Title: METHOD FOR THE PROGNOSIS AND TREATMENT OF METASTASIS IN BREAST CANCER

Abstract: The present invention relates to the diagnosis and prognosis of metastasis in breast cancer based in the determination of the expression level of PRDX2 gene. It also relates to a method for diagnosis and prognosis of metastasis in breast cancer based in the determination of the expression level of ERp57/GRP58 gene. Lastly, the invention refers to the use of PRDX2 and/or PRDX3 inhibitors and a ROS-generating agent for the treatment and/or prevention of lung metastasis in breast cancers as well as to the use of ERp57/GRP58 inhibitors for the treatment and/or prevention of bone metastasis in breast cancers.
METHOD FOR THE PROGNOSIS AND TREATMENT OF METASTASIS IN BREAST CANCER

FIELD OF THE INVENTION

The present invention relates to the diagnosis and prognosis of metastasis in breast cancer based in the determination of the expression level of PRDX2 gene. It also relates to a method for diagnosis and prognosis of metastasis in breast cancer based in the determination of the expression level of ERp57/GRP58 gene. Lastly, the invention refers to the use of PRDX2 and/or PRDX3 inhibitors and a ROS-generating agent for the treatment and/or prevention of lung metastasis in breast cancer.

BACKGROUND OF THE INVENTION

Breast cancer is the third most common cancer, and the most common cancer in women, as well as a cause of disability, psychological trauma, and economic loss. Breast cancer is the second most common cause of cancer death in women in the United States, in particular for women between the ages of 15 and 54. Prognosis and survival rate varies greatly depending on cancer type and staging. With best treatment and dependent on staging, 5-year relative survival varies from 98% to 23, with an overall survival rate of 85%. Despite recent advances, one challenge of cancer treatment remains to target specific treatment regimens to pathogenically distinct tumor types, and ultimately personalize tumor treatment in order to maximize outcome.

One of the most frequent complications of breast cancer is the metastasis to distal tissues. The presence or absence of metastasis often determines treatment as well as survival. Therefore, the prediction of metastatic potential is thus an important component of cancer management. After bone, lung is the second main target of breast cancer metastasis (Weigelt B et al. Cancer Res 2005;65(20):9155-8). Transcriptomic analysis of a variety of cell lines has identified genes that mediate metastasis to bone or lungs (Minn AJ et al. Nature 2005;28:518-24; Minn AJ et al. Proc Natl Acad Sci USA 2007;17:6740-5; Gupta GP et al. Proc Natl Acad Sci USA 2007;104(49):19506-1 1). Different tropisms to bone and lungs have recently been associated with discrete
variations in overall gene expression patterns. Thus, specialized gene sets have been defined that mediate metastasis to these target organs (Nguyen DX et al. cited ad supra; Minn AJ et al. 2007 cited ad supra; Padua D et al. Cell 2008;133:66-77).

The patent application EP1961825-A1 describes a method for predicting metastasis to bone, lung, liver or brain in breast cancer subjects, comprising determining in a tumour sample the expression level of one or more markers with respect to the expression level in a reference sample. However, the method described in this document requires that the determination of the expression levels of several genes in order to obtain statistically significant prediction.

The patent application US2005/0181375 describes methods for the detection of metastasized breast cancer based on the determination of the expression levels of some underexpressed or overexpressed genes in a metastasis tumour sample.

The patent application WO20 10/000907 describes a genetic signature useful as a genomic predictor of distal metastasis in breast cancer subjects.

Espana et al. (American Journal of Pathology, 2005, 167: 1125-37) describe that the expression of the \( \text{BclX}_{L} \) in breast cancer cells promotes metastasis. The authors have also identified several proteins whose expression is increased in breast cancer cell lines expressing \( \text{BclX}_{L} \) which have metastasized to lung.

Chang et al. (Breast Cancer Research 2007, 9 No 6) describe that peroxiredoxin 6 (PRDX6) is overexpressed in a highly lung metastatic breast cell line. The authors have also demonstrated that peroxiredoxin 6 knockdown breast cancer cells grew more slowly and had fewer pulmonary metastases.

WO2006/074367 describes a gene signature which allows predicting the risk of metastasis to the lung in subjects suffering from breast cancer.

However, up to now there are not genetic markers in the state of the art suitable for the efficient diagnosis and/or prognosis of lung metastasis in a breast cancer patient, allowing the design of a proper therapy to the subject. Hence, a need exists for tests that provide predictive information about patient responses to the variety of treatment options. The identification of new prognostic factors will serve as a guide for selecting suitable and efficient treatments.

**SUMMARY OF THE INVENTION**
The present invention is based, at least in part, on the observation that expression of PRDX2 gene in primary breast cancer tissues is associated to the risk of suffering metastasis to lung. Thus is seen for instance in the examples of the present invention, wherein PRDX2 levels are higher in lung metastatic variants than in lymph node or bone metastatic variants (see Example 1) and wherein the analysis of expression in breast cancer tissues from patients revealed that there is a significant association between lung metastasis progression and high expression of PRDX2 in breast carcinomas (see Example 5).

The authors of the invention have also shown that the ERp57/GRP58 gene is overexpressed in breast cancer metastasis, particularly in liver metastasis. Thus, as seen in the examples of the present invention, the analysis of expression in breast cancer tissues from patients revealed that there is a significant association between liver metastasis progression and high expression of ERp57/GRP58 in breast carcinomas (see Example 7 and Figure 9).

Moreover, the inventors have also shown that a metastatic variant of MDA-MB-435 breast cancer cells that specifically metastatize to lungs (435-L3) transduced with shRNAs to specifically silence PRDX2 or PRDX3, showed significantly higher sensitivity to $\text{H}_2\text{O}_2$-induced oxidative stress than the parental and scrambled transfected cells. Moreover, they also have shown that PRDX2 knockdown inhibited the growth of 435-L3 cells in the lungs, whereas the orthotopic growth of these cells in the mammary gland remained unaffected. Thus, an inhibitor of the expression of the PRDX2 and/or the PRDX3 gene or of the protein encoded by said gene/s may be used in the treatment and/or prevention of lung metastasis in breast cancer.

The authors of the present invention have shown that the inhibition of PRDX2 or PRDX3 in a metastatic variant of breast cancer cells that specifically metastatize to lungs showed significantly increased intracellular ROS levels when cells were exposed to $\text{H}_2\text{O}_2$ than the parental and scrambled transfected cells. Thus, a ROS generating agent may be used in the treatment and/or prevention of lung metastasis in breast cancer.

Thus, in a first aspect, the invention relates to an *in vitro* method for determining the risk of developing lung metastasis in a subject suffering from breast cancer comprising determining the expression level of PRDX2 in a tumour sample of said
subject, wherein if the expression level of said gene is increased with respect to a reference value, said subject has an increased risk of developing lung metastasis.

In a second aspect, the invention relates to an in vitro method for designing a personalized therapy for a subject suffering from breast cancer comprising determining the expression level of PRDX2 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, said subject is a candidate for receiving a therapy directed to prevent and/or treat lung metastasis.

In another aspect, the invention relates to an in vitro method for determining the risk of developing liver metastasis in a subject suffering from breast cancer comprising determining the expression level of ERp57/GRP58 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, said subject has an increased predisposition of developing liver metastasis.

In another aspect, the invention relates to a PRDX2 and/or PRDX3 inhibitor for use in the treatment and/or prevention of lung metastasis in breast cancer.

In another aspect, the invention relates to a ROS-generating agent for use in the treatment and/or prevention of lung metastasis in breast cancer.

In a last aspect, the invention relates to a composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Characterization of the MDA-MB-435 lung metastatic phenotype. (a) Left-hand panel: western blots of peroxiredoxin (PRDX) isoforms 1-6 in 435 parental cells and metastatic variants. Total protein extracts (50 μg per lane) were fractioned by sodium dodecyl sulfate - polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. β-Actin or α-tubulin expression was measured as the loading control. The expression of PRDXs in lung metastatic variants (435-L2 and
435-L3) was assessed with regard to parental 435-P cells. Lymph nodes (435-N) and bone (435-B) metastatic variants are included for comparison. Right-hand panel: subcellular localization of PRDX2 and PRDX3. The 435-P and 435-L3 cells on coverslips were washed, fixed and stained individually with anti-PRDX2 and anti-PRDX3 antibodies, and mitochondria were stained with MitoTracker. Confocal microscopy images of 435-L3 cells show that PRDX2 is localized in the cytosol, while PRDX3 is mitochondrial in basal conditions (images for 435-P cells not shown). Under oxidative stress, PRDX2 translocates to the nucleus. The subcellular distribution was similar in both cell types. (b) Viability of 435-P cells and lung metastatic variants stimulated with 0.4-3 mM \( \text{H}_2\text{O}_2 \) for 48 h at 5 \( \times \) 10\(^3\) per well after starving them for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 5 mg/ml was added before measuring the optical density at 540 nm. Results are expressed as the percentage of cell viability of untreated control cells (absorbance of stimulated cells/absorbance of control cells) and are the mean of three independent experiments. 435-L2 and 435-L\(_3\) cells show higher cell viability than parental cells under oxidative stress conditions. *P < 0.01 for 435-P vs 435-L3 and 435-L2, respectively, using one-way analysis of variance followed by Dunnnett's multiple comparison test. (c) Internal ROS production. Cells were incubated with 7 \( \mu \text{M} \) DCFH\(_2\)-DA in Hanks's balanced salt solution (Invitrogen) for 40 min or with 2 \( \mu \text{M} \) dihydroethidium (DHE) in Hanks's balanced salt solution for 30 min at 37 °C, according to the manufacturer's instructions, with or without \( \text{H}_2\text{O}_2 \) (0.4-4mM). Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, Biogen, Spain) at 485-520 or 530-562 nm (DCF and ethidium, respectively) to assess the fluorescence intensity relative to protein concentration. The results are the mean of three independent experiments. 435-L\(_3\) cells produced less ROS than parental cells under basal conditions, and when cells were treated with 3mM H\(_2\)O\(_2\) *P*<0.05 for 435-L\(_3\) vs 435-P in both conditions using the Student's t-test. (d) Western blot images of PRDX-S0\(_3\) expression in 435-P and 435-L\(_3\) cells (left panel). Relative expression of PRDX-S0\(_3\) was assessed by band densitometry. The overoxidized, inactive form of PRDX, PRDX-S0\(_3\), accumulated less in lung metastatic cells than in parental cells under oxidative stress conditions. The comparative expression of PRXD2 is showed at the right panel.
Figure 2. ROS generating agents prevent lung metastasis. PRDX2 expression in intramammary fat path tumors (i.m.f.p.) induced orthotopically with 1x10^6 of 435-L3 cells. Mice were treated or not with 500 mg/Kg VPA (Sigma, St. Louis, MO) dissolved in PBS 5 days when i.m.f.p. tumor reach 1 cm^3. Mice were sacrificed when controls died. H&E of lungs from corresponding mice show metastatic progression. The table at the bottom describes tumor weigh and metastasis incidence in treated and not treated mice.

Figure 3. Transient knockdown of PRDX2 and PRDX3 in 435 lung metastatic cells. Stealth RNAi oligonucleotide duplexes that target the open reading frame sequences of PRDX2 or PRDX3 and a non-targeting Stealth RNAi negative control (Invitrogen) were used for transient protein knockdown: PRDX2, 5'-ACA AAG GGA AGU ACG UGG UCC UCU U-3' (SEQ ID NO:28), PRDX3, 5'-GGA CAC CGG AUU CUC CUA CGA UCA A-3' (SEQ ID NO:29). RNA duplexes were introduced into 435-L3 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. (A) PRDX2 and PRDX3 expression in mock-transfected 435-L3 cells and cells silenced using siRNA oligos 48 hours after transfection. RNA expression of PRDX2 and PRDX3 was measured by qPCR (upper panels) and protein expression was assessed by Western blot (lower panels); tubulin expression was used as the housekeeping gene. (B) The viability of 435 parental and lung metastatic cells and silenced variants under basal and oxidative stress conditions. 435-L3 cells are more resistant than 435-P cells to oxidative stress. Lung metastatic cells that are deprived of PRDX2 or PRDX3 show a lower viability when exposed to 3 mM H_2O_2 than control 435-L3 cells. *P <0.01 using one-way ANOVA followed by Dunnett's Multiple Comparison test. (C) Internal ROS production of 435 lung cells and cells depleted of PRDX2 or PRDX3. Silenced variants show higher internal ROS production than 435-L3 cells in basal conditions and when stressed with 3 mM H_2O_2, indicating that deprivation of PRDX leads to an accumulation of ROS. ***P <0.0005 using one-way ANOVA and Dunnett's Multiple Comparison test.

