexin protein, for instance of at least one extracellular annexin.
Furthermore the present invention allows for the diagnosis of cancer, in particular of the urogenital and/or intestinal tract, and/or the discrimina-

tion between cancerous and non-cancerous tissue. This is particularly achieved by the determination of the ratios of intra- versus extracellular abundances and extra- versus intracellular abundances respectively of annexin proteins which may be, if appropriate, combined with the determination of the corresponding ratios for other proteins. Preferably, diagnosis of cancer and/or discrimination between cancerous and non-cancerous tissue is based on the extracellular abundance of at least one annexin protein. The determined protein ratios and abundances respectively reveal differences between cancerous and non-cancerous tissues, thus allowing for a patient's profiling. Therefore, annexin proteins, in particular annexin A3, are reliable diagnostic markers that may even completely substitute tumor markers that are conventionally applied in cancer diagnosis.

For a more detailed description of the present invention, reference will now be made to the accompanying tables and figures:

Table:1

# of samples	Cancer 40	BPH 40	Controls 40	False neg (% of 120)	False pos (% of 120)
ANXA3-P (% of 40)					
Biomarker down	84	23	40		
Biomarker up	16	77	60	6	13.3
ANXA3-S (% of 40)					
Biomarker down	5	20	91		
Biomarker up	95	80	9		
Ratio ANXA3-S/ ANXA3-P and sorting					
Cancer	92	12	3		
BPH	6	80	6	1.5	7.5

Control	2	8	91		

Table 1: Summary of diagnostic results, annexin A3 abundances are low in pellets (annexin A3-P) of exprimate urine samples of cancer patients and comparatively high in corresponding pellets of exprimate urine samples of BPH patients and healthy patients. For annexin A3 abundances in supernatants (annexin A3-S) of exprimate urine samples , there is a different picture: they are low for healthy patients and high for cancer and BPH patients. The combined read-out correctly assorts the three cases with numbers indicated in the lower part of the table. Concerning further details it is referred to the above specification.

Table: 2

Name	Description
P_ug_tot	Pellet: Total Protein amount (µg)
U_ug_tot	Supernatant*: Total Protein amount (µg
P_ANX_ug	Pellet: Annexin signal level per µg protein
P_ANX_tot	Pellet: Annexin signal level per total patient sample
U_ANX_ug	Supernatant: Annexin signal level per µg protein
U_ANX_tot	Supernatant: Annexin signal level per total patient sample
PU_ANX_ug	Pellet + Supernatant: Annexin-level per µg protein
PU_ANX_tot	Pellet + Supernatant: Annexin-level per total patient

	sample
PU_ANX_ug_ratio	Ratio of Pellet / Supernatant: Annexin level per ug protein
PU_ANX_tot_ratio	Ratio Pellet / Supernatant: Annexin level per total patient sample
P_NGAL_ug	Pellet: NGAL signal level per µg protein
P_NGAL_tot	Pellet: NGAL signal level per total patient sample
U_NGAL_ug	Supernatant: NGAL signal level per µg protein
U_NGAL_tot	Supernatant: NGAL signal level per total patient sample
PU_NGAL_ug	Pellet + Supernatant: NGAL-level per µg protein
PU_NGAL_tot	Pellet + Supernatant: NGAL-level per total patient sample
PU_NGAL_ug_ratio	Ratio of Pellet / Supernatant: NGAL level per ug protein
PU_NGAL_tot_ratio	Ratio Pellet / Supernatant: NGAL level per total patient sample
P_ANX_NGAL_ug_ratio	Pellet: Annexin/NGAL ratio per µg protein
U_ANX_NGAL_ug_ratio	Supernatant: Annexin/NGAL ratio per µg protein
PU_ANX_NGAL_ug_ratio	Pellet+Supernatant: Annexin/NGAL ratio per µg protein

('U' = 'supernatant')

Table 2. Protein parameters measured from exprimate urine supernatant and pellets fractions.

Table 3:

Variable	Description
P.ug.tot	Pellet: Total Protein Quantity (µg)

U.ug.tot	Supernatant: Total Protein Quantity (µg)
P.ANX.ug	Pellet: Annexin-Level per µg Protein
P.ANX.tot	Pellet: Annexin-Level per total Sample
U.ANX.ug	Supernatant: Annexin-Level per µg Protein
U.ANX.tot	Supernatant: Annexin-Level per total Sample
PU.ANX.ug	Supernatant+Pellet: Annexin-Level per µg Protein
PU.ANX.tot	Supernatant+Pellet: Annexin-Level per total Sample
PU.ANX.ug.ratio	Ratio Pellet / Supernatant: Annexin Level per ug Protein
PU.ANX.tot.ratio	Ratio Pellet / Supernatant: Annexin Level per total Sample
psa.ini	Blood PSA levels
perc.free.psa	Percentage free PSA

Table 3: description of abbreviations and variables used in example 4

Table 4:

Test	AUROC	Patients
anx.comb.var	0.78	112
comb.var.anx.psa 1	0.76	112
u.anx.tot	0.76	112
pu.anx.tot	0.75	112
u.anx.ug	0.74	112
perc.free.psa	0.72	103
psa.ini	0.57	109

Table 4.: ROC curve analysis results for the indicated variable parameters (see Table 3 for description), performed for patients grouped ac-

cording to PSA values of 4-10 ng/mL. The test parameters, resulting AUROC values, and number of patients included per analysis are tabularised.

Table: 5

Test	AUROC	Patients
comb.var.anx.psa 2	0.79	109
comb.var.anx.psa 1	0.77	109
pu.anx.tot	0.73	109
anx.comb.var	0.71	109
u.anx.tot	0.71	109
pu.anx.ug	0.71	109
u.anx.	0.71	109
p.anx.tot	0.69	109
psa.ini	0.69	109
perc.free.psa	0.69	109

Table 5. ROC curve analysis results for the indicated variable parameters (see Table 3 for description), performed for patients grouped according to PSA values 2-6 ng/mL. The test parameters, resulting AUROC values, and number of patients included per analysis are tabularised.

Table 6:

Test	AUROC	Patients
comb.var.anx.psa 1	0.75	226
comb.var.anx.psa 2	0.73	244
perc.free.psa	0.70	227
psa.ini	0.68	239
anx.comb.var	0.67	244
pu.anx.tot	0.66	245

Table 6: ROC curve analysis results for the indicated variable parameters (see Table 3 for description), performed for all patients, including all PSA values. The test parameters, resulting AUROC values, and number of patients included per analysis are tabularised.

Table 7:

Criterion	Sens. (95% C.I.)	Spec. (95% C.I.)	+LR	-LR	+PV	-PV
>=-3.5155	100.0 (93.7-100.0)	0.0 (0.0- 6.9)	1.00		52.3	
> -3.5155	98.2 (90.6- 99.7)	0.0 (0.0- 6.9)	0.98		51.9	0.0
> -2.6907	98.2 (90.6- 99.7)	1.9 (0.3- 10.3)	1.00	0.91	52.3	50.0
> -2.5444	98.2 (90.6- 99.7)	3.8 (0.6- 13.2)	1.02	0.46	52.8	66.7
> -2.0663	98.2 (90.6- 99.7)	5.8 (1.3- 16.0)	1.04	0.30	53.3	75.0
> -1.9579	98.2 (90.6- 99.7)	7.7 (2.2- 18.6)	1.06	0.23	53.8	80.0
> -1.9096	98.2 (90.6- 99.7)	9.6 (3.2- 21.0)	1.09	0.18	54.4	83.3
> -1.6827	98.2 (90.6- 99.7)	11.5 (4.4- 23.5)	1.11	0.15	54.9	85.7
> -1.6673	98.2 (90.6- 99.7)	13.5 (5.6- 25.8)	1.14	0.13	55.4	87.5
> -1.5429	96.5 (87.9- 99.5)	13.5 (5.6- 25.8)	1.12	0.26	55.0	77.8
> -1.4419	96.5 (87.9- 99.5)	15.4 (6.9- 28.1)	1.14	0.23	55.6	80.0
> -1.3903	94.7 (85.4- 98.8)	15.4 (6.9- 28.1)	1.12	0.34	55.1	72.7
> -1.273	94.7 (85.4- 98.8)	17.3 (8.3- 30.3)	1.15	0.30	55.7	75.0
> -1.2619	94.7 (85.4- 98.8)	19.2 (9.6- 32.5)	1.17	0.27	56.2	76.9
> -1.1825	93.0 (83.0- 98.0)	19.2 (9.6- 32.5)	1.15	0.36	55.8	71.4
> -1.1791	93.0 (83.0- 98.0)	21.2 (11.1- 34.7)	1.18	0.33	56.4	73.3
> -1.0689	91.2 (80.7- 97.1)	21.2 (11.1- 34.7)	1.16	0.41	55.9	68.7
> -1.0621	91.2 (80.7- 97.1)	23.1 (12.5- 36.8)	1.19	0.38	56.5	70.6
> -1.0192	91.2 (80.7- 97.1)	25.0 (14.0- 38.9)	1.22	0.35	57.1	72.2
> -1.0041	91.2 (80.7- 97.1)	26.9 (15.6- 41.0)	1.25	0.33	57.8	73.7
> -0.9502	91.2 (80.7- 97.1)	28.8 (17.1- 43.1)	1.28	0.30	58.4	75.0
> -0.8458	89.5 (78.5- 96.0)	28.8 (17.1- 43.1)	1.26	0.36	58.0	71.4
> -0.8339	89.5 (78.5- 96.0)	30.8 (18.7- 45.1)	1.29	0.34	58.6	72.7
> -0.8285	87.7 (76.3- 94.9)	30.8 (18.7- 45.1)	1.27	0.40	58.1	69.6
> -0.8253	87.7 (76.3- 94.9)	32.7 (20.3- 47.1)	1.30	0.38	58.8	70.8
> -0.8237	87.7 (76.3- 94.9)	34.6 (22.0- 49.1)	1.34	0.35	59.5	72.0
> -0.8044	87.7 (76.3- 94.9)	36.5 (23.6- 51.0)	1.38	0.34	60.2	73.1
> -0.7935	87.7 (76.3- 94.9)	38.5 (25.3- 53.0)	1.43	0.32	61.0	74.1
> -0.7836	87.7 (76.3- 94.9)	40.4 (27.0- 54.9)	1.47	0.30	61.7	75.0
> -0.7431	87.7 (76.3- 94.9)	42.3 (28.7- 56.8)	1.52	0.29	62.5	75.9
> -0.7371	86.0 (74.2- 93.7)	42.3 (28.7- 56.8)	1.49	0.33	62.0	73.3
> -0.6841	86.0 (74.2- 93.7)	44.2 (30.5- 58.7)	1.54	0.32	62.8	74.2
> -0.6759	86.0 (74.2- 93.7)	46.2 (32.2- 60.5)	1.60	0.30	63.6	75.0

Criterion	Sens. (95% C.I.)	Spec. (95% C.I.)	+LR	-LR	+PV	-PV
> -0.6297	86.0 (74.2- 93.7)	48.1 (34.0- 62.4)	1.66	0.29	64.5	75.8
> -0.6205	86.0 (74.2- 93.7)	50.0 (35.8- 64.2)	1.72	0.28	65.3	76.5
> -0.6095	86.0 (74.2- 93.7)	51.9 (37.6- 66.0)	1.79	0.27	66.2	77.1
> -0.5341	86.0 (74.2- 93.7)	53.8 (39.5- 67.8)	1.86	0.26	67.1	77.8
> -0.5077	86.0 (74.2- 93.7)	55.8 (41.3- 69.5)	1.94	0.25	68.1	78.4
> -0.503	86.0 (74.2- 93.7)	57.7 (43.2- 71.3)	2.03	0.24	69.0	78.9
> -0.4942	86.0 (74.2- 93.7)	59.6 (45.1- 73.0)	2.13	0.24	70.0	79.5
> -0.4548	84.2 (72.1- 92.5)	59.6 (45.1- 73.0)	2.09	0.26	69.6	77.5
> -0.4346	82.5 (70.1- 91.2)	59.6 (45.1- 73.0)	2.04	0.29	69.1	75.6
> -0.4211	80.7 (68.1- 89.9)	59.6 (45.1- 73.0)	2.00	0.32	68.7	73.8
> -0.3763	78.9 (66.1- 88.6)	59.6 (45.1- 73.0)	1.95	0.35	68.2	72.1
> -0.201	78.9 (66.1- 88.6)	61.5 (47.0- 74.7)	2.05	0.34	69.2	72.7
> -0.1764	78.9 (66.1- 88.6)	63.5 (49.0- 76.4)	2.16	0.33	70.3	73.3
> -0.1476	78.9 (66.1- 88.6)	65.4 (50.9- 78.0)	2.28	0.32	71.4	73.9
> -0.1049	77.2 (64.2- 87.2)	65.4 (50.9- 78.0)	2.23	0.35	71.0	72.3
> -0.0809	75.4 (62.2- 85.9)	65.4 (50.9- 78.0)	2.18	0.38	70.5	70.8
> -0.0773	75.4 (62.2- 85.9)	67.3 (52.9- 79.7)	2.31	0.36	71.7	71.4
> -0.0323	73.7 (60.3- 84.5)	67.3 (52.9- 79.7)	2.25	0.39	71.2	70.0
> -0.0007	71.9 (58.5- 83.0)	67.3 (52.9- 79.7)	2.20	0.42	70.7	68.6
> 0.007	71.9 (58.5- 83.0)	69.2 (54.9- 81.3)	2.34	0.41	71.9	69.2
> 0.0841	70.2 (56.6- 81.6)	69.2 (54.9- 81.3)	2.28	0.43	71.4	67.9
> 0.085	68.4 (54.8- 80.1)	69.2 (54.9- 81.3)	2.22	0.46	70.9	66.7
> 0.1274	68.4 (54.8- 80.1)	71.2 (56.9- 82.9)	2.37	0.44	72.2	67.3
> 0.1395	68.4 (54.8- 80.1)	73.1 (59.0- 84.4)	2.54	0.43	73.6	67.9
> 0.1475	68.4 (54.8- 80.1)	75.0 (61.1- 86.0)	2.74	0.42	75.0	68.4
> 0.1672	68.4 (54.8- 80.1)	76.9 (63.2- 87.5)	2.96	0.41	76.5	69.0
> 0.1764	68.4 (54.8- 80.1)	78.8 (65.3- 88.9)	3.23	0.40	78.0	69.5
> 0.1803	68.4 (54.8- 80.1)	80.8 (67.5- 90.4)	3.56	0.39	79.6	70.0
> 0.1808	66.7 (52.9- 78.6)	80.8 (67.5- 90.4)	3.47	0.41	79.2	68.9
> 0.2017	64.9 (51.1- 77.1)	80.8 (67.5- 90.4)	3.38	0.43	78.7	67.7
> 0.2682	63.2 (49.3- 75.5)	80.8 (67.5- 90.4)	3.28	0.46	78.3	66.7
> 0.2761	63.2 (49.3- 75.5)	82.7 (69.7- 91.7)	3.65	0.45	80.0	67.2
> 0.3249	61.4 (47.6- 74.0)	82.7 (69.7- 91.7)	3.55	0.47	79.5	66.2
> 0.3445	59.6 (45.8- 72.4)	82.7 (69.7- 91.7)	3.45	0.49	79.1	65.2
> 0.4091	57.9 (44.1- 70.9)	82.7 (69.7- 91.7)	3.35	0.51	78.6	64.2
> 0.476	57.9 (44.1- 70.9)	84.6 (71.9- 93.1)	3.76	0.50	80.5	64.7
> 0.4824	57.9 (44.1- 70.9)	86.5 (74.2- 94.4)	4.30	0.49	82.5	65.2
> 0.4969	57.9 (44.1- 70.9)	88.5 (76.5- 95.6)	5.02	0.48	84.6	65.7
> 0.5666	57.9 (44.1- 70.9)	90.4 (79.0- 96.8)	6.02	0.47	86.8	66.2
> 0.6639	57.9 (44.1- 70.9)	92.3 (81.4- 97.8)	7.53	0.46	89.2	66.7
> 0.683	56.1 (42.4- 69.3)	92.3 (81.4- 97.8)	7.30	0.48	88.9	65.8
> 0.6877	56.1 (42.4- 69.3)	94.2 (84.0- 98.7)	9.73	0.47	91.4	66.2
> 0.6934	54.4 (40.7- 67.6)	94.2 (84.0- 98.7)	9.43	0.48	91.2	65.3
> 0.7038 *	54.4 (40.7- 67.6)	96.2 (86.8- 99.4)	14.14	0.47	93.9	65.8
> 0.7226	52.6 (39.0- 66.0)	96.2 (86.8- 99.4)	13.68	0.49	93.8	64.9
> 0.8531	50.9 (37.3- 64.4)	96.2 (86.8- 99.4)	13.23	0.51	93.5	64.1
> 0.8868	49.1 (35.6- 62.7)	96.2 (86.8- 99.4)	12.77	0.53	93.3	63.3

Criterion	Sens. (95% C.I.)	Spec. (95% C.I.)	+LR	-LR	+PV	-PV
> 0.9511	47.4 (34.0- 61.0)	96.2 (86.8- 99.4)	12.32	0.55	93.1	62.5
> 0.9527	45.6 (32.4- 59.3)	96.2 (86.8- 99.4)	11.86	0.57	92.9	61.7
> 1.0734	43.9 (30.7- 57.6)	96.2 (86.8- 99.4)	11.40	0.58	92.6	61.0
> 1.116	42.1 (29.1- 55.9)	96.2 (86.8- 99.4)	10.95	0.60	92.3	60.2
> 1.1278	40.4 (27.6- 54.2)	96.2 (86.8- 99.4)	10.49	0.62	92.0	59.5
> 1.1707	38.6 (26.0- 52.4)	96.2 (86.8- 99.4)	10.04	0.64	91.7	58.8
> 1.1763	36.8 (24.5- 50.7)	96.2 (86.8- 99.4)	9.58	0.66	91.3	58.1
> 1.2415	35.1 (22.9- 48.9)	96.2 (86.8- 99.4)	9.12	0.68	90.9	57.5
> 1.267	33.3 (21.4- 47.1)	96.2 (86.8- 99.4)	8.67	0.69	90.5	56.8
> 1.3195	31.6 (19.9- 45.2)	96.2 (86.8- 99.4)	8.21	0.71	90.0	56.2
> 1.3976	29.8 (18.4- 43.4)	96.2 (86.8- 99.4)	7.75	0.73	89.5	55.6
> 1.4368	28.1 (17.0- 41.5)	96.2 (86.8- 99.4)	7.30	0.75	88.9	54.9
> 1.4579	26.3 (15.5- 39.7)	96.2 (86.8- 99.4)	6.84	0.77	88.2	54.3
> 1.4781	24.6 (14.1- 37.8)	96.2 (86.8- 99.4)	6.39	0.78	87.5	53.8
> 1.6158	22.8 (12.8- 35.8)	96.2 (86.8- 99.4)	5.93	0.80	86.7	53.2
> 1.6423	21.1 (11.4- 33.9)	96.2 (86.8- 99.4)	5.47	0.82	85.7	52.6
> 1.6559	19.3 (10.1- 31.9)	96.2 (86.8- 99.4)	5.02	0.84	84.6	52.1
> 1.7477	17.5 (8.8- 29.9)	96.2 (86.8- 99.4)	4.56	0.86	83.3	51.5
> 1.7524	17.5 (8.8- 29.9)	98.1 (89.7- 99.7)	9.12	0.84	90.9	52.0
> 1.7628	17.5 (8.8- 29.9)	100.0 (93.1-100.0)		0.82	100.0	52.5
> 1.8054	15.8 (7.5- 27.9)	100.0 (93.1-100.0)		0.84	100.0	52.0
> 1.8153	14.0 (6.3- 25.8)	100.0 (93.1-100.0)		0.86	100.0	51.5
> 1.8787	12.3 (5.1- 23.7)	100.0 (93.1-100.0)		0.88	100.0	51.0
> 2.1994	10.5 (4.0- 21.5)	100.0 (93.1-100.0)		0.89	100.0	50.5
> 2.4311	8.8 (2.9- 19.3)	100.0 (93.1-100.0)		0.91	100.0	50.0
> 2.472	7.0 (2.0- 17.0)	100.0 (93.1-100.0)		0.93	100.0	49.5
> 2.5544	5.3 (1.2- 14.6)	100.0 (93.1-100.0)		0.95	100.0	49.1
> 2.5595	3.5 (0.5- 12.1)	100.0 (93.1-100.0)		0.96	100.0	48.6
> 2.8088	1.8 (0.3- 9.4)	100.0 (93.1-100.0)		0.98	100.0	48.1
> 3.6467	0.0 (0.0- 6.3)	100.0 (93.1-100.0)		1.00		47.7

Sens. = Sensitivity

Spec. = Specificity

+LR = Positive likelihood ratio

-LR = Negative likelihood ratio

+PV = Positive predictive value

-PV = Negative predictive value

Table 7: Data corresponding to the ROC curve of Fig. 9. The maximum likelihoods estimate model is described in Tables 8 and 9.

Table 8:

Parameter	DF	Estimate	Error (Standard)	Chi-Square (Wald)	Pr > ChiSq
Intercept	1	-2.3860	1.7461	1.8673	0.1718
Log PU ANX tot	1	-0.4747	0.1394	11.5908	0.0007
Log PSA ini	1	3.2942	1.0198	10.4343	0.0012

Table 8 Condensed SAS Output for the logit model which leads to comb.var.anx.psa2: Analysis of Maximum Likelihood Estimates

Table 9:

Effect	Estimate (Point)	Confidence (95%)	Limits
log PU ANX tot	0.622	0.473	0.818
log PSA ini	26.956	3.652	198.934

Table 9 Condensed SAS Output for the logit model which leads to comb.var.anx.psa2: Odds Ratio Estimates

Figure 1: Characterisation of the anti-annexin A3 rabbit polyclonal antiserum by Western blot. (A) Western blot of 1-D SDS gel. Lane 1: Magic Mark (Invitrogen) molecular weight markers 3 µl per lane, gives masses in kDa of 120, 100, 80, 60, 50, 40, 30, and 20. Replicate lanes contain 15 µg whole tissue protein extract from cancer (Lanes 2-4) and benign (Lanes 5-7) prostate from patient 29. The filter was incubated using anti annexin 3 polyclonal serum (1:20,000). Lanes 1-7 show a false colour image of signal from the entire filter. The boxed insert (Lanes 1' and 2') shows the single colour depiction of lanes 1 and 2. The positions of annexin A3 and resumed annexin A6 bands are indicated. (B) 2D-PAGE

Western blot of protein 100 µg extract from the cancer sample of patient 29 in false colour shows the distribution of proteins with cross reactivity to the polyclonal serum. The identity of three strongest (red coloured) annexin A3 spots was confirmed by MALDI-TOF PMF (data not shown), the strongest of which corresponds to the protein spot detected in our proteomics analysis.

Figure 2:

Immune histochemistry of Annexin 3: annexin A3 immunoreactivity of the anti-annexin A3 polyclonal serum (dilution: 1:100) was found in the epithelial cells of A) benign prostate tissue. In B) cribbriform prostatic intraepithelial neoplasis (PIN) and C) cancer tissue, epithelial and cancer cells were stained. An elevated level of diffuse extra-epithelial localisation was also observed. Brown colour indicates annexin A3-specific peroxidase staining. Blue colour represents counter staining of the tissue with Gill's hematoxylin solution (Sigma).