Figure 4. Stable downregulation of PRDX2 expression in MDA-MB-435-L3 lung metastatic cells. (a) Stable downregulation of PRDX2 in 435-L3 cells infected with
GFP/LUC. PRDX2 expression levels as determined by immunoblot analysis are shown for several shPRDX2 clones, 435-L3 cells, mock-transfected (scbl) control cells and a pool of shPRDX2 clones (pool A). Further analyses were done with lung metastatic cells stably downregulated for PRDX2 (shPRDX2, clone #7) and scramble control cells (scbl) (b) Tumor cells were treated with 3mM H$_2$O$_2$ for 24 h. The cell viability of tumor cells under oxidative stress conditions was calculated as a percentage of treated cells with regard to untreated cells. Downregulation of PRDX2 in lung metastatic cells (shPRDX2, clone #7) made them more susceptible to oxidative stress than the scbl control cells and parental 435-L3 cells. ***P<0.0005, using the Student’s t-test. (c) Internal ROS production in stably downregulated shPRDX2 cells in basal conditions or treated with 3mM H$_2$O$_2$ for 24 h. Under oxidative stress, shPRDX2 (Clone #7) showed significantly higher ROS production than the scbl control and 435-L3 cells. *P<0.05 using the Student's t test. (d) Cell cycle response to oxidative stress. We cocultured the following cells with or without H$_2$O$_2$ (3mM) for 24 h: 435-P, 435-L3, stably downregulated shPRDX2 and scbl control cells. Ethanol-fixed cells were incubated with 5 µl RNase (10 mg/ml) and 50 µl propidium iodide (PI) in phosphate-buffered saline (0.5 mg/ml, Sigma) for 30 min at 37 °C, and PI-stained cells were analyzed for DNA content by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ,USA). At least 20000 events were collected in each sample and evaluated using the ModFit LT (Verity Software, BD Biosciences) program. The percentages of cells present in each cell phase are shown. No significant differences in cell cycle characteristics were found between shPRDX2 and mock-transfected scbl or untransfected 435-L3 cells, respectively, under basal or oxidative stress conditions. The mean±s.d. of three experiments are shown.

**Figure 5. PRDX2 is a cause-effect player in lung metastasis.** (A) Tumor growth curves from athymic Nude/Balb-c female mice (n = 7 animals per group) after intramammary fat pad inoculation (1x1 10$^6$ cells) of scbl control or PRDX2 knockdown (shPRDX2, clone #7) cells. Tumor volumes were calculated at the indicated times after cell injection using the formula [volume (mm$^3$) = L$^2$ x W$^3$/2], where L and W are the major and minor diameters in millimeters, respectively. Tumor growth did not differ statistically significantly between both groups (P = 0.165 using the Mann-Whitney test).
(B) Representative images of primary breast tumors and spontaneous lung metastases on day 40 and day 82 after intra-mammary fat pad implantation. The standard rainbow color scale was used to depict relative light intensities (red = highest; blue = lowest). Tumor volume and lung metastasis incidence are shown in the insets. (C) IHC analysis of paraffin-embedded histological sections of tumors and lung metastases stained for PRDX2, PCNA, Casp-3 (x20 magnification) and H&E stained (x10) in light microscopy. Representative images of tumors (at day 40) and respective metastasis (at day 82) from intra-mammary fat pad implantation are shown. (D) The in vivo angiogenic effect of PRDX2 downregulation in 435 lung tumors. Representative images of CD31 stained frozen tissues sections of tumors and lungs from mice injected with shPRDX2 (clone #7) and scbl control cells (x10) are shown (upper panel).

Figure 6. Lung metastasis development. (A) The progression curve of lung metastasis development after i.m.f.p. inoculation of downregulated shPRDX2 cells (clone #7) and mock-transfected scbl control cells. The metastatic burden is represented as a logarithm from the quantification of normalized photon flux (NPF) periodically taken as of day 40 after i.m.f.p. injection of cells. While 6/7 mice in the control group had developed visible lung metastases by the end of the experiment, this was the case of only 1/7 mice injected with shPRDX2 cells. However, some animals from this group developed micrometastatic lesions that were detectable by in vivo bioluminescence imaging. The NPF differences between control animals and mice bearing shPRDX2 tumors were significant by the end of the experiment at day 82. **P < 0.005 using a linear mixed model. (B) Metastatic potential. The global incidence of lung metastasis in each group relative to the primary tumor size at the time of tumor exeresis was evaluated by calculating the NPF ratios of metastasis/tumor for each animal. The difference in metastatic capacity between groups was statistically significant by the end of the experiment. **P < 0.005 using a linear mixed model.

Figure 7. PRDX2 expression in human breast carcinomas. Representative tabulation of protein expression in breast cancer tissues from 104 patients with or without metastasis
progression. From them, 27 patients had lung and 44 had bone metastases, including among them 10 patients with both, other metastasis were also represented (13 brain and 23 liver metastasis). Tissues are shown as viewed by light microscopy (x 10). Anti-PRDX2 antibody (LabFrontier, Seoul, Korea) was used at 1/1000 in samples retrieved in Na-citrate buffer. Low and medium cytoplasmic intensities of staining were considered as negative for semi-quantitative purposes, and only tumors with high cytoplasmic intensity staining were taken into account as positive samples. Small square is a high stained breast cancer used as a standard positive control tissue sample.

Figure 8. Lung metastasis development after intravenous injection of 435 lung metastatic cells. (A) Pulmonary tumor burden in mice bearing shPRDX2, clone #7, and scbl tumors, as judged by fluorescence measurement. Left-hand panel: monitoring of tumor growth development in animals from day 0 to day 55 after tumor cell inoculation. Results are expressed as normalized photon flux (NPF). Metastatic progression varied between groups ($P = 0.056$ using a mixed linear model) and the number of shPRDX2 cells retained in the lungs was higher than in scbl controls. Right-hand panel: representative fluorescent images of mice on day 55 after tumor cell inoculation. (B) Left-hand panel: comparison of light production (NPF) between lungs and lymph nodes on day 48 after tumor cell inoculation. There was a trend toward less light production in the lungs of animals injected with shPRDX2 cells than in those injected with scbl control cells ($P = 0.09$, Student's t test). The light production in lymph nodes was similar between groups ($P = 0.59$). Right hand panel: representative images of mice on day 48 after tumor cell inoculation. (C) Representative images of cryostat sections (30 μm) from H&E stained (10x magnification) and GFP-labeled (10x) lung metastases, as well as lymph node tissues stained with H&E (10x) and labeled with an antibody for PRDX2 expression (20x), respectively.

Figure 9. Overexpression of PRDX2 in B02 breast cancer metastatic cells impairs tumor growth in bone. (a) Left-hand panel: WB of PRDX2 expression in MDA-MB-231 breast cancer cells (231) and the highly bone metastatic B02/GFP cell line, as well as in B02-GFP/tTA cells overexpressing PRDX2 (B02/PRDX2). For PRDX2
overexpression, we used the pBIL vector system for protein and luciferase coexpression. Protein expression levels of PRDX2 in B02/PRDX2 cells (clones #10 and #88), parental B02-GFP cells and 231 cells are shown. Right-hand panel: internal ROS production in B02/GFP control and B02/PRDX2 cells. Under both basal and oxidative stress conditions, B02/PRDX2 cells produced significantly less ROS than parental B02/GFP cells. *P<0.05 and **P<0.005 using the Student's t-test. (b) Effects of overexpression of PRDX2 in B02 cells on bone metastasis formation. B02/PRDX2 cells and B02/GFP control cells were inoculated intracardiacally into nude mice (n = 6 animals per group). Left-hand panel: mice bearing B02/PRDX2 tumors developed significantly smaller osteolytic lesions than mice injected with B02/GFP cells on day 36 and day 40 after tumor cell inoculation (**P<0.005 using the Student's t-test). Right-hand panel: representative radiographic images of hind legs on day 40 after tumor cell inoculation. Arrows indicate osteolytic lesions. (c) Tumor burden in mice bearing B02/GFP control and B02/PRDX2 tumors, as judged by bioluminescence measurement. Left-hand panel: monitoring of tumor growth development in animals from day 14 to day 35 after tumor cell inoculation. Results are expressed as relative light units (RLUs) in photons per second. *P<0.05 using a mixed linear model. Right-hand panel: representative bioluminescent images of mice on day 28 after tumor cell inoculation. (d) Left-hand panel: representative bioluminescent image (BLI) of mice bearing B02/GFP tumors on day 28 after tumor cell inoculation. Middle- and right-hand panels: representative whole-body microPET images of B02/GFP tumor-bearing mice on day 28 after tumor cell inoculation, 1 h after administration of 400 µCi of 2-deoxy-2[18F]fluoro-D-glucose (FDG) or sodium [18F]fluoride ion (NAF) via the tail vein. FDG is used to target tumors cells that have a high glycolytic metabolism, whereas NAF is a radiopharmaceutical that has a high affinity to bone. The PET scan of animals did not show any accumulation of FDG in metastatic hind limbs, despite the presence of BLI-positive tumor cells. In contrast, there was far greater accumulation of NAF, which confirms the presence of osteolytic lesions.

**Figure 10. PRDX2 regulates the metabolic stress response of metastatic cells,** (a) Viability of MDA-MB-435 and MDA-MB-231 metastatic variants was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay maintaining cells
for 48, 72 or 96 h in glucose-free culture medium, with no serum or pyruvate. Results are expressed as the percentage of cell viability of untreated control cells (absorbance of stimulated cells/absorbance of control cells) and are the mean of three independent experiments. *P=0.016, **P=0.001 and P=0.003 and ***P=0.0005, using one-way analysis of variance and Dunnett's multiple comparison test. (b) Schematic representation of metastatic progression in lungs. For metastatic cells to grow in lungs, the cancer cells must possess high levels of redox scavenging molecules that allow adaptation to a microenvironment with functional oxidative phenotype. The overexpression of PRDX2 in lung metastatic cells has pathogenic role acting through its ROS scavenger function. Its unique ability to remove ROS subtends the use of glucose, exerting an effective antioxidant defense system needed to protect metastatic cells from free radicals and ROS. As PRDX2 knockdown dramatically decreases lung metastasis formation, PRXD2 might be a 'metabolic adaptor', which stabilizes the redox state required for cell survival, then allowing metastasis to progress and shortening the metastasis dormancy period.

Figure 11. ERp57/GRP58 is a putative biomarker for liver metastasis in breast cancer. Representative tabulation of protein expression in breast cancer tissues for ERp57/GRP58. Anti-ERp57/GRP58 antibody at 1/1000 60min citrate buffer (Acris Antibodies GmbH, Germany) was used. Tissues are shown as viewed by light microscopy (x 10). Low and medium cytoplasmic intensities of staining were considered as negative for semi-quantitative purposes, and only tumors with high intensity staining were considered as positive samples.

Figure 12. ERp57/GRP58/ERp57 is a putative biomarker for liver metastasis in breast cancer. The area under the ROC curve obtained with the integrated predictive indexes. The potential biomarker was assessed in a multivariate logistic regression model using a forward stepwise procedure to identify the best to predict liver metastasis. ROC curve obtained for ERp57/GRP58, aROC=0.680, is represented.

Figure 13. ERp57/GRP58/ERp57 downregulation impairs bone metastasis formation. A) B02 cells were stably transfected to underexpress ERp57. IF validation
of ERp57 underexpression and HLA I overexpression in B02sh21 and 32 cells vs.
controls (shB02ct4). B) Clones were injected in NOD/SCID mice and tumor formation
was followed by bioluminescence analysis. First, in vitro linear regression was
performed between bioluminescence (ph/s) and cell number. Then, conversion to cell
number was performed after bioluminescence follow-up in injected mice. (**=p<0.01,
***=p<0.001). Right panel: representative image showing bioluminescence
in animals at day 47. In the case of sh21, the animal shown was the only one affected.
C) H&E and immunohistochemical analysis of bone metastasis using ERp57, PCNA
and Caspase 3 antibodies. All images at X20.

DETAILED DESCRIPTION

METHOD FOR DETERMINING THE RISK OF METASTASIS IN BREAST
CANCER BASED ON THE EXPRESSION LEVEL OF PRDX2

The inventors have shown that the PRDX2 gene is overexpressed in breast
cancer metastasis, particularly in lung metastasis. Thus, as seen in the examples of the
present invention, PRDX2 levels are higher in lung metastatic variants than in lymph
node or bone metastatic variants (see Example 1). The analysis of expression in breast
cancer tissues from patients revealed that there is a significant association between lung
metastasis progression and high expression of PRDX2 in breast carcinomas (see
Example 5).

Thus, in one aspect, the invention relates to an in vitro method for determining
the risk of developing lung metastasis in a subject suffering from breast cancer,
hereinafter first method of the invention, comprising determining the expression level of
PRDX2 in a tumour sample of said subject, wherein if the expression level of said gene
is increased with respect to a reference value, said subject has an increased risk of
developing lung metastasis.

The term "determining the risk of developing lung metastasis in a subject", as
used herein, is understood as knowing, based on the signs detected, if the breast cancer
that said subject suffers will metastasize to the lung in the future. In the context of the
present invention, the sign is PRDX2 gene overexpression in the primary tumor tissue.
The PRDX2 gene, also known as Peroxiredoxin 2, encodes a member of the peroxiredoxin family of antioxidant enzymes (EC 1.1.1.15), which reduces hydrogen peroxide and alkyl hydroperoxides. The encoded protein may play an antioxidant protective role in cells, and may contribute to the antiviral activity of CD8(+) T-cells.

This protein may have a proliferative effect and play a role in cancer development or progression. PRDX2 not only plays a protective role against oxidative damage, but also inhibits the immune cell responsiveness, which may be regulated by scavenging the low amount of reactive oxygen species (ROS) (Moon et al. 2005. Immunol. Lett. 102:184-190). Transcript variants encoding distinct isoforms have been identified for this gene in humans: isoform a, described in the NCBI data base under the access number NM_005809.4 (SEQ ID NO:1) and isoform c, described in the NCBI data base under the access number NM_181738.1 (SEQ ID NO:2). The PRDX2 gene is conserved in chimpanzee, dog, cow, mouse, rat, zebrafish, fruit fly, mosquito, C.elegans, S.pombe, S.cerevisiae, A.thaliana, rice and Pfalciparum.

In the context of the present invention, "metastasis" is understood as the propagation of a cancer from the organ where it started to a different organ. It generally occurs through the blood or lymphatic system. When the cancer cells spread and form a new tumor, the latter is called a secondary or metastatic tumor. The cancer cells forming the secondary tumor are like those of the original tumor. If a breast cancer, for example, spreads (metastasizes) to the lung, the secondary tumor is formed of malignant breast cancer cells. The disease in the lung is metastatic breast cancer and not lung cancer.

The first method of the invention comprises in a first step quantifying the PRDX2 gene expression level in a tumor tissue sample from a subject.

As used herein, the term "subject" or "patient" refers to all animals classified as mammals and includes but is not limited to domestic and farm animals, primates and humans, for example, human beings, non-human primates, cows, horses, pigs, sheep, goats, dogs, cats, or rodents. Preferably, the subject is a human man or woman of any age or race.

In the present invention "tumor tissue sample" is understood as the tissue sample originating from the primary breast cancer tumor. Said sample can be obtained by conventional methods, for example biopsy, using methods well known by the persons skilled in related medical techniques. The methods for obtaining a biopsy sample...
include splitting a tumor into large pieces, or microdissection, or other cell separating methods known in the art. The tumor cells can additionally be obtained by means of cytology through aspiration with a small gauge needle. To simplify sample preservation and handling, samples can be fixed in formalin and soaked in paraffin or first frozen and then soaked in a tissue freezing medium such as OCT compound by means of immersion in a highly cryogenic medium which allows rapid freezing.

As understood by the person skilled in the art, the gene expression levels can be quantified by measuring the messenger RNA levels of said gene or of the protein encoded by said gene.

For this purpose, the biological sample can be treated to physically or mechanically break up the tissue or cell structure, releasing the intracellular components into an aqueous or organic solution for preparing nucleic acids. The nucleic acids are extracted by means of commercially available methods known by the person skilled in the art (Sambrook, J., et al., "Molecular cloning: a Laboratory Manual", 3rd ed., Cold Spring Harbor Laboratory Press, N.Y., Vol. 1-3.)

Thus, the PRDX2 gene expression level can be quantified from the RNA resulting from the transcription of said gene (messenger RNA or mRNA) or, alternatively, from the complementary DNA (cDNA) of said gene. Therefore, in a particular embodiment of the invention, the quantification of the PRDX2 gene expression levels comprises the quantification of the messenger RNA of the PRDX2 gene or a fragment of said mRNA, complementary DNA of the PRDX2 gene or a fragment of said cDNA or the mixtures thereof.

Virtually any conventional method can be used within the scope of the invention for detecting and quantifying the mRNA levels encoded by the PRDX2 gene or of the corresponding cDNA thereof. By way of non-limiting illustration, the mRNA levels encoded by said gene can be quantified using conventional methods, for example, methods comprising mRNA amplification and the quantification of said mRNA amplification product, such as electrophoresis and staining, or alternatively, by Southern blot and using suitable probes, Northern blot and using specific probes of the mRNA of the gene of interest (PRDX2) or of the corresponding cDNA thereof, mapping with SI nuclease, RT-PCR, hybridization, microarrays, etc., preferably by means of real time quantitative PCR using a suitable marker. Likewise, the cDNA levels corresponding to
said mRNA encoded by the PRDX2 gene can also be quantified by means of using conventional techniques; in this case, the method of the invention includes a step for synthesizing the corresponding cDNA by means of reverse transcription (RT) of the corresponding mRNA followed by the amplification and quantification of said cDNA amplification product. Conventional methods for quantifying expression levels can be found, for example, in Sambrook et al., 2001. (cited ad supra).

In a particular embodiment, the PRDX2 gene expression levels are quantified by means of quantitative polymerase chain reaction (PCR) or a DNA or RNA array.

In addition, the PRDX2 gene expression level can also be quantified by means of quantifying the expression levels of the protein encoded by said gene, i.e.: the PRDX2 protein (peroxiredoxin 2) or any functionally equivalent variant of the PRDX2 protein. Transcript variants encoding two protein isoforms have been identified for the PRDX2 gene: isoform a (NCBI, NP_005800.3), consisting of 198 amino acids (SEQ ID NO: 3) and isoform c (NCBI, NP_859428.1), consisting of 142 amino acids (SEQ ID NO: 4).

The PRDX2 gene expression level can be quantified by means of quantifying the expression levels of any of the PRDX2 protein isoforms. Thus, in a particular embodiment, the quantification of the levels of the protein encoded by the PRDX2 genes comprises the quantification of the PRDX2 proteins.

In the context of the present invention, "functionally equivalent variant of the PRDX2 protein" is understood as (i) variants of the PRDX2 protein (SEQ ID NO: 5 or SEQ ID NO: 6 for PRDX2 depending on the isoform) in which one or more of the amino acid residues are substituted by a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), wherein such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) variants comprising an insertion or a deletion of one or more amino acids and having the same function as the PRDX2 protein, i.e., it is involved in redox regulation of the cell, reducing peroxides with reducing equivalents provided through the thioredoxin system. PRDX2 variants can be determined according to any method known in the art. For example, the PRDX2 variants can be identified using methods based on the antioxidant activity of PRDX2, as shown in Jacobson, F.S. et al. (1989) J. Biol. Chem. 264, 1488-1496, wherein the
activity is assayed by observing the reduction of hydrogen peroxide to water and the corresponding alcohol.

The variants according to the invention preferably have sequences similarity with the amino acid sequence of any of the PRDX2 protein isoforms (SEQ ID NO: 5 or SEQ ID NO: 6 for PRDX2, depending on the isoform) of at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%. The degree of similarity between the variants and the specific PRDX2 protein sequences defined previously is determined using algorithms and computer processes which are widely known by the persons skilled in the art. The similarity between two amino acid sequences is preferably determined using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

The PRDX2 protein expression levels can be quantified by any conventional method which allows detecting and quantifying said protein in a sample from a subject. By way of non-limiting illustration, said protein levels can be quantified, for example, by using antibodies with PRDX2 binding capacity (or a fragment thereof containing an antigenic determinant) and the subsequent quantification of the complexes formed. The antibodies used in these assays may or may not be labeled. Illustrative examples of markers that can be used include radioactive isotopes, enzymes, fluorophores, chemiluminescence reagents, enzyme substrates or cofactors, enzyme inhibitors, particles, dyes, etc. There is a wide range of known assays that can be used in the present invention which use unlabeled antibodies (primary antibody) and labeled antibodies (secondary antibody); these techniques include Western-blot or Western transfer, ELISA (enzyme-linked immunosorbent assay), RIA (radioimmunoassay), competitive EIA (competitive enzyme immunoassay), DAS-ELISA (double antibody sandwich ELISA), immunocytochemical and immunohistochemical techniques, techniques based on the use of protein microarrays or biochips including specific antibodies or assays based on colloidal precipitation in formats such as dipsticks. Other ways for detecting and quantifying said PRDX2 protein include affinity chromatography techniques, ligand binding assays, etc. When an immunological method is used, any antibody or reagent that is known to bind to the PRDX2 protein
with a high affinity can be used for detecting the amount thereof. Nevertheless, the use of an antibody, for example, polyclonal sera, supernatants of hybridomas or monoclonal antibodies, antibody fragments, Fv, Fab, Fab' and F(ab')2, scFv, humanized diabodies, triabodies, tetrabodies and antibodies. There are commercially available antibodies against PRDX2, which can be used in the context of the present invention, for example, the SIGMA ALDRICH anti-PRDX2 antibodies SAB1404456 (human monoclonal antibody produced in mouse clone S2), GW21018 (human antibody produced in chicken, affinity isolated antibody) WH0007001M1 (human monoclonal antibody produced in mouse clone 4E10-2D2), SAB2500777 (human antibody produced in goat, affinity isolated antibody), SAB1406520 (human antibody produced in mouse, purified immunoglobulin), SAB2101878 (human antibody produced in rabbit, affinity isolated antibody) and R8656 (human, mouse, rat C-terminal antibody produced in rabbit).

In a particular embodiment, the PRDX2 protein levels are quantified means of western blot, ELISA or a protein array.

The first method of the invention comprises in a second step comparing the PRDX2 gene expression levels obtained in the tumor sample from the subject with a reference value.

In a preferred embodiment, the reference value is the expression level of the PRDX2 gene in a control or reference sample.

Once the PRDX2 gene expression levels in a tumor tissue sample from a subject with breast cancer have been measured and compared with the control sample, if the expression levels of said gene/s is/are increased with respect to its expression levels in the control sample, then it can be concluded that said subject has a greater tendency to develop metastasis, specifically lung metastasis.

The determination of the PRDX2 gene expression levels must be correlated with values of a control sample or reference sample. Depending on the type of tumor to be analyzed, the exact nature of the control sample may vary. Thus, in the event that a diagnosis is to be evaluated, then the reference sample is a tumor tissue sample from a subject with breast cancer that has not metastasized or that corresponds to the median value of the PRDX2 gene expression levels measured in a tumor tissue collection in biopsy samples from subjects with breast cancer which have not metastasized.
Said reference sample is typically obtained by combining equal amounts of samples from a subject population. Generally, the typical reference samples will be obtained from subjects who are clinically well documented and in whom the absence of metastasis is well characterized. In such samples, the normal concentrations (reference concentration) of the biomarker (PRDX2 gene) can be determined, for example by providing the mean concentration over the reference population. Various considerations are taken into account when determining the reference concentration of the marker. Among such considerations are the age, weight, sex, general physical condition of the patient and the like. For example, equal amounts of a group of at least 2, at least 10, at least 100 to preferably more than 1000 subjects, preferably classified according to the foregoing considerations, for example according to various age categories, are taken as the reference group. The sample collection from which the reference level is derived will preferably be formed by subjects suffering from the same type of cancer as the patient object of the study.

Once this median value has been established, the level of this marker expressed in tumor tissues from patients with this median value can be compared and thus be assigned to the "increased" expression level. Due to the variability among subjects (for example, aspects referring to age, race, etc.) it is very difficult (if not virtually impossible) to establish absolute reference values of PRDX2 expression. Thus, in particular embodiment the reference values for "increased" or "reduced" expression of the PRDX2 expression are determined by calculating the percentiles by conventional means which involves performing assays in one or several samples isolated from subjects whose disease is well documented by any of the methods mentioned above the PRDX2 expression levels. The "reduced" levels of PRDX2 can then preferably be assigned to samples wherein the PRDX2 expression levels are equal to or lower than 50th percentile in the normal population including, for example, expression levels equal to or lower than the 60th percentile in the normal population, equal to or lower than the 70th percentile in the normal population, equal to or lower than the 80th percentile in the normal population, equal to or lower than the 90th percentile in the normal population, and equal to or lower than the 95th percentile in the normal population. The "increased" PRDX2 gene expression levels can then preferably be assigned to samples wherein the PRDX2 gene expression levels are equal to or greater than the 50th percentile in the
normal population including, for example, expression levels equal to or greater than the 60th percentile in the normal population, equal to or greater than the 70th percentile in the normal population, equal to or greater than the 80th percentile in the normal population, equal to or greater than the 90th percentile in the normal population, and equal to or greater than the 95th percentile in the normal population.