Figure 3:

Western blot quantification of annexin A3 in exprimate pellets of urine obtained after prostatic massage of cancer patients. The top panels show the chemiluminescent annexin A3 signal from blotted proteins. The bottom panels show the loaded protein stained with Ponceau S ('Protein'). Each gel contains a molecular weight ladder (M) as well as duplicates of 7.5 µg of total cell protein lysate from a prostate tumour containing annexin A3 as a positive control (+C).

Figure 4:

Western blot quantification of annexin A3 in exprimate pellets of urine obtained after prostatic massage of BPH patients. Other details follow fig. 3.

Figure 5:
Western blot quantification of annexin A3 in exprimate pellet of urine obtained after prostatic massage of non-cancer control patients. Other details follow fig. 3.

Figure 6:
Normalised annexin A3 signals from cell pellets of urine of patients following prostate massage as shown in fig. 3 to fig. 5. Annexin A3 values from different gels are normalised in arbitrary units of 7.5 µg of total cell protein lysate from a prostate tumour (PR-26CA) containing annexin A3 as a positive control in each of Figure 3 to Figure 5.

Figure 7:
Ratio of annexin A3 content between EDTA-treated supernatants and cell pellets of exprimate urine samples of patients following prostatic massage (additional patient material, not contained in fig. 6), a.u. are arbitrary units.

Figure 8:
ROC Curve – Two-Step Procedure 1:
"Use U_ANX_tot if 2.5 ≤ PSA_ini ≤ 12, most obvious decision otherwise"

Legend:
VARIABLE = IF(PSA_ini<2.5;100000;IF(PSA_ini<=12;u_anx_tot;0))
twostepvar1
CLASSIFICATION VARIABLE: PCa
POSITIVE GROUP: PCa = 1
Sample size = 137
NEGATIVE GROUP: PCa = 0
Sample size = 101
Disease prevalence (%) = 57.6
Area under the ROC curve = 0.740

Standard error = 0.033
95% Confidence interval = 0.679 to 0.794
P (Area=0.5) < 0.0001

Fig. 9:
ROC Curve – T comb.var.anx.psa2:
≡ −2.386 + 3.294 log(1+PSA_ini) − 0.475 log(1+PU_ANX tot)

Legend:
select : AND(PSA_ini>=2;PSA_ini<=6)
classification variable PCa
positive group: PCa = 1, Sample size = 57
negative group: PCa = 0, Sample size = 52
Disease prevalence (%) = 52.3
Area under the ROC curve = 0.791
Standard error = 0.043
95% Confidence interval = 0.703 to 0.863
P (Area=0.5) < 0.0001

Examples

While the present invention is described in more detail with reference to examples, the present invention is by no means restricted to these examples.

Example 1:

Processing of post-prostatic massage urine:
Prostatic massage exprimate urine was obtained from patients undergoing clinical examination, after screening for blood prostate specific antigen (PSA) abundances had indicated elevated risk of cancer. 47 ml of urine obtained following vigorous prostate massage by rectal finger insertion was added to 3 ml 0.5 M EDTA, pH 8 precooled to 0°C, and im-

mediately cooled to 0°C. If urine volume was < 47 ml, the volume was made up with ice cooled phosphate buffered saline (PBS) solution. The cooled samples were centrifuged at 3000 rpm at 0°C for 30 minutes to create a cell pellet. 1 ml aliquots of the supernatant were removed, frozen in liquid nitrogen, and stored at –80°C until use. The cell pellets were gently resuspended in 2 ml ice cold PBS and transferred to Eppendorf tubes on ice, followed by centrifugation at 12000 rpm for 5 minutes at 4°C. The supernatant was removed, and the pellet frozen in liquid nitrogen and stored at –80°C until use.

Example 2:

Western blotting:

SDS-PAGE gels for Western blotting were prepared using a BioRad-Mini gel apparatus and 12% T polyacrylamide gels with 1 mm spacers and 15 wells, according to manufacturer's instructions. Anti annexin A3 (annexin A3) was the same antibody described below. Diluted 1:20,000. Recombinant GST- annexin A3 protein was purchased from Abnova Corporation (#ABV 0040710002; Lot: T04G01-ANNEXINA3, 0.05 µg/µl, 61 kDa). Antibody binding was visualised with a goat anti-rabbit IgG (Sigma A 3937, lot# 121K9151) diluted 1:1000, using the ECL detection method (Pierce) and a DIANA III CCD camera-based chemiluminescence detector (Raytest, Straubenhardt, Germany). A rabbit polyclonal serum against recombinant bacterially expressed annexin A3 exhibits primary specificity for annexin A3 and some cross reactivity for annexin A6.

Example 3:

Immune histochemistry:

Immune histochemistry was performed with 5 µm paraffin tissue sections employing polyclonal anti annexin A3 serum, according to a standard Horse Radish Peroxidase immunohistochemistry protocol using the

Zymed PicTure PLUS Kit (Broad Spectrum, DAB, Zymed, South San Fransisco, CA). After immunostaining sections were counterstained with Gill's hematoxylin solution (Sigma).

Example: Clinical study using exprimate urine of 250 patients

Annexin A3 levels were determined in the exprimate urine of clinical patients, diagnosed as being either positive or negative for the presence of prostate cancer (PCa). A variety of additional paramters were examined, such as prostate specific antigen (PSA) levels in the blood, and other variables listed below.

Sample collection was as per example 1, but without the addition of EDTA to urine. Following prostate massage the entire exprimate urine volume was collected and recorded. A Combur-10-Test® (Roche Diagnostics Cat. No. 11 203 479) was performed immediately on an aliquot of the urine to record specific gravity, pH, Leukocyte count, and the levels of nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and erythrocytes. Urine was then centrifuged at room temperature for 15 min at 1000 xg. The cell pellet and supernatant of this supernatant were handled separately. After removal of the last traces of supernatant, the pellet was resuspended in 1 ml ice cold phosphate buffered saline and frozen in liquid nitrogen or on frozen CO_2. Separate aliquots of 2 x 1.8 ml and up to 2 x 50 ml of supernatant were similarly frozen.

Frozen protein samples were thawed, and 1/100 volumes of 2% deoxycholate were added, followed by vortexing then addition of 1/10 volume of trichloroacetic acid, vortexing, and 10 minutes incubation at 0°C. This was followed by centrifugation at 10000 x g for 15 min at 4°C to precipitate proteins. The supernatant was removed, and the pellet was washed three times with ice-cold 80% acetone by vigorously vortexing the pellet

to remove remaining TCA completely, followed by recentrifugation at 10000 x g as before after each wash. After the final centrifugation the supernatant was removed and the pellet was left to air dry for 2 minutes, paying attention not to completely dehydrate the pellet. The pellets were resuspended in boiling XT- sample buffer (1x XT-Buffer: 141 mM Tris-Base; 106 mM Tris-HCl; 2% SDS; BPB; pH about 8.5; 50 mM DTT; 35% Glycerol).

The protein concentration of each sample was estimated by loading defined volumes of each sample to a one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Criterion XT-precast gel: Biorad, Cat# 345-0119, lot# CX070706B2), which contained a serial dilution of calibrated amounts of rat proteins from whole liver cell lysates and was electrophoresed in a BioRad Criterion electrophoresis device according to manufacturer's instruction. The gel was stained using Sypro Ruby according to manufacturer's instructions. Briefly, the gels were fixed 2 x 30 min in aqueous solution containing 50% methanol, 7% acetic acid, followed by staining overnight in Sypro Ruby solution (Molecular Probes, # S12001). Gels were washed for 30 min in 10% methanol, 7% acetic acid, and then for 2x 5 min in water. Protein staining with Sypro Ruby was quantified with a Diana III CCD-based digital imager (Raytest Isotopenmessgeräte GmbH, Straubenhard Germany: Sypro Filter, 605nm).

The intensity of protein staining of Sypro Ruby-stained gel lanes was compared between standard proteins and patient urine samples. For supernatants the whole lane was used for determination. For urine Pellet samples only the lane area below the dominating Uromodulin band was considered, resulting in a "uromodulin corrected protein concentration".