In the present invention "increased expression levels" is understood as the expression level when it refers to the levels of the PRDX2 gene greater than those in a reference sample or control sample. Particularly, a sample can be considered to have high PRDX2 expression levels when the expression levels in the reference sample are at least 1.1 times, 1.5 times, 5 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times or even more with respect to the sample isolated from the patient.

In the context of the present invention, it is understood that "a subject has an increased predisposition of developing lung metastasis" when the probabilities that the breast cancer suffered by the subject will metastasize in the future to lung are high.

The person skilled in the art will understand that the prediction of the tendency for a primary breast tumor to metastasize is not intended to be correct for all the subjects to be identified (i.e., for 100% of the subjects). Nevertheless, the term requires enabling the identification of a statistically significant part of the subjects (for example, a cohort in a cohort study). Whether a part is statistically significant can be determined in a simple manner by the person skilled in the art using various well known statistical evaluation tools, for example, the determination of confidence intervals, determination of p values, Student's T test, Mann-Whitney test, etc. Details are provided in Dowdy and Wearden, Statistics for Research, John Wiley and Sons, New York 1983. The preferred confidence intervals are at least 90%\textgreater, at least 95%, at least 97%, at least 98%\textgreater or at least 99%. The p values are preferably 0.1, 0.05, 0.01, 0.005 or 0.0001. More preferably, at least 60%\textgreater, at least 70%\textgreater, at least 80%\textgreater or at least 90%\textgreater of the subjects of a population can be suitably identified by the method of the present invention.

METHOD FOR DETERMINING THE RISK OF METASTASIS IN BREAST CANCER BASED ON THE EXPRESSION LEVEL OF ERp57/ERp57/GRP58
The authors of the invention have shown that the ERp57/GRP58 gene is overexpressed in breast cancer metastasis, particularly in liver metastasis. Thus, as seen in the examples of the present invention, the analysis of expression in breast cancer tissues from patients revealed that there is a significant association between liver metastasis progression and high expression of ERp57/GRP58 in breast carcinomas (see Example 7 and Figure 9).

Thus, in another aspect, the invention relates to an in vitro method for determining the risk of developing liver metastasis in a subject suffering from breast cancer, hereinafter second method of the invention, comprising determining the expression level of ERp57/GRP58 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, said subject has an increased predisposition of developing liver metastasis.

The ERp57/GRP58 gene, also known as PDIA3, encodes a protein disulfide isomerase (EC 5.3.4.1) found in the endoplasmic reticulum lumen. The encoded protein catalyzes the rearrangement of both intrachain and interchain disulfide bonds in proteins to form the native structures. It is an essential component of the peptide-loading complex of the major histocompatibility complex class I pathway. The ERp57/GRP58 gene is described in the NCBI data base under the access number NM_005313.4 (SEQ ID NO:5)

The terms and expressions "subject", "tumor tissue sample", "metastasis", "determination of expression levels", "increased expression levels" and "control sample" have been described in detail in relation to the first method of the invention and are equally applicable to the second method of the invention.

In the present invention "risk of developing liver metastasis in a subject" is understood as knowing, based on the signs, if the breast cancer that said subject suffers will metastasize to the liver in the future. In the context of the present invention, the sign is ERp57/GRP58 gene overexpression in the primary tumor tissue.

In the context of the present invention, it is understood that "a subject has an increased predisposition of developing liver metastasis" when the probabilities that the breast cancer suffered by the subject will metastasize in the future to liver are high.

In a particular embodiment, the determination of the expression level comprises the determination of the mRNA level of said gene or a fragment thereof, the
determination of the cDNA level or a fragment thereof or determining the protein level
encoded by said gene or a variant thereof.

In a particular embodiment, the determination of the mRNA or cDNA expression level is carried out by quantitative PCR or by a DNA or RNA array.

In a particular embodiment, the determination of the protein level is carried out by western blot, ELISA or a protein array.

The different methods and details for quantification of the ERp57/GRP58 gene expression levels have been described for PRDX2 in the first method of the invention and are equally applicable to the second method of the invention.

The ERp57/GRP58 gene expression level can also be quantified by means of quantifying the expression levels of the protein encoded by said gene, i.e.: the ERp57/GRP58 protein (or PDIA3) or any functionally equivalent variant of the ERp57/GRP58 protein. The sequence protein encoded by the ERp57/GRP58 gene has been described in the NCBI data base under the access number NP_005304.3 (SEQ ID NO: 6).

There are commercially available antibodies against ERp57/GRP58, which can be used in the context of the present invention for the determination of the protein level, for example, the SIGMA ALDRICH human anti-PDIA3 antibody produced in rabbit (HPA002645), SIGMA ALDRICH human anti-PDIA3 antibody produced in goat (SAB2500769), SIGMA ALDRICH human anti-PDIA3 antibody produced in mouse (SAB 1405897), SIGMA ALDRICH monoclonal anti-PDIA3 antibody produced in mouse (E5031), MILLIPORE monoclonal anti-PDIA3 antibody produced in mouse (05-728), etc.

METHOD FOR DESIGNING A PERSONALIZED THERAPY IN A SUBJECT SUFFERING FROM BREAST CANCER USING PRDX2

As is known in the state of the art, the treatment to be administered to a subject suffering from cancer depends on whether the latter is a malignant tumor, i.e., whether it has high probabilities of undergoing metastasis, or whether the latter is a benign tumor. In the first assumption, the treatment of choice is a systemic treatment such as
chemotherapy and in the second assumption, the treatment of choice is a localized treatment such as radiotherapy.

Therefore, as described in the present invention, knowing that the PRDX2 gene overexpression in breast cancer cells is related to the presence of lung metastasis, then the most suitable therapy for the subject suffering from said cancer can be decided depending on the PRDX2 gene expression levels.

Thus, in another aspect the invention relates to an *in vitro* method for designing a personalized therapy for a subject suffering from breast cancer, hereinafter third method of the invention, comprising determining the expression level of PRDX2 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, said subject is a candidate for receiving a therapy directed to prevent and/or treat lung metastasis.

The terms and expressions "subject", "tumor tissue sample", "metastasis", "determination of expression levels", "PRDX2 gene", "increased expression levels" and "control sample or reference value" have been described in detail in relation to the first method of the invention and are equally applicable to the third method of the invention.

The third method of the invention comprises in a first step quantifying the PRDX2 gene expression level in a tumor tissue sample in a subject suffering from breast cancer. In the case of the third method of the invention the sample is a primary tumor tissue sample of the subject. In a second step, the PRDX2 gene expression level obtained in the tumor sample of the subject is compared with the expression level of said gene in a control sample. The determination of the PRDX2 gene expression levels must be related to values of a control sample or reference sample. Depending on the type of tumor to be analyzed, the exact nature of the control sample may vary. Thus preferably the reference sample is a tumor tissue sample of subject with breast cancer that has not metastasized or that correspond to the median value of the PRDX2 gene expression levels measured in a tumor tissue collection in biopsy samples of subjects with breast cancer which has not metastasized.

The different methods and details for quantification of the ERp57/GRP58 gene expression levels have been described for PRDX2 in the first method of the invention and are equally applicable to the second method of the invention.
Once the PRDX2 gene expression levels in the sample have been measured and compared with the reference value, if the expression levels of said gene are increased with respect to the reference value in a control sample, then it can be concluded that said subject is susceptible to receiving therapy aiming to prevent (if the subject has yet to undergo metastasis) and/or treat lung metastasis (if the subject has already experienced metastasis).

When the cancer has metastasized, systemic treatments including but not limited to chemotherapy, hormone treatment, immunotherapy, or a combination thereof are used. Additionally, radiotherapy and/or surgery can be used. The choice of treatment generally depends on the type of primary cancer, the size, the location of the metastasis, the age, the general health of the patient and the types of treatments used previously.

The systemic treatments are those that reach the entire body:

- Chemotherapy is the use of medicaments to destroy cancer cells. The medicaments are generally administered through oral or intravenous route. Sometimes, chemotherapy is used together with radiation treatment.

- Hormone therapy is based on the fact that some hormones promote some cancer growth. For example, estrogen in women produced by the ovaries sometimes promotes the breast cancer growth. There are several ways for stopping the production of these hormones. A way is to remove the organs producing them: the ovaries in the case of women, the testicles in the case of the men. More frequently, medicaments to prevent these organs from producing the hormones or to prevent the hormones from acting on the cancer cells can be used.

- Immunotherapy is a treatment that aids the immune system itself of the patient to combat cancer. There are several types of immunotherapy which are used to treat metastasis patients. These include but are not limited to cytokines, monoclonal antibodies and antitumor vaccines.
Therefore, as described in the present invention, knowing that the ERp57/GRP58 gene overexpression in breast cancer cells is related to the presence of liver metastasis, then the most suitable therapy for the subject suffering from said cancer can be decided depending on the ERp57/GRP58 gene expression levels.

Thus, in another aspect the invention relates to an in vitro method for designing a personalized therapy for a subject suffering from breast cancer, hereinafter fourth method of the invention, comprising determining the expression level of ERp57/GRP58 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value said subject is a candidate for receiving a therapy directed to prevent and/or treat liver metastasis.

The terms and expressions "subject", "tumor tissue sample", "metastasis", "determination of expression levels", "ERp57/GRP58 gene", "increased expression levels" and "reference value or control sample" have been described in detail in relation to the first and second method of the invention and are equally applicable to the fourth method of the invention.

The different methods and details for quantification of the ERp57/GRP58 gene expression levels have been described for PRDX2 in the first method of the invention and are equally applicable to the fourth method of the invention.

Once the ERp57/GRP58 gene expression levels in the sample have been measured and compared with the control sample, if the expression levels of said gene are increased with respect to their expression levels in the control sample, then it can be concluded that said subject is susceptible to receiving therapy aiming to prevent (if the subject has yet to undergo metastasis) and/or treat liver metastasis (if the subject has already experienced metastasis).

When the cancer has metastasized, systemic treatments including but not limited to chemotherapy, hormone treatment, immunotherapy, or a combination thereof are used. Additionally, radiotherapy and/or surgery can be used. The choice of treatment generally depends on the type of primary cancer, the size, the location of the metastasis, the age, the general health of the patient and the types of treatments used previously.

Suitable treatments for metastatic cancer that can be provided for patients identified according to the present method include, without limitation:
Chemotherapy: The term "chemotherapy", as used herein, refers to medicaments to destroy cancer cells. The medicaments are generally administered through oral or intravenous route. Sometimes, chemotherapy is used together with radiation treatment. Any chemotherapeutic agent exhibiting anticancer activity can be used according to the invention. In certain embodiments, the chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors, vinca alkaloids, epipodophyllotoxins, antibiotics, L-Asparaginase, topoisomerase inhibitor, interferons, platinum coordination complexes, anthracenedione substituted urea, methyl hydrazine derivatives, adrenocortical suppressant, adrenocorticosteroids, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog. In certain embodiments, the chemotherapeutic agent is for example, capecitabine, taxane, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane(R)), gemcitabine, vinorelbine or combinations thereof. In certain embodiments, the chemotherapeutic agent is for example, capecitabine, taxane, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane(R)), gemcitabine, or combinations thereof. In certain embodiments, the chemotherapeutic agent is for example, capecitabine, taxane, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane(R)), or combinations thereof. Two or more chemotherapeutic agents can be used in a cocktail to be administered in combination with administration of the anti-VEGF antibody. Clinical benefits of the treatments according to the invention can be measured by, for example, duration of progression free survival (PFS), time to treatment failure, objective response rate and duration of response.

Hormone therapy: The term "hormone therapy", as used herein, refers to the use of hormonal analogs in ER+ cancers which modulate the levels of hormone that promote cancer growth. For example, estrogen in women produced by the ovaries sometimes promotes the breast cancer growth. There are several ways for stopping the production of these hormones. A way is to remove the organs producing them: the ovaries in the case of women, the testicles in the case of the men. More frequently, medicaments to prevent these organs from producing the
hormones or to prevent the hormones from acting on the cancer cells can be used. Suitable anti-hormonal agents for the treatment of hormone-dependent breast cancer include anti-estrogens such as tamoxifen, toremifene, raloxifene, droloxifene, iodoxyfene, as well as selective estrogen receptor modulators (SERMS) such those described in US 5,681,835, 5,877,219, and 6,207,716.

Immunotherapy is a treatment that aids the immune system itself of the patient to combat cancer. There are several types of immunotherapy which are used to treat metastasis patients. These include but are not limited to cytokines, monoclonal antibodies and antitumor vaccines.

THERAPEUTIC METHODS OF THE INVENTION

PRDX2 AND/OR PRDX3 INHIBITORS FOR THE TREATMENT AND/OR PREVENTION OF LUNG METASTASIS IN BREAST CANCER

The authors of the present invention have shown that a metastatic variant of MDA-MB-435 breast cancer cells that specifically metastatize to lungs (435-L3) transduced with shRNAs to specifically silence PRDX2 or PRDX3 showed significantly higher sensitivity to $H_2O_2$-induced oxidative stress than the parental and scrambled transfected cells. Moreover, they also have shown that PRDX2 knockdown inhibited the growth of 435-L3 cells in the lungs, whereas the orthotopic growth of these cells in the mammary gland remained unaffected. Thus, an inhibitor of the expression of the PRDX2 and/or the PRDX3 gene or of the protein encoded by said gene/s may be used in the treatment and/or prevention of lung metastasis in breast cancer.

Thus, in another aspect the invention relates to a PRDX2 and/or PRDX3 inhibitor, hereinafter inhibitor of the invention, for use in the treatment and/or prevention of lung metastasis in breast cancer. Alternatively, the invention relates to the use of a PRDX2 and/or PRDX3 inhibitor in the manufacture of a medicament for the treatment and/or prevention of lung metastasis in breast cancer. Alternatively, the invention relates to a method for the treatment and/or prevention of lung metastasis in a subject suffering from breast cancer comprising the administration of a PRDX2 and/or a PRDX3 inhibitor to said subject.
A "PRDX2 and/or PRDX3 inhibitor", as used in the present invention, refers to any molecule capable of totally or partially inhibiting the expression of PRDX2 and/or PRDX3, reducing therefore the activity of PRDX2 and/or PRDX3. The inhibition can be carried out either by preventing the generation of the expression product of said gene/s (by interrupting the PRDX2 and/or PRDX3 gene/s transcription and/or blocking the translation the mRNA coming from the PRDXs gene expression) or by directly inhibiting or reducing the PRDX2 and/or PRDX3 activity.

The PRDX2 gene, also known as Peroxiredoxin 2, encodes a member of the peroxiredoxin family of antioxidant enzymes (EC 1.1.1.15), which reduces hydrogen peroxide and alkyl hydroperoxides. The encoded protein may play an antioxidant protective role in cells, and may contribute to the antiviral activity of CD8(+) T-cells. This protein may have a proliferative effect and play a role in cancer development or progression. PRDX2 not only plays a protective role against oxidative damage, but also inhibits the immune cell responsiveness, which may be regulated by scavenging the low amount of reactive oxygen species (ROS) (Moon et al. 2005. Immunol. Lett. 102:184-190). Transcript variants encoding distinct isoforms have been identified for this gene in humans: isoform a, described in the NCBI data base under the access number NM_005809.4 (SEQ ID NO:1) and isoform c, described in the NCBI data base under the access number NM_181738.1 (SEQ ID NO:2). The PRDX2 gene is conserved in chimpanzee, dog, cow, mouse, rat, zebrafish, fruit fly, mosquito, C.elegans, S.pombe, S.cerevisiae, A.thaliana, rice and Pfalciparum. Transcript variants encoding two protein isoforms have been identified for the PRDX2 gene: isoform a (NCBI, NP_005800.3), consisting of 198 amino acids (SEQ ID NO: 3) and isoform c (NCBI, NP_859428.1), consisting of 142 amino acids (SEQ ID NO: 4).

The PRDX3 gene, also known as Peroxiredoxin 3, encodes a member of the peroxiredoxin family of antioxidant enzymes (EC 1.1.1.15) localized in the mitochondrion. It is involved in redox regulation of the cell and protects radical-sensitive enzymes from oxidative damage by a radical-generating system. The Peroxiredoxin 3 protein acts synergistically with MAP3K13 to regulate the activation of NF-kappa-B in the cytosol. Two transcript variants encoding two different isoforms have been found for this gene: isoform a, described in the NCBI data base under the access number NM_006793.2 and the protein under the access number NP_006784.1
(SEQ ID NO:7 and SEQ ID NO: 8, respectively) and isoform b, described in the NCBI
data base under the access number NM_014098.2 and NP_054817.2 (SEQ ID NO:9 and
SEQ ID NO: 10, respectively). The PRDX3 gene is conserved in chimpanzee, dog, cow,
mouse, rat, chicken, zebrafish, fruit fly, mosquito, and C.elegans.

Methods suitable for determining those compounds which are inhibitors of the
expression of PRDX2 and/or PRDX3 have previously been described and comprise
both the methods based on determining the levels of the PRDX2 and/or PRDX3 polypeptides or of mRNA encoding PRDX2 and/or PRDX3 and those based on the
capacity of the PRDX2 and PRDX3 inhibitors of sensitizing cells against increased
ROS levels (ROS scavenger function) (e.g. when cells are exposed to H₂O₂, as shown in
Example 3 of the present invention). Moreover, inhibitors of PRDX2 and/or PRDX3
can also be identified based in their ability to suppress metastatic progression in lungs
of breast cancer cells lines (e.g. by non-invasive bioluminescence imaging, as shown in
Example 5 of the present invention).

In a particular embodiment, the PRDX2 and/or PRDX3 inhibitory agent suitable
for use thereof in the present invention is selected from the group of:

(i) an interfering RNA specific for PRDX2 or PRDX3,
(ii) a polynucleotide encoding an interfering RNA specific for PRDX2 or PRDX3,
(iii) an antisense oligonucleotide specific for PRDX2 or PRDX3,
(iv) an inhibitory antibody specific for PRDX2 or PRDX3,
(v) an inhibitory peptide specific for PRDX2 or PRDX3, and
(vi) a ribozyme or DNA enzyme specific for PRDX2 or PRDX3.

In a preferred embodiment, the siRNA specific for ERp57/GRP58 is directed to
positions 2378-2396 of the sequence identified as SEQ ID NO:24. In a particular
embodiment, the different inhibitory agents (i) to (vi) may be combined between them
for its administration.

(i) Interfering RNA specific for PRDX2 or PRDX3 (siRNA/shRNA)

Small interference RNA or siRNA are agents which are capable of inhibiting the
expression of a target gene by means of RNA interference. A siRNA can be chemically
synthesized, can be obtained by means of in vitro transcription or can be synthesized in
vivo in the target cell. Typically, the siRNA consist of a double stranded RNA between 15 and 40 nucleotide long and may contain a 3' and/or 5' protruding region of 1 to 6 nucleotides. The length of the protruding region is independent of the total length of the siRNA molecule. The siRNA acts by means of degrading or silencing the target messenger after transcription.

The siRNA of the invention are substantially homologous to the mRNA of the PRDX2 and/or PRDX3 encoding gene or to the gene sequence which encodes said proteins. "Substantially homologous" is understood as having a sequence which is sufficiently complementary or similar to the target mRNA such that the siRNA is capable of degrading the latter through RNA interference. The siRNA suitable for causing said interference include siRNA formed by RNA, as well as siRNA containing different chemical modifications such as:

- siRNA in which the bonds between the nucleotides are different than those appear in nature, such as phosphorothionate bonds.
- Conjugates of the RNA strand with a functional reagent, such as a fluorophore.
- Modifications of the ends of the RNA strands, particularly of the 3' end by means of the modification with different hydroxyl functional groups in 2' position.
- Nucleotides with modified sugars such as O-alkylated residues on 2' position like 2'-0-methylribose or 2'-0-fluororibose.
- Nucleotides with modified bases such as halogenated bases (for example 5-bromouracil and 5-iodouracil), alkylated bases (for example 7-methylguanosine).

The siRNA can be used as is, i.e., in the form of a double stranded RNA with the aforementioned characteristics. Alternatively, the use of vectors containing the sense and antisense strand sequence of the siRNA is possible under the control of suitable promoters for the expression thereof in the cell of interest.

The specific sequence utilized in design of the interfering RNA for use according to the present invention may be any contiguous sequence of nucleotides contained within the expressed PRDX2 and/or PRDX3 gene message. Programs and algorithms, known in the art, may be used to select appropriate target sequences. In addition, optimal sequences may be selected utilizing programs designed to predict the

Messenger RNA (mRNA) is generally thought of as a linear molecule which contains the information for directing protein synthesis within the sequence of ribonucleotides, however studies have revealed a number of secondary and tertiary structures that exist in most mRNAs. Secondary structure elements in RNA are formed largely by Watson-Crick type interactions between different regions of the same RNA molecule. Important secondary structural elements include intramolecular double stranded regions, hairpin loops, bulges in duplex RNA and internal loops. Tertiary structural elements are formed when secondary structural elements come in contact with each other or with single stranded regions to produce a more complex three dimensional structure. A number of researchers have measured the binding energies of a large number of RNA duplex structures and have derived a set of rules which can be used to predict the secondary structure of RNA (see, e.g., Jaeger et al, Proc. Natl. Acad. Sci. USA 86: 7706, 1989; and Turner et al, Annu. Rev. Biophys. Biophys.Chem. 17:167, 1988). The rules are useful in identification of RNA structural elements and, in particular, for identifying single stranded RNA regions which may represent preferred segments of the mRNA to target for silencing RNAi, ribozyme or antisense technologies. Accordingly, preferred segments of the mRNA target can be identified for design of the RNAi mediating dsRNA oligonucleotides as well as for design of appropriate ribozyme and hammerhead ribozyme compositions of the invention.

PRDX2 specific siRNAs or shRNA include, but are not limited to, the siRNA which binds to the positions 712 to 730 of the PRDX2 cDNA of the sequence identified as SEQ ID NO:1 (specific siRNA sequence of the 5'-3' strand is shown in SEQ ID NO: 22), the siRNA which binds to the positions 748 to 766 of the PRDX2 cDNA of the sequence identified as SEQ ID NO:1, the siRNA specific for PRDX2 commercially available: human shRNA from Origene (TG310194), human siRNA from Origene.
(SR304771), the shRNA from GeneCopoeia (HSH018002), the shRNA from Santa Cruz Biotechnology (sc-106456-V), etc.

PRDX3 specific siRNAs or shRNA include, but are not limited to, the siRNA which binds to positions 741 to 765 of the PRDX3 cDNA of the sequence identified as SEQ ID NO:7 (specific siRNA sequence of the 5’-3’ strand is shown in SEQ ID NO: 23), the siRNA shown in US2003228294, the siRNA specific for PRDX3 commercially available the siRNA from Sigma-Aldrich (SASI_Hs01_00141994), the human shRNA from Sigma-Aldrich (SHCLNG-NM_006793), human siRNA from OriGene (SR307465), from GeneCopoeia (HSH001068), from Santa Cruz Biotechnology (sc-40833), etc.

(ii) A polynucleotide encoding an interfering RNA specific for PRDX2 or PRDX3

Vectors suitable for expressing siRNA are those in which the two DNA regions encoding the two strands of siRNA are arranged in tandem in one and the same DNA strand separated by a spacer region which, upon transcription, forms a loop and wherein a single promoter directs the transcription of the DNA molecule giving rise to shRNA.

Alternatively, the use of vectors in which each of the strands forming the siRNA is formed from the transcription of a different transcriptional unit is possible. These vectors are in turn divided into divergent and convergent transcription vectors. In divergent transcription vectors, the transcriptional units encoding each of the DNA strands forming the siRNA are located in tandem in a vector such that the transcription of each DNA strand depends on its own promoter which may be the same or different (Wang, J. et al., 2003, Proc. Natl. Acad. Sci. USA., 100:5103-5106 and Lee, N.S., et al, 2002, Nat. Biotechnol., 20:500-505). In convergent transcription vectors, the DNA regions giving rise to the siRNA form the sense and antisense strands of a DNA region which are flanked by two reverse promoters. After the transcription of the sense and antisense RNA strands, the latter will form the hybrid for forming a functional siRNA. Vectors with reverse promoter systems in which 2 U6 promoters (Tran, N. et al., 2003, BMC Biotechnol., 3:21), a mouse U6 promoter and a human H1 promoter (Zheng, L., et al, 2004, Proc. Natl. Acad. Sci. USA., 135-140 and WO 2005026322) and a human U6
promoter and a mouse HI promoter (Kaykas, A. and Moon, R., 2004, BMC Cell Biol, 5:16) are used have been described.