Annexin A3 and neutrophil gelatinase-associated lipocalin (NGAL, SWISSPROT Accession P80188, a marker for Neutrophils) levels in each sample were quantified by loading normalised protein amounts to

SDS-PAGE gels as described above, whereby each gel contained three replicate lanes of 2µg of a standardized protein extract from PC3 human prostate cancer cell line, which contained a convenient reference amount of both annexin A3 and NGAL. Proteins from these gels were western blotted onto PVDF (polyvinylidene fluoride) membranes according to standard methods for 1.5 h at 15V constant voltage and a limit of 3 mA/cm².

The non-specific protein binding sites on blotted membranes were blocked by 2 h incubation with gentle shaking in TBS (175 mM NaCl, 3.5 mM KCl, 20 mM Tris, pH 7.4) containing 5% redissolved dried milk powder. Primary antibodies were added specific for annexin A3 (1:20000 dilution, polyclonal rabbit anti-human Annexin A3) or NGAL (1:500 dilution, anti-human Lipocalin, polyclonal, from goat, R&D Systems, Nr. AF1757, lot JBH025051). After incubation at room temperature for 2 h the buffer was removed, washed three times for 10 min with TBS, and then incubated with the appropriate respective second antibodies against rabbit IgG (goat anti-rabbit-IgG pre-absorbed with human IgG and mouse IgG, couples to horse radish peroxidase. Santa Cruz, #sc-2054, lot# G2005. 1:5000 dilution) or goat IgG (Anti-goat IgG, from rabbit, pre-absorbed with human IgG and mouse IgG, coupled to horse radish peroxidise. Santa Cruz Biotechnology, #sc-2922 lot# C1405. 1:5000 dilution). Enhanced chemiluminescence (ECL) was measured afer addtion of Super Signal West Dura, Pierce (0.1ml/cm²).

Values of NGAL or annexin A3 signals were normalised to the average signal from each of the three reference PC3 lanes on each gel, and the normalised annexin A3 or NGAL values were used for the statistical analysis. From these values, the levels of both proteins were calculated relative to the absolute sample volume, and also normalised to protein concentration. These values were calculated separately for pellet and supernatant, as well as for the ratio of pellet:supernatant. Parameters

that were correlated to cancer and compared to PSA values are summarised in Table 2.

Clinical parameters recorded included blood PSA levels, free total PSA levels, and complexed PSA levels, as well as histological evaluation of prostate tissue biopsies that were obtained following donation of exprimate urine, during the course of a standard digital rectal examination (DRE). In cases where high levels of serum PSA and DRE indicated necessity of prostectomy, the histological evaluation was performed on that material without prior biopsy.

The above data parameters were included in the statistical analysis, that included also clinical data recorded in the hospital. Prostate biopsies or prostectomy were obtained from all patients, and the clinician made a diagnosis of positive or negative for PCa based on histological examination of said tissue. Blood PSA levels were also obtained according to standard clinical practices. The U.S. Food and Drug Administration (FDA) has approved the PSA test for annual screening of prostate cancer in men of age 50 and older. PSA levels between 4 and 10 ng/mL (nanograms per milliliter) are considered to be suspicious and should be followed by rectal ultrasound imaging and, if indicated, biopsy. PSA is false positive- and false negative-prone. Biopsy-detected prostate cancer, including high-grade cancers, is not rare among men with PSA levels of 4.0 ng/mL or less – levels generally thought to be in the normal range.

The dataset consists of composite data files from 250 patients. Initial PSA values were available in 243 of the 250 patients and were missing for 7 patients. Furthermore, two of the latter seven patients and five other patients lacked the histological results of the biopsy (prostate cancer yes/no). Hence, all results presented here only use the data on those 243 patients whose PCa status is known: 140 patients with positive and

103 with negative PCa diagnosis. In the figures 8 and 9, the Receiver Operating Characteristic (ROC) curves generated using the clinical results are presented using inverse values for all ROC curves other than for PSA; i.e. the areas under the ROC curves were observed to correlate with higher PSA values in cancer patients, and with a lower average annexin A3 signal measured for cancer patients.

The area under the ROC curve (AUROC) for PSA was 0.684, which is significantly different from an AUROC of 0.5, but somewhat artificially high due to patient recruitment in participating clinics, (as known to every expert in the field). However we do note that our patient collective had an unusually high proportion of cancer patients (57%) because some of the patients had been examined by our test clinics after having providing high PSA readings at other centres. The AUROC values for the individual annexin A3-based variables measured are in the same range (the maximum is attained by PU_ANX_tot, AUROC 0.666; data not shown), and also significantly different from 0.5. Thus annexin A3 could also be used to replace PSA entirely, because in the crucial grey zone of PSA (2-6 ng/ml and 4-10 ng/ml), annexin 3 offers considerable advantages (see ROC curves 0.78 and 0.791 in Tables 4-6) with high specificities at acceptable sensitivities, and shows a similar overall performance, considering the whole range of PSA-values.

There was no preselection for annexin A3 in our patient selection, and there was no correlation between annexin A3 values and PSA values, indicating that annexin A3 expression/secretion and PSA entry to the bloodstream are regulated by separate mechanisms. We observed no correlation between either PSA or annexin A3 levels with patient age. Presumably the high proportion of cancer-positive patient, some of whom were preselected on the basis of suspiciously high PSA values measured at other centres, upset the expected higher abundance of PSA levels that would be expected with increasing age. However as an-

nexin A3 was not preselected we conclude that its levels are probably not age-related.

Particular emphasis is placed on the statistical analysis of the subpopulation of patients with initial PSA values in the interval 2 ng/mL to 6 ng/mL. Again, all seven AUROC values for the measured individual parameters are significantly different from 0.5, although only 57 PCa patients and 52 non-PCa patients meet the PSA criterion defining the subpopulation. In contrast to the overall results, the AUROC of PSA_ini is no longer the largest one, but is superceded by five of the six AUROC values of annexin A3 based variables (P_ANX_ug being the only exception). The highest AUROC value, 0.735, is attained by PU_ANX_tot.

Besides the PSA range 2 ng/mL – 6 ng/mL the PSA range 2.5 ng/mL – 12 ng/mL is of special interest: In this subpopulation PSA_ini itself performs poorly (AUROC 0.580), while U_ANX_tot performs best (AUROC 0.693). Thus, this PSA range seems appropriate to assess the characteristics of the following two-step procedure, which is presented by way of methodical demonstration:

In the first step patients are assigned to one of three classes depending on their initial PSA value:

PSA_ini < 2.5 → low PCa risk,
PSA_ini > 12 → high PCa risk,
PSA_ini in [2.5, 12] → application of a test based on U_ANX_tot as a second step deciding whether or not invasive diagnostic procedures are indicated.

(The first two cases are referred to as the "most obvious decision" in the caption of Fig. 8.)

This two-step procedure is then incorporated into a single variable, here called twostepvar1, by defining

$$\text{twostepvar1} := \begin{cases} 100000, & \text{if PSA_ini} < 2.5 \\ \text{U_ANX_tot}, & \text{if PSA_ini} \in [2.5, 12] \\ 0, & \text{if PSA_ini} > 12. \end{cases}$$

Low values of U_ANX_tot indicate an increased risk of prostate cancer and high values a lower risk. The value 100000 is chosen in order to ensure that it should always be greater than the largest U_ANX_tot value actually measured. The performance of twostepvar1 can be seen in Fig. 8. (The constancy of twostepvar1 for PSA_ini values below 2.5 and beyond 12 causes the ROC curve to start and end with a noticeable straight line segment.) The AUROC of 0.740 is of course highly significantly different from 0.5. Moreover, a comparison with the conventional PSA test is enlightening: For example, the criterion "PSA_ini > 4" leads—in this analysis data set—to a sensitivity of 80.3 % and a specificity of 49.5 %. The same sensitivity, 80.3 %, is gained by using the criterion "twostepvar1 < 450", but now a specificity of 57.4 % is achieved.

The example of Fig. 8 demonstrates the principles of stepped AUROC values which rely on different meausurements (PSA or annexin A3 values) depending upon the PSA level. These principles, as will be obvious to experts, are the same as used in several results presented in Tables 4-6, and which provide higher AUROC values. The excellent performance of cancer prediction of the annexin A3 variables in patients with intermediate levels of serum PSA is further evidenced by considering the annexin A3-based multiple variables for patients with intermediate PSA values.

According to the methods and strategies demonstrated above, ROC curve analysis was performed for the parameters shown in Tables 3-6.

Additionally to those variables, ROC curve analysis was performed using the following parameters generated by logical regression analysis.

anx.comb.var is based upon logical regression analysis using the combined U_ANX_ug und U_ANX_tot as variables according to the following relationship.

≡ 4.463 + 2.906 log(1+U_ANX_ug) − 0.790 log(1+U_ANX_tot)

comb.var.anx.psa1 is based upon logical regression analysis using the combined PSA_ini und U_ANX_tot as variables according to the following relationship.

≡ 0.254 + 1.046 log(1+PSA_ini) − 0.342 log(1+PU_ANX_tot)

comb.var.anx.psa2 is based upon logical regression analysis using the combined PSA_ini und PU_ANX_tot as variables according to the following relationship.

≡ −2.386 + 3.294 log(1+PSA_ini) − 0.475 log(1+PU_ANX_tot)

While extremely high or extremely low PSA values provide relatively reliable assignment of cancer/non-cancer status, the results of Tables 4-6 clearly demonstrate that various combinations of annexin A3-based parameters outperform PSA for intermediate values of PSA. Thus, the measurement of annexin A3 levels in exprimate urine supernatants or pellets, preferably including supernatant to pellet ratios, provides improved diagnostic reliability.

To demonstrate the utility of these results, the ROC curve for comb.var.anx.psa2 is presented in detail in Fig. 9, and Table 7. This ROC curve is based only on patients with PSA values between 2 ng/mL and 6 ng/mL, and gives a highly significant AUROC value of 0.791 despite the use of only 109 patients in this range for the analysis. Furthermore, this ROC curve exhibits an extremely steep climb in sensitivity

(true positive fraction) relative to specificity (true negative fraction), which is quite advantageous regarding predictive value. For instance at a sensitivity level of 54% the specificity is 96% (Fig. 9, Table 7). The ROC curve for anx.comb.var performed similarly, with sensitivity of 38% having specificity of 91% (AUROC 0.78) for the PSA range 4 ng/mL to 10 ng/mL. The data disclosed here demonstrate convincingly that annexin A3 is a novel and powerful marker for prostate cancer, that is especially powerful in those patients where PSA values are the least reliable.

Taken together, the third comprehensive study which was double-blinded and multi-center, showed, that the most robust and statistically significant diagnostic read-out was the annexin A3-amount in supernatants of exprimate urines after prostatic massage which have been obtained during a standard clinical procedure (DRE) due for potential prostate cancer patients with a standard low speed centrifugation. This is very favourable because it allows direct access to ELISA-based or other antibody-based assays without prior solubilisation of pelleted samples (danger of interference by detergents, salts, chemicals etc.). This annexin A3-amount in supernatants is inversely correlated with cancer, in non-cancers, annexin A3- amounts are higher, with certain indications that additional and sequential profiling of non-cancer cases can even improve the overall diagnostic value.

The results are completely in line with the first two studies, which were smaller and in some aspects incomplete concerning sample collection and sample control. The first study only included pellets (Figure 6), yet nevertheless here it was found the inverse correlation for cancer patients. In this study a group of healthy volunteers were included, which was not the case for subsequent studies. During the second study which did take into account supernatant-annexin A3 and pellet-annexin A3 (albeit with sample numbers which were too small to come to statistically

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significant solutions), there was a trend to higher supernatant to pellet ratios in cancer patients as compared to BPH and other non-cancers. This is perfectly in line with the first and third study, because obviously the low pellet annexin A3-amounts combine in cancer patients with a slightly higher annexin A3-amount to bigger ratios (Figure 7). In non-cancers (like e.g. BPH, fibrosis and others), obviously in total the considerably larger annexin A3-amounts in pellets and supernatants combine to lower overall ratios. The robustness of the annexin A3-signal in supernatants provides an experimental and clinical advantageous and easy diagnostic read-out.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as, an acknowledgement or admission or any form of suggestion that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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The claims defining the invention are as follows:

1. A method for diagnosing prostate cancer by discrimination between cancerous and non-cancerous prostate tissue, the method comprising:
   (a) determining the abundance of at least one intracellular annexin A3 protein in a urine sample or fraction thereof; and/or
   (b) determining the abundance of at least one extracellular annexin A3 protein in a urine sample or fraction thereof;
   wherein the levels of annexin A3 observed in the urine or fraction thereof from a subject with cancerous prostate tissue are lower than in urine or fraction thereof from a subject with non-cancerous prostate tissue and wherein the urine samples or fractions thereof are obtained from exprimate urine.

2. The method according to claim 1, wherein the abundance of the annexin A3 protein is determined together with the abundance of at least one further annexin protein.

3. The method according to claim 1 or claim 2, wherein the abundance of the annexin A3 protein is determined together with the abundance of at least one further protein selected from the group consisting of: serum amyloid P, isopeptidase T, muscle-type fatty acid binding protein, galectin 1, heat shock protein 90, BiP, protein disulfide isomerise, epidermal-type fatty acid binding protein, enoyl coenzyme A hydratase, nucleophosmin, a 14-3-3 protein, proteasome, activator subunit 2, cytokeratin family, KNP-I alpha protein and KNP-I beta protein.

4. The method according to any one of claims 1 to 3, wherein the abundance of the annexin A3 protein is determined together with the abundance of at least one blood marker, serum marker or epithelial cell marker.

5. The method according to claim 2, wherein the further annexin protein is annexin A8.

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6. The method according to any one of claims 1 to 5, wherein the urine sample or fraction thereof is subjected to a separation process to yield cell a pellet and supernatant prior to determining the abundance of the annexin A3 protein, the further annexin protein or the further protein.

7. The method according to claim 6, wherein the cell pellet is used for determining the intracellular abundance of the annexin A3 protein, the further annexin protein or the further protein.

8. The method according to claim 6, wherein the supernatant is used for determining the extracellular abundance of the annexin A3 protein, the further annexin protein or the further protein.

9. The method according to any one of claims 1 to 8, wherein a cation chelator is added to the urine sample a fraction thereof prior to determining the abundance of the annexin A3 protein, the further protein or the further annexin protein.

10. The method according to claim 9 wherein the cation chelator is EDTA and/or EGTA.

11. The method according to any one of claims 1 to 10, wherein the exprimate urine sample or fraction thereof is recovered subsequent to prostate massage.

12. The method according to any one of claims 1 to 11, wherein the urine sample or fraction thereof is purified.

13. The method according to any one of claims 1 to 12, wherein the urine sample is a sample of morning urine or a fraction thereof.

14. The method according to any one of claims 1 to 11, further comprising measuring the abundance of the annexin in faeces or in epithelial cells of the intestinal tract.

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15. The method according to any one of claims 1 to 14, wherein the abundance of the annexin in a fraction or preparation of faeces is used to further diagnose epithelial cancers of the gastrointestinal tract or any potentially exosome-producing epithelial surface.

16. The method according to claim 15, wherein the abundance of the annexin in a fraction or preparation of faeces is used to further diagnose colorectal cancer.

17. The method according to any one of claims 1 to 16, further comprising determination of neutrophil levels to discriminate inflammatory conditions from cancers.

18. The method according to claim 17, wherein the inflammatory condition is Crohn's disease or colitis ulcerosa.

19. A method for diagnosing prostate cancer comprising determining the abundance of at least one intracellular and/or extracellular annexin A3 in a sample of exprimate urine or fraction thereof substantially as hereinbefore described with reference to the Examples.