Promoters suitable for use thereof in the expression of siRNA from convergent or divergent expression vectors include any promoter or pair of promoters compatible with the cells in which the siRNA is to be expressed. Thus, promoters suitable for the present invention include but are not necessarily limited to constitutive promoters such as those derived from the genomes of eukaryotic viruses such as the polyoma virus, adenovirus, SV40, CMV, avian sarcoma virus, hepatitis B virus, the metallothionein gene promoter, the thymidine kinase gene promoter of the herpes simplex virus, retrovirus LTR regions, the immunoglobulin gene promoter, the actin gene promoter, the EF-1 alpha gene promoter as well as inducible promoters in which the protein expression depends on the addition of a molecule or an exogenous signal such as the tetracycline system, the NFkappaB/UV light system, the Cre/Lox system and the heat shock gene promoter, the regulatable RNA polymerase II promoters described in WO/2006/135436 as well as specific tissue promoters (for example, the PSA promoter described in WO2006012221). In a preferred embodiment, the promoters are RNA polymerase III promoters which act constitutively. The RNA polymerase III promoters are found in a limited number of genes such as 5S RNA, tRNA, 7SL RNA and U6 snRNA. Unlike other RNA polymerase III promoters, type III promoters do not require any intragenic sequence but rather need sequences in 5’ direction comprising a TATA box in positions -34 and -24, a proximal sequence element or PSE between -66 and -47 and, in some cases, a distal sequence element or DSE between positions -265 and -149. In a preferred embodiment, the type III RNA polymerase III promoters are the human or murine HI and U6 gene promoters. In a yet more preferred embodiment, the promoters are 2 human or murine U6 promoters, a mouse U6 promoter and a human HI promoter or a human U6 promoter and a mouse HI promoter. In the context of the present invention, the U6 promoter is especially suitable and therefore it is especially preferred to specifically express the siRNA or shRNA of interest in breast tumors.

The siRNA can be generated intracellularly from the so called shRNA (short hairpin RNA) characterized in that the antiparallel strands forming the siRNA are connected by a loop or hairpin region. The shRNAs can be encoded by plasmids or viruses, particularly retroviruses, and are under the control of a promoter. Promoters
suitable for expressing shRNA are those indicated in the paragraph above for expressing siRNA.

Vectors suitable for expressing siRNA and shRNA include prokaryotic expression vectors such as pUC18, pUC19, Bluescript and the derivatives thereof, mpl8, mpl9, pBR322, pMB9, CoIE1, pCRI, RP4, phages and shuttle vectors such as pSA3 and pAT28, yeast expression vectors such as 2-micron plasmid type vectors, integration plasmids, YEP vectors, centromeric plasmids and the like, insect cell expression vectors such as pAC series vectors and pVL series vectors, plant expression vectors such as pIBI, pEarleyGate, pAVA, pCAMBIA, pGSA, pGWB, pMDC, pMY, pORE series vectors and the like and viral vector-based (adenovirus, viruses associated with adenoviruses as well as retroviruses and particularly lentiviruses) higher eukaryotic cell expression vectors or non-viral vectors such as pcDNA3, pHCMV/Zeo, pCR3.1, pEFl/His, pIND/GS, pRC/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1, pZeoSV2, pCI, psiSTRIKE, pSVL and pKSV-10, pBPV-1, pML2d and pTDT1.

The siRNA and shRNA of the invention can be obtained using a series of techniques known by the person skilled in the art. The region of the nucleotide sequence taken as a basis for designing the siRNA is not limiting and it may contain a region of the coding sequence (between the start codon and the end codon) or it may alternatively contain sequences of the non-translated 5' or 3' region preferably between 25 and 50 nucleotides long and in any position in 3' direction position with respect to the start codon. One way of designing an siRNA involves the identification of the AA(N19)TT motifs wherein N can be any nucleotide in the PRDX2 and/or PRDX3 gene sequence, and the selection of those having a high G/C content. If said motif is not found, it is possible to identify the NA(N21) motif wherein N can be any nucleotide.

(iii) An antisense oligonucleotide specific for PRDX2 or PRDX3

An additional aspect of the invention relates to the use of isolated "antisense" nucleic acids to inhibit expression, for example, for inhibiting transcription and/or translation of a nucleic acid which encodes PRDX2 and/or PRDX3 the activity of which is to be inhibited. The antisense nucleic acids can be bound to the target potential of the drug by means of conventional base complementarity or, for example, in the case of
biding to double stranded DNA through specific interaction in the large groove of the
double helix. Generally, these methods refer to a range of techniques generally used in
the art and they include any method which is based on the specific binding to
oligonucleotide sequences.

An antisense construct of the present invention can be distributed, for example,
as an expression plasmid which, when is transcribed in cell, produces RNA
complementary to at least one unique part of the cellular mRNA encoding PRDX2
and/or PRDX3. Alternatively, the antisense construct is a oligonucleotide probe
generated \textit{ex vivo} which, when introduced into the cell, produces inhibition of gene
expression hybridizing with the mRNA and/or gene sequences of a target nucleic acid.

Such oligonucleotide probes are preferably modified oligonucleotides which are
resistant to endogenous nuclease, for example, exonucleases and/or endonucleases and
are therefore stable \textit{in vivo}. Examples of nucleic acids molecules for use thereof as
antisense oligonucleotides are DNA analogs of phosphoramidate, phosphothionate and
methylphosphonate (see also US patent Nos. 5176996; 5264564; and 5256775). Additionally, the general approximations for constructing oligomers useful in the
antisense therapy have been reviewed, for example, in Van der Krol \textit{et al.},

With respect to the antisense oligonucleotide, the oligodeoxyribonucleotide
regions derived from the starting site of the translation, for example, between -10 and
+10 of the target gene are preferred. The antisense approximations involve the
oligonucleotide design (either DNA or RNA) that are complementary to the mRNA
encoding the target polypeptide. The antisense oligonucleotide will be bound to the
transcribed mRNA and translation will be prevented.

The oligonucleotides which are complementary to the 5' end of the mRNA, for
example the non translated 5' sequence up to and including the start codon AUG must
function in the most efficient manner to inhibit translation. Nevertheless, it has been
shown recently that the sequences complementary to the non translated 3' sequences of
the mRNA are also efficient for inhibiting mRNA translation (Wagner, Nature 372:
333, 1994). Therefore, complementary oligonucleotides could be used at the non
translated 5' or 3' regions, non coding regions of a gene in an antisense approximation
to inhibit the translation of that mRNA. The oligonucleotides complementary to the non
translated 5' region of the mRNA must include the complement of the start codon AUG. The oligonucleotides complementary to the coding region of the mRNA are less efficient translation inhibitors but they could also be used according to the invention. If they are designed to hybridize with the 5' region, 3' region or the coding region of the mRNA, the antisense nucleic acids must have at least six nucleotides long and preferably have less than approximately 100 and more preferably less than approximately 50, 25, 17 or 10 nucleotides long.

 Preferably, *in vitro* studies are performed first to quantify the capacity of the antisense oligonucleotides for inhibiting gene expression. Preferably these studies use controls which distinguish between antisense gene inhibition and non specific biological effects of the oligonucleotides. Also preferably these studies compared the levels of target RNA or protein with that of an internal control of RNA or protein. The results obtained using the antisense oligonucleotides can be compared with those obtained using a control oligonucleotide. Preferably the control oligonucleotide is approximately of the same length as the oligonucleotide to be assayed and that the oligonucleotide sequence does not differ from the antisense sequence more than it is deemed necessary to prevent the specific hybridization to the target sequence.

The antisense oligonucleotide can be a single or double stranded DNA or RNA or chimeric mixtures or derivatives or modified versions thereof. The oligonucleotide can be modified in the base group, the sugar group or the phosphate backbone, for example, to improve the stability of the molecule, its hybridization capacity etc. The oligonucleotide may include other bound groups, such as peptides (for example, for directing them to the receptors of the host cells) or agents for facilitating transport through the cell membrane (see, for example, Letsinger *et al.*, Proc. Natl. Acad. Sci. U.S.A. 86: 6553-6556, 1989; Lemaitre *et al*, Proc. Natl. Acad. Sci. 84: 648-652, 1987; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, for example, PCT Publication No. WO 89/10134), intercalating agents (see, for example, Zon, Pharm. Res. 5: 539-549, 1988). For this purpose, the oligonucleotide can be conjugated to another molecule, for example, a peptide, a transporting agent, hybridization triggered cleaving agent, etc.

The antisense oligonucleotides may comprise at least one group of modified base. The antisense oligonucleotide may also comprise at least a modified sugar group
selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose. The antisense oligonucleotide may also contain a backbone similar to a neutral peptide. Such molecules are known as peptide nucleic acid (PNA) oligomers and are described, for example, in Perry-O'Keefe et al., Proc. Natl. Acad. Sci. U.S.A. 93: 14670, 1996, and in Eglom et al., Nature 365: 566, 1993. In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone. In yet another embodiment, the antisense oligonucleotide is an alpha-anomeric oligonucleotide.

While antisense oligonucleotides complementary to the coding region of the target mRNA sequence can be used, those complementary to the transcribed nontranslated region can also be used.

In some cases, it may be difficult to reach the sufficient intracellular concentrations of the antisense to suppress the endogenous mRNA translation. Therefore, a preferred approximation uses a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter.

Alternatively, the target gene expression can be reduced by directing deoxyribonucleotide sequences complementary to the gene regulating region (i.e., the promoter and/or enhancers) to form triple helix structures preventing gene transcription in the target cells in the body (see in general, Helene, Anticancer Drug Des. 6(6): 569-84, 1991). In certain embodiments, the antisense oligonucleotides are antisense morpholines.

(iv) A DNA Enzyme specific for PRDX2 or PRDX3

On the other hand, the invention also contemplates the use of DNA enzymes to inhibit the expression of the PRDX2 and/or PRDX3 genes of the invention. DNA enzymes incorporate some of the mechanistic features of both antisense and ribozyme technologies. DNA enzymes are designed such that they recognize a particular target nucleic acid sequence similar to the antisense oligonucleotide, nevertheless like the ribozyme they are catalytic and specifically cleave the target nucleic acid.
(v) A ribozyme specific for PRDX2 or PRDX3

Ribozyme molecules designed for catalytically cleaving transcription products of a target mRNA to prevent the translation of the mRNA which encodes PRDX2 and/or PRDX3 the activity of which is to be inhibited, can also be used. Ribozymes are enzymatic RNA molecules capable of catalyzing specific RNA cleaving. (For a review, see, Rossi, Current Biology 4: 469-471, 1994). The mechanism of ribozyme action involves a specific hybridization of a ribozyme molecule sequence to a complementary target RNA followed by an endonucleolytic cleavage event. The composition of the ribozyme molecules preferably includes one or more sequences complementary to the target mRNA and the well known sequence responsible for cleaving the mRNA or a functionally equivalent sequence (see, for example, US patent No. 5093246).

The ribozymes used in the present invention include hammer-head ribozymes, endoribonuclease RNA (hereinafter "Cech type ribozymes") (Zaug et al., Science 224:574-578, 1984).

The ribozymes can be formed by modified oligonucleotides (for example to improve the stability, targeting, etc.) and they should be distributed to cells expressing the target gene in vivo. A preferred distribution method involves using a DNA construct which "encodes" the ribozyme under the control of a strong constitutive pol III or pol II promoter such that the transfected cells will produce sufficient amounts of the ribozyme to destroy the endogenous target messengers and to inhibit translation. Since the ribozymes are catalytic, unlike other antisense molecules, a low intracellular concentration is required for its efficiency.

(vi) An inhibitory antibody specific for PRDX2 or PRDX3

In the context of the present invention, "inhibitory antibody" is understood as any antibody capable of binding specifically to the PRDX2 and/or PRDX3 protein and inhibiting one or more of the functions of said protein, preferably those related to transcription. The antibodies can be prepared using any of the methods which are known by the person skilled in the art, some of which have been mentioned above. Thus, the polyclonal antibodies are prepared by means of immunizing an animal with
the protein to be inhibited. The monoclonal antibodies are prepared using the method described by Kohler, Milstein et al. (Nature, 1975, 256: 495). In the context of the present invention, suitable antibodies include intact antibodies comprising a variable antigen binding region and a constant region, "Fab", "F(ab')2" and "Fab"', Fv, scFv fragments, diabodies and bispecific antibodies. Once antibodies with PRDX2 and/or PRDX3 protein binding capacity are identified, those capable of inhibiting the activity of this protein will be selected using an inhibitory agent identification assay.