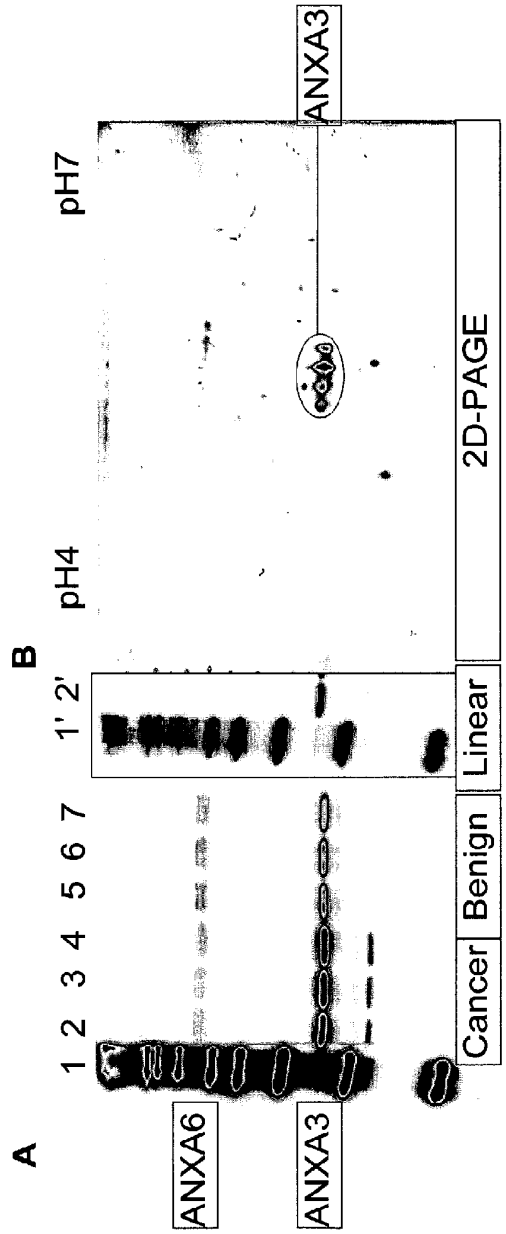
A
1 2 3 4 5 6 7
ANXA6
ANXA3
Cancer
Benign
B
1' 2'
pH4
pH7
ANXA3
Linear
2D-PAGE

Figure 1:

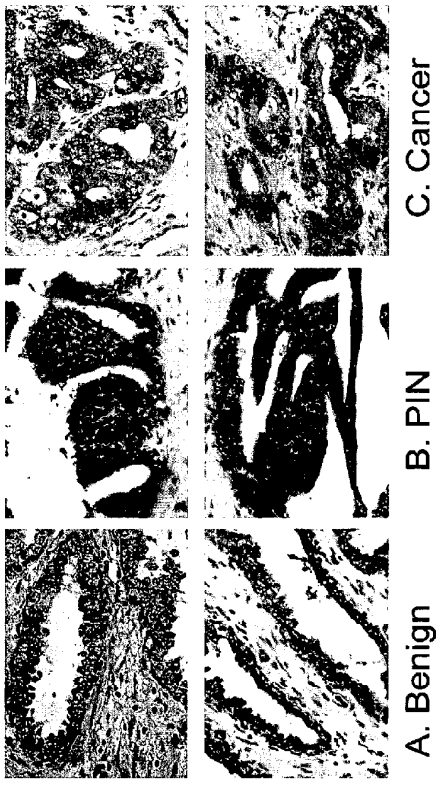

Figure 2:

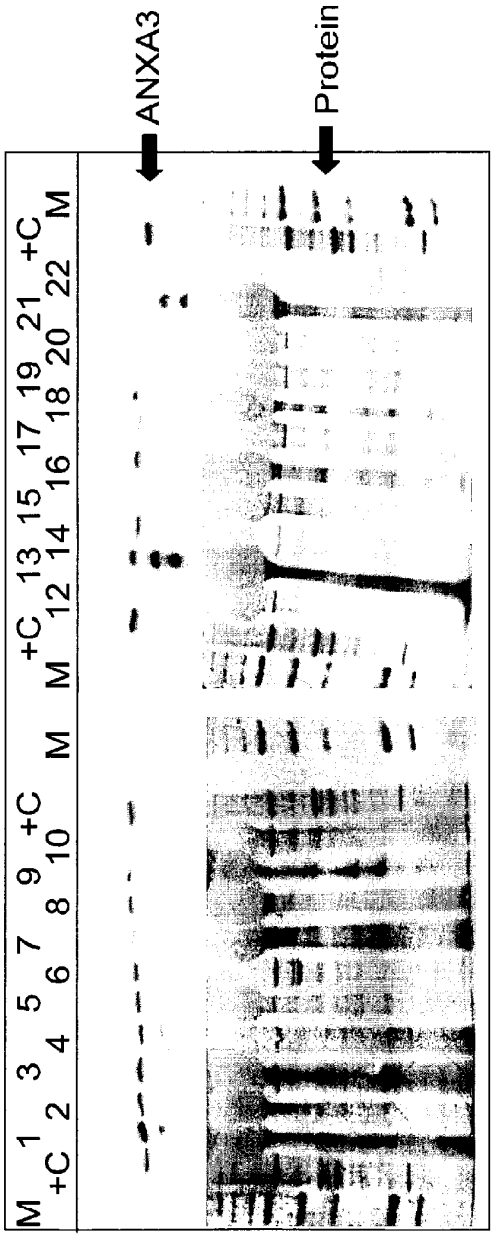
ANXA3
Protein
M +C 1 2 3 4 5 6 7 8 9 10 +C M +C 12 13 14 15 16 17 18 19 20 21 22 +C M

Figure 3

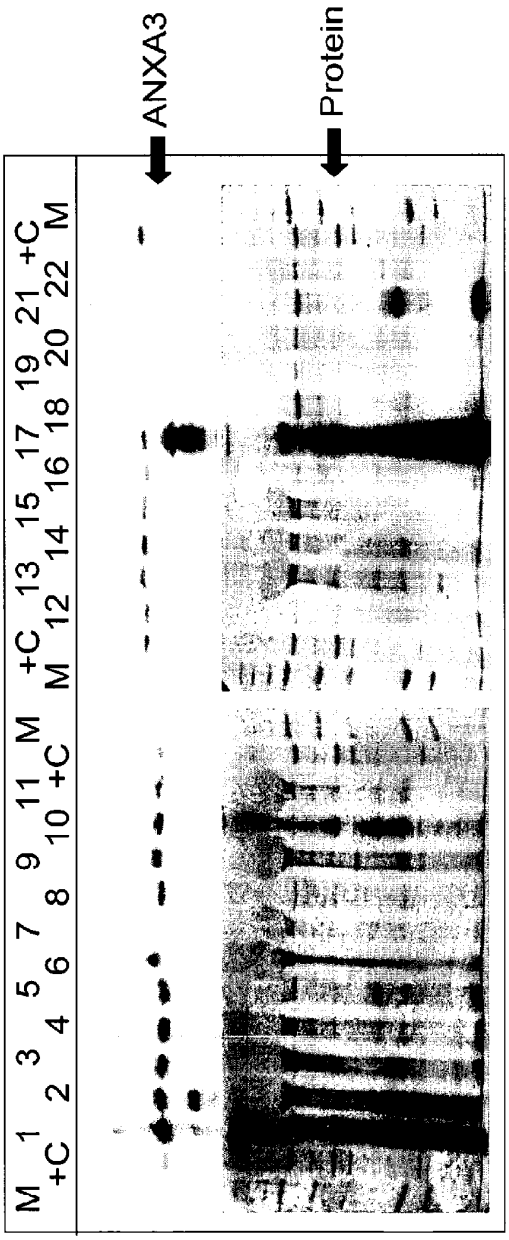
ANXA3
Protein
M +C 1 2 3 4 5 6 7 8 9 10 +C 11 M +C 12 13 14 15 16 17 18 19 20 21 22 +C M

Figure 4

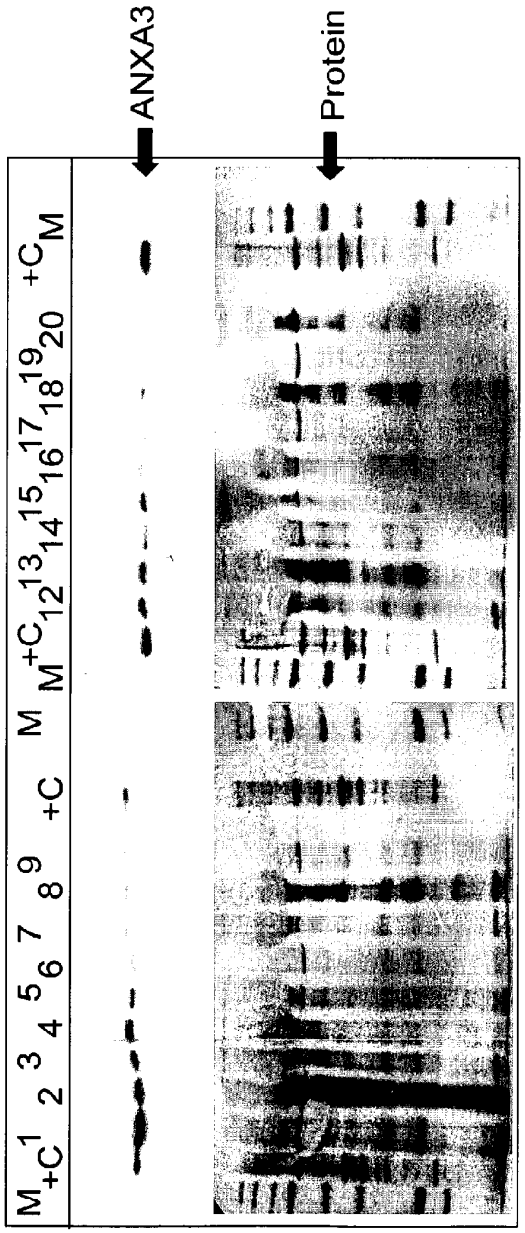

Figure 5

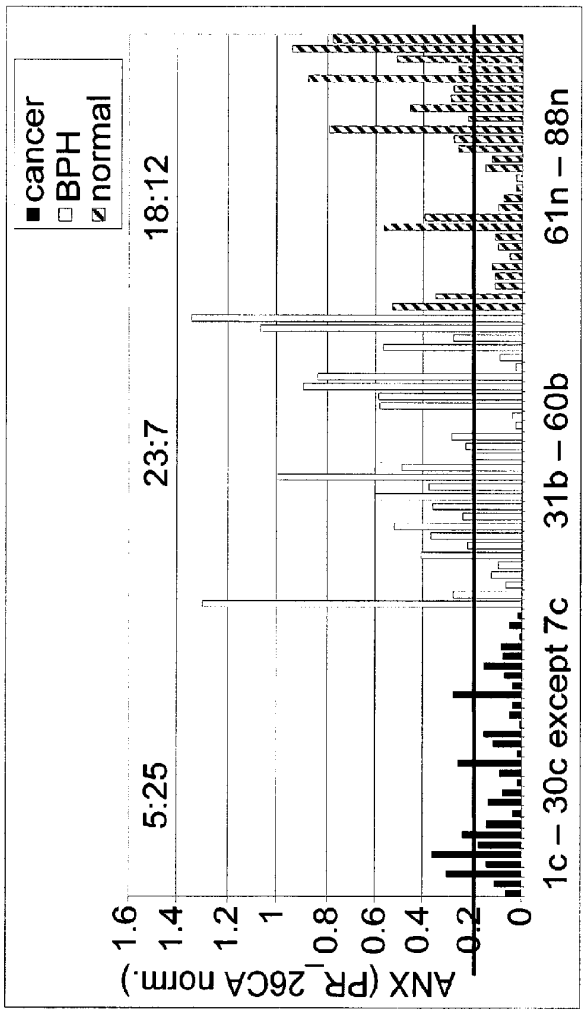
cancer
BPH
normal
ANX (PR_26CA norm.)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5:25
23:7
18:12
1c – 30c except 7c
31b – 60b
61n – 88n

Figure 6

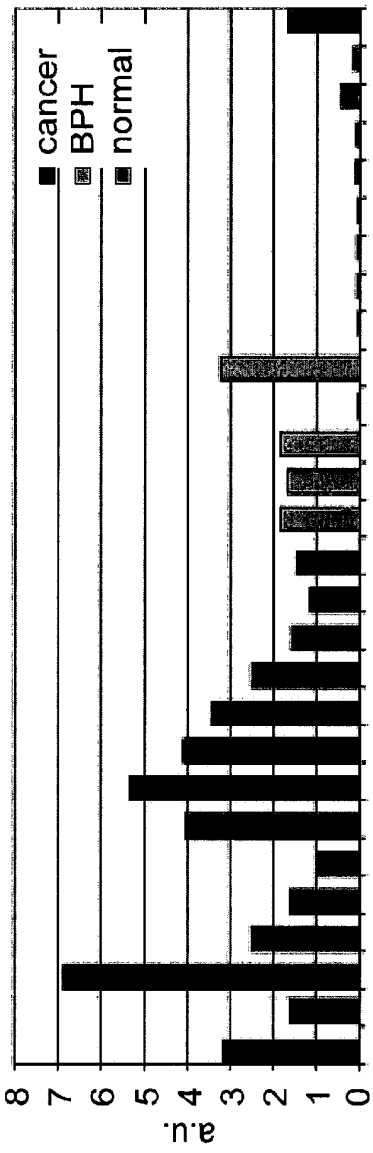

Figure 7

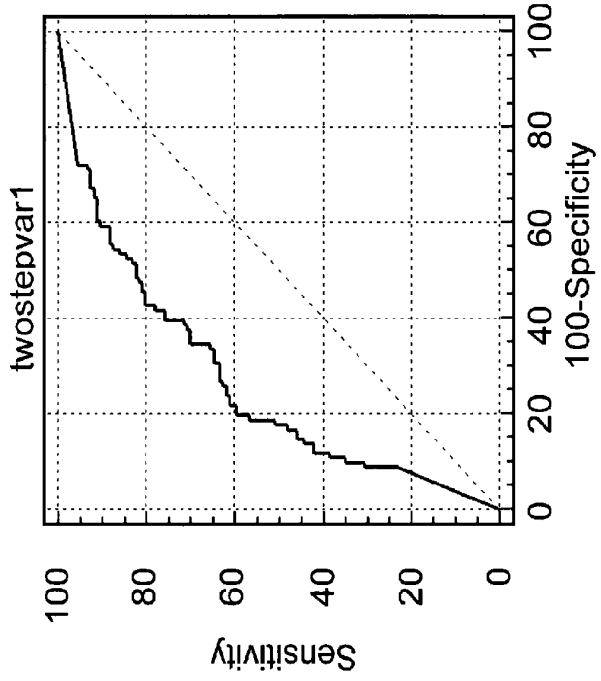
twostepvar1
Sensitivity
100
80
60
40
20
0
0
20
40
60
80
100
100-Specificity

Figure 8

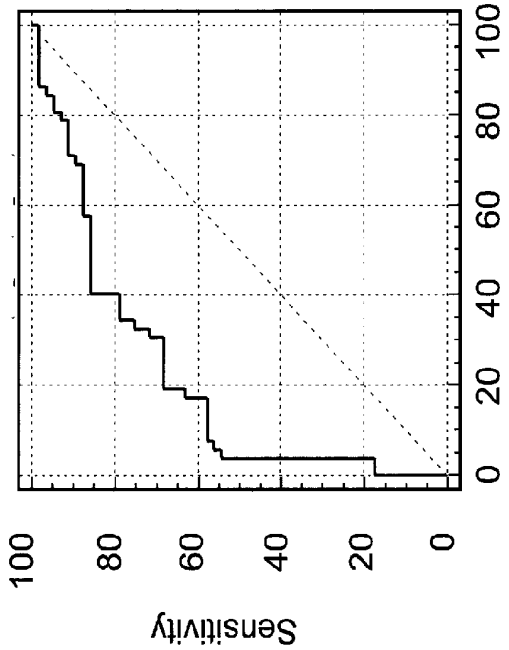

Figure 9