There are commercially available antibodies against PRDX2, which can be used in the context of the present invention, for example, the SIGMA ALDRICH anti-PRDX2 antibodies SAB1404456 (human monoclonal antibody produced in mouse clone S2), GW21018 (human antibody produced in chicken, affinity isolated antibody) WH0007001M1 (human monoclonal antibody produced in mouse clone 4E10-2D2), SAB2500777 (human antibody produced in goat, affinity isolated antibody), SAB1406520 (human antibody produced in mouse, purified immunoglobulin), SAB2101878 (human antibody produced in rabbit, affinity isolated antibody) and R8656 (human, mouse, rat C-terminal antibody produced in rabbit). There are also commercially available antibodies against PRDX3, which can be used in the context of the present invention, for example, from SIGMA ALDRICH, the anti-PRDX3 antibodies AV52341 (human antibody produced in rabbit, affinity isolated), WH0010935M1 (human monoclonal antibody produced in mouse clone 1E3-B2), GW22208F (human Anti-PRDX3 antibody produced in chicken, affinity isolated), SAB1407075 (human antibody produced in mouse, purified immunoglobulin), P1247 (human antibody produced in rabbit IgG fraction of antiserum) and A7674 (human, mouse, rat monoclonal antibody, clone AOP-38, produced in mouse).

(vii) An inhibitory peptide specific for PRDX2 or PRDX3

As used herein, the term "inhibitory peptide" refers to those peptides capable of binding to the PRDX2 and/or PRDX3 protein and inhibiting its activity as has been explained above, i.e., preventing the PRDX2 and/or PRDX3 from being able to activate gene transcription.
Other inhibitory compounds of PRDX2 and/or PRDX3 suitable for use in the present invention include, but are not limited to:

- Conoidin A, which is known to be covalent inhibitor of PRDX2 of *Toxoplasma gondii* (Haraldsen, J.D. *et al.* (2009) Organic and Biomolecular Chemistry, 7 (15): 3040-3048);  
- Any compound which blocks the oligomerization of PRDX2, which inhibits the PRDX2 activity.


- Any compound which phosphorylates a conserved threonin in PRDX2 or in PRDX3 and reduces its activity (Chang, T.S. *et al.* (2002) J. Biol. Chem. 277: 25370-25376)

- Oxidation activators, which avoid the regeneration of PRDX2 or PRDX3, e.g. which decreases the thioredoxin reductase activity.

- An inhibitor compound of the acetylation of PRDX2 or PRDX3, which increases the overoxidation resistance. Acetylation of PRDX2 protein increases its activity in reducing H₂O₂ and increases their relative resistance to superoxidation and to transition to high-molecular mass complexes (R. B. Parmigiani *et al.* PNAS 2008; vol. 105:9633-9638).  

The different PRDX2 and/or PRDX3 inhibitory agents are typically administered in combination with a pharmaceutically acceptable carrier.

The term "carrier" refers to a diluent or an excipient whereby the active ingredient is administered. Such pharmaceutical carriers can be sterile liquids such as water and oil, including those of a petroleum, animal, plant or synthetic origin such peanut oil, soy oil, mineral oil, sesame oil and the like. Water or aqueous saline solutions and aqueous dextrose and glycerol solutions, particularly for injectable
solutions, are preferably used as carriers. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin, 1995. Preferably, the carriers of the invention are approved by the state or federal government regulatory agency or are listed in the United States Pharmacopeia or other pharmacopeia generally recognized for use thereof in animals and more particularly in human beings.

The carriers and auxiliary substances necessary for manufacturing the desired pharmaceutical dosage form of the pharmaceutical composition of the invention will depend, among other factors, on the pharmaceutical dosage form chosen. Said pharmaceutical dosage forms of the pharmaceutical composition will be manufactured according to the conventional methods known by the person skilled in the art. A review of the different methods for administering active ingredients, excipients to be used and processes for producing them can be found in "Tratado de Farmacia Galenica", C. Fauli i Trillo, Luzan 5, S.A. 1993 Edition. Examples of pharmaceutical compositions include any solid composition (tablets, pills, capsules, granules, etc.) or liquid composition (solutions, suspensions or emulsions) for oral, topical or parenteral administration. Furthermore, the pharmaceutical composition may contain, as deemed necessary, stabilizers, suspensions, preservatives, surfactants and the like.

For use in medicine, the PRDX2 and/or PRDX3 inhibitory agents can be found in the form of a prodrug, salt, solvate or clathrate, either isolated or in combination with additional active agents and can be formulated together with a pharmaceutically acceptable excipient. Excipients preferred for use thereof in the present invention include sugars, starches, celluloses, rubbers and proteins. In a particular embodiment, the pharmaceutical composition of the invention will be formulated in a solid pharmaceutical dosage form (for example tablets, capsules, pills, granules, suppositories, sterile crystal or amorphous solids that can be reconstituted to provide liquid forms etc.), liquid pharmaceutical dosage form (for example solutions, suspensions, emulsions, elixirs, lotions, ointments etc.) or semisolid pharmaceutical dosage form (gels, ointments, creams and the like). The pharmaceutical compositions of the invention can be administered by any route, including but not limited to the oral route, intravenous route, intramuscular route, intraarterial route, intramedulary route, intrathecal route, intraventricular route, transdermal route, subcutaneous route, intraperitoneal route, intranasal route, enteric route, topical route, sublingual route or
rectal route. A review of the different ways for administering active ingredients, of the excipients to be used and of the manufacturing processes thereof can be found in Tratado de Farmacia Galenica, C. Fauli i Trillo, Luzan 5, S.A., 1993 Edition and in Remington's Pharmaceutical Sciences (A.R. Gennaro, Ed.), 20th edition, Williams & Wilkins PA, USA (2000). Examples of pharmaceutically acceptable carriers are known in the state of art and include phosphate buffered saline solutions, water, emulsions such as oil/water emulsions, different types of wetting agents, sterile solutions, etc. The compositions comprising said carriers can be formulated by conventional processes known in the state of the art.

In the event that nucleic acids (siRNA or polynucleotides encoding siRNA or shRNA) are administered, the invention contemplates pharmaceutical compositions particularly prepared for administering said nucleic acids. The pharmaceutical compositions can comprise said naked nucleic acids, i.e., in the absence of compounds protecting the nucleic acids from degradation by the nucleases of the body, which entails the advantage that the toxicity associated with the reagents used for transfection is eliminated. Administration routes suitable for naked compounds include the intravascular route, intratumor route, intracranial route, intraperitoneal route, intrasplenic route, intramuscular route, subretinal route, subcutaneous route, mucosal route, topical route and oral route (Templeton, 2002, DNA Cell Biol, 21:857-867).

Alternatively, the nucleic acids can be administered forming part of liposomes conjugated to cholesterol or conjugated to compounds capable of promoting the translocation through cell membranes such as the Tat peptide derived from the HIV-1 TAT protein, the third helix of the homeodomain of the D. melanogaster antennapedia protein, the herpes simplex virus VP22 protein, arginine oligomers and peptides as described in WO07069090 (Lindgren, A. et al, 2000, Trends Pharmaco. Sci, 21:99-103, Schwarze, S.R. et al., 2000, Trends Pharmaco. Sci., 21:45-48, Lundberg, M et al, 2003, Mol Therapy 8:143-150 and Snyder, E.L. and Dowdy, S.F., 2004, Pharm. Res. 21:389-393). Alternatively, the polynucleotide can be administered forming part of a plasmid vector or viral vector, preferably adenovirus-based vectors, in adeno-associated viruses or in retroviruses such as viruses based on murine leukemia virus (MLV) or on lentivirus (HIV, FIV, EIAV).
The PRDX2 and/or PRDX3 inhibitory agents or the pharmaceutical compositions containing them can be administered at a dose of less than 10 mg per kilogram of body weight, preferably less than 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of body weight. The unit dose can be administered by injection, inhalation or topical administration.

The dose depends on the severity and the response of the condition to be treated and it may vary between several days and months or until the condition subsides. The optimal dosage can be determined by periodically measuring the concentrations of the agent in the body of the patient. The optimal dose can be determined from the EC50 values obtained by means of previous in vitro or in vivo assays in animal models. The unit dose can be administered once a day or less than once a day, preferably less than once every 2, 4, 8 or 30 days. Alternatively, it is possible to administer a starting dose followed by one or several maintenance doses, generally of a lesser amount than the starting dose. The maintenance regimen may involve treating the patient with a dose ranging between 0.01 μg and 1.4 mg/kg of body weight per day, for example 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of body weight per day. The maintenance doses are preferably administered at the most once every 5, 10 or 30 days. The treatment must be continued for a time that will vary according to the type of disorder the patient suffers, the severity thereof and the condition of the patient. After treatment, the progress of the patient must be monitored to determine if the dose should be increased in the event that the disease does not respond to the treatment or the dose is reduced if an improvement of the disease is observed or if unwanted side effects are observed.

The efficacy of PRDX2 and/or PRDX3 inhibitory agents on the metastasis of breast cancer in lungs can be assessed using Clinical Endpoints available to the skilled person such as one or more of the following: (1) a reduction in the circulating concentrations of VEGF relative to pretreatment baseline circulating concentrations of VEGF; (2) antiangiogenic or anti-inflammatory activity as documented by changes in circulating concentrations of either angiogenic mediators other than VEGF (e.g., VEGF165b, VEGFR, VEGF-C, VEGF-D, PIGF), inflammatory cytokines (e.g., IL-6, IL-8) or both relative to pretreatment baseline circulating concentrations of angiogenic mediators and inflammatory cytokines; (3) a reduction in tumor perfusion as assessed by DCE-MRI; (4) a change in tumor metabolism as assessed by changes in 18F-2-
fluorodeoxyglucose positron emission tomography (FDG-PET) standardized uptake value (SUV) in a target tumor lesion; (5) a reduction in tumor size relative to pretreatment tumor size; (6) an increase in progression-free survival (PFS); and (7) a reduction in tumor markers relative to pretreatment baseline tumor markers.

5

ROS-GENERATING AGENTS FOR THE TREATMENT AND/OR PREVENTION OF LUNG METASTASIS IN BREAST CANCER

The authors of the present invention have shown that a metastatic variant of MDA-MB-435 breast cancer cells that specifically metastasize to lungs (435-L3) transduced with shRNAs to specifically silence PRDX2 or PRDX3 showed significantly increased intracellular ROS levels when cells were exposed to ¾ O₂ than the parental and scrambled transfected cells. Thus, a ROS generating agent may be used in the treatment and/or prevention of lung metastasis in breast cancer.

Thus, in another aspect the invention relates to a ROS generating agent for use in the treatment and/or prevention of lung metastasis in breast cancer. Alternatively, the invention relates to the use of a PRDX2 and/or PRDX3 inhibitor in the manufacture of a medicament for the treatment and/or prevention of lung metastasis in breast cancer. Alternatively, the invention relates to a method for the treatment and/or prevention of lung metastasis in a subject suffering from breast cancer comprising the administration of a PRDX2 and/or a PRDX3 inhibitor to said subject.

A "ROS generating agent", as used in the present invention, refers to any molecule capable of increasing the production of intracellular reactive oxygen species (ROS) (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical), either by increasing the expression or activity of enzymes which generate reactive oxygen species (e.g., NADPH oxidase and dual oxidases), or by depleting or inactivating protective reducing metabolites or enzymes (e.g., superoxide dismutase (SOD) isoenzymes (CuZnSOD, MnSOD and ecSOD), glutathione, glutathione peroxidase, catalase, nicotinamide adenine dinucleotide phosphate (NADPH), thioredoxin, thioredoxin reductase).

Methods suitable for identifying agents which generate ROS have previously been described (Vanden Hoek TL et al. J Mol Cell Cardiol 1997; 29:2571-83) and
comprise, for example, a method wherein a ROS scavenger is used (i.e. 2,7-dichlorofluorescein diacetate (DCFH$_2$-DA, Molecular Probes) or dihydroethidium (DHE, Sigma-Aldrich)). These ROS scavenger compounds react specifically with H$_2$O$_2$ produced by the ROS-generating agent to induce the highly fluorescent DCF or ethidium (Et). Cells are then incubated with 7 µM DCFH2-DA in HBSS (Invitrogen) for 40 min or with 2 µM DHE in HBSS for 30 min at 37°C, according to the manufacturer's instructions, with or without H$_2$O$_2$ (0.4-4 mM). Fluorescence is finally measured in a fluorescence microplate reader at 485-520 nm or 530-562 nm (DCF and Et, respectively) to assess the fluorescence intensity relative to protein concentration.

In a particular embodiment, the cancer to be treated has increased levels of PRDX2 and/or PRDX3. The ROS generating agent is preferably used in the treatment and/or prevention of lung metastasis in breast cancer of a subject, wherein the expression levels of PRDX2 and/or PRDX3 are increased in a cancer subject, either in a primary breast cancer sample or in a lung metastasis sample of said subject.

Any pro-oxidant agent capable of inducing oxidative stress through creating reactive oxygen species may be used in the context of the present invention as ROS generating agents. Illustrative examples of pro-oxidant agents which may be used are, but not limited to:

- Acetaminophen is considered a safe analgesic and antipyretic when dosed properly. Arnaiz et al. (Free Radic. Biol. Med. 1995; 19: 303-310) found a decrease in catalase and glutathione peroxidase activities along with an increase in H$_2$O$_2$ concentration in the livers of acetaminophen-treated mice.

- Elesclomol [N-malonyl-bis (N’-methyl-NT-thiobenzoyl hydrazide)] (codenamed STA-4783) is a first-in-class investigational drug, believed to exert anticancer activity through the elevation of reactive oxygen species (ROS) levels leading to the activation of the mitochondrial apoptosis pathway (Jessica R. Kirshner et al. Mol. Cancer Ther. 2008 7: 2319).

- 2,2'-dithio- bis-ethane sulfonate compounds (and possibly mesna) act to enhance oxidative stress or compromise the anti-oxidative response of cancerous tumor cells, or both, which may thereby enhance their oxidative biological and physiological state. This may serve to subsequently increase the amount of oxidative damage (e.g., as mediated by reactive oxygen species (ROS), reactive
nitrogen species (RNS), or other mechanisms) in tumor cells exposed to chemotherapy, thereby enhancing cytotoxicity/apoptosis of chemotherapy agents.

- Vitamin C has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide (Duarte TL et al. (2005). Free Radic. Res. 39 (7): 671-86); however, it can also reduce metal ions which leads to the generation of free radicals through the Fenton reaction.

- Glutamate increases the formation of reactive oxygen species (ROS) by the cerebral cortex and cause sustained cerebral vascular endothelial injury (Helena Parfenova et al. Am J Physiol Cell Physiol May 2006 vol. 290 no. 5).

- Sulindac sulfide is the active metabolite of sulindac and induce both oxidative stress and the expression of COX-2, particularly in dying cells (Yu Sun et al. Carcinogenesis (2009) 30 (1): 93-100).

- Buthionine-sulfoximine, nitrofurantoin and phorone are known to cause liver oxidative stress (increasing ROS levels) through different mechanisms (Tacchini et al. Biochemical Pharmacology 2002; Volume 63, Issue 2:139-148).

- Transition metals, as used in the present invention, comprise the metals as defined by the IUPAC definition, which states that a transition metal is "an element whose atom has an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell". Group 12 elements are not transition metals in this definition. However, other authors describe a "transition metal" as any element in the d-block of the periodic table, which includes groups 3 to 12 on the periodic table. All elements in the d-block are metals. In actual practice, the f-block is also included in the form of the lanthanide and actinide series. Examples of transition metals are Chromium, Molybdenum, Manganese, Techtenium, Rhenium, Bohrium, Iron, Ruthenium, Osmium, Cobalt, Rhodium, Iridium, Meitnerium, Nickel, Palladium, Platinum, Copper, Silver, Gold, Roentgenium, etc. Transition metals are known to generate ROS (Vidrio et al. Atmospheric Environment, 42 (18): 4369-4379).

- Uric acid can also mediate the production of active oxygen species and, thus, act as a pro-oxidant. Some researchers think urate-induced oxidative stress is causative in stroke (Bos et al. 2006. Stroke 37 (6): 1503).
Homocysteine is a powerful reducing agent and, like most such agents, can induce oxidative stress through reducing molecular oxygen to its radical forms. It is known that homocysteine in a dose- and time-dependent manner induces ROS accumulation. It has also been described that homocysteine activates PAR-4, which induces production of reactive oxygen species by increasing NADPH oxidase and decreasing thioredoxin expression. (Tyagi et al. Am. J Physiology 2005 vol. 289 no. 6; Signorello et al. Annals of the New York Academy of Sciences 2002; Vol 973: 546-549)

Anthracyclines are a class of drugs used in cancer chemotherapy derived from Streptomyces bacteria, which may promote the formation of ROS through redox cycling of their aglycones as well as their anthracycline-iron complexes (Simunek T et al. Pharmacol Rep. 2009;61(1): 154-71). Illustrative examples of anthracyclines which may be used in the present invention as ROS generating agents, are Daunorubicin (Daunomycin), Daunorubicin (liposomal), Doxorubicin (Adriamycin), Doxorubicin (liposomal), Epirubicin, Idarubicin, Valrubicin, Mitoxantrone, etc.

Bleomycin is a glycopeptide antibiotic produced by the bacterium Streptomyces verticillus. Bleomycin is known to cause an increase of reactive oxygen species (ROS) resulting in oxidative stress, mitochondrial leakage, and apoptosis (Shulamit et al. Am. J Physiology 2006; vol. 290 no. 4).

Cisplatin is a chemotherapy drug, which is used to treat various types of cancers. It has been described that cisplatin promote ROS production, which in turn contributes to Fas receptor aggregation and cell death (Huang et al. Oncogene (2003) 22, 8168-8177).

Histone deacetylase inhibitors: It has been found that histone deacetylase inhibitors cause an accumulation of reactive oxygen species in transformed cells (Ungerstedt et al. PNAS, 102(3):673-78). Examples of histone deacetylase inhibitors which can be used in the context of the present invention, includes, but are not limited to, the suberoylanilide hydroxamic acid (SAHA), MS-275, valproic acid (VPA), etc.
ERp57/GRP58 INHIBITORS FOR THE TREATMENT AND/OR PREVENTION OF METASTASIS IN BREAST CANCER

The authors of the present invention have shown that overexpression of ERp57/GRP58 in breast cancer cells results in an improved growth of the cancer cells in bones. Moreover, stable knockdown of ERp57/GRP58 in in bone metastasis resulted in a proliferation decrease in bone metastasis of breast cancer cells as well as in induced. Thus, an inhibitor of the expression of the ERp57/GRP58 gene or of the protein encoded by said gene may be used in the treatment and/or prevention of metastasis in breast cancer.

Thus, in another aspect the invention relates to an ERp57/GRP58 inhibitor for use in the treatment and/or prevention of lung metastasis in breast cancer. Alternatively, the invention relates to the use of an ERp57/GRP58 inhibitor in the manufacture of a medicament for the treatment and/or prevention of metastasis in breast cancer. Alternatively, the invention relates to a method for the treatment and/or prevention of metastasis in a subject suffering from breast cancer comprising the administration of an ERp57/GRP58 inhibitor to said subject.

A "ERp57/GRP58 inhibitor", as used in the present invention, refers to any molecule capable of totally or partially inhibiting the expression of ERp57/GRP58, reducing therefore the activity of ERp57/GRP58. The inhibition can be carried out either by preventing the generation of the expression product of said gene/s (by interrupting the transcription of the ERp57/GRP58 gene and/or blocking the translation the mRNA resulting from the ERp57/GRP58 gene expression) or by directly inhibiting or reducing ERp57/GRP58 activity.

The term "ERp57/GRP58" gene, also known as protein disulfide isomerase family A, member 3, endoplasmic reticulum resident protein 60, 58 kDa glucose-regulated protein, ERp57, 58 kDa microsomal protein, ERp60, ER protein 57, ERp61, ER protein 60, GRP57, HsT17083, ER60, P58, endoplasmic reticulum P58, PI-PLC, phospholipase C-alpha, glucose regulated protein, 58kDa, protein disulfide-isomerase A3, protein disulfide isomerase-associated 3, ERp57, disulfide isomerase ER-60, ERp60, Endoplasmic reticulum resident protein 57 and p58, refers to a protein of the endoplasmic reticulum having protein disulfide isomerase activity that interacts with
lectin chaperones calreticulin and calnexin to modulate folding of newly synthesized glycoproteins.

Methods suitable for determining those compounds which are inhibitors of the expression of ERp57/GRP58 have previously been described and comprise both the methods based on determining the levels of the ERp57/GRP58 polypeptides or of mRNA encoding ERp57/GRP58 and those based on determining the ability of the inhibitor compound to inhibit ERp57/GRP58-mediated reduction of insulin as described by Hirano et al. (Eur. J. Biochem., 1995, 234: 336-342). Moreover, inhibitors of ERp57/GRP58 can also be identified based in their ability to suppress metastatic progression in lungs of breast cancer cells lines (e.g. by non-invasive bioluminescence imaging, as shown in Example 5 of the present invention).

In a particular embodiment, the ERp57/GRP58 inhibitory agent suitable for use thereof in the present invention is selected from the group of:

(i) an interfering RNA specific for ERp57/GRP58,
(ii) a polynucleotide encoding an interfering RNA specific for ERp57/GRP58,
(iii) an antisense oligonucleotide specific for ERp57/GRP58,
(iv) an inhibitory antibody specific for ERp57/GRP58,
(v) an inhibitory peptide specific for ERp57/GRP58, and
(vi) a ribozyme or DNA enzyme specific for ERp57/GRP58.

In a preferred embodiment, the siRNA specific for ERp57/GRP58 is directed to positions 2378-2396 of the sequence identified as SEQ ID NO:24. In a particular embodiment, the different inhibitory agents (i) to (vi) may be combined between them for its administration.

In a preferred embodiment, the breast cancer metastasis is bone metastasis.

COMPOSITION COMPRISING PRDX2 AND/OR PRDX3 INHIBITORS AND ROS-GENERATING AGENTS

The authors of the present invention have shown that a metastatic variant of MDA-MB-435 breast cancer cells that specifically metastatize to lungs (435-L3) transduced with shRNAs to specifically silence PRDX2 or PRDX3 showed significantly
higher sensitivity against increased ROS levels than the parental and scrambled transfected cells (Example 3).

Thus, in another aspect, the invention relates to a composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent, hereinafter composition of the invention.

The term "PRDX2 and/or PRDX3 inhibitor" has already been described in detail in relation to the inhibitor of the invention and is equally applicable to the composition of the invention.

In a particular embodiment, the PRDX2 and/or PRDX3 inhibitory agent suitable for use in the present invention is selected from the group of:

(i) an interfering RNA specific for PRDX2 or PRDX3,
(ii) a polynucleotide encoding an interfering RNA specific for PRDX2 or PRDX3,
(iii) an antisense oligonucleotide specific for PRDX2 or PRDX3,
(iv) an inhibitory antibody specific for PRDX2 or PRDX3,
(v) an inhibitory peptide specific for PRDX2 or PRDX3,
(vi) a ribozyme or DNA enzyme specific for PRDX2 or PRDX3.

Specific details of the PRDX2 and/or PRDX3 inhibitors which may be used in the context of the present invention have already been described with respect to the inhibitor of the invention.

The term "ROS-generating agent" has already been described in detail in relation to the ROS-generating agents of the invention and is equally applicable to the composition of the invention.

In a particular embodiment, the ROS-generating agent is selected from the group of acetaminophen, elesclomol, 2,2'-dithio- bis-ethane sulfonate compounds, vitamin C, glutamate, sulindac sulfide, buthionine-sulfoximine, nitrofurantoin, phorone, transition metals, uric acid, homocysteine, anthracycline, bleomycin and cisplatin.

Illustrative examples of "PRDX2 and/or PRDX3 inhibitor" and "ROS-generating agent" have been defined with respect to the inhibitor of the invention and the ROS-generating agents of the invention and are equally applicable to the composition of the invention.

The composition of the invention is typically administered in combination with a pharmaceutically acceptable carrier. The pharmaceutically effective carrier can be solid
or liquid. A solid carrier can include one or more substances that can also act as flavoring agents, lubricants, solubilizers, suspension agents, fillers, sliding agents, compression agents, binders or tablet disintegrating agents; they can also be encapsulating material. In powders, the carrier is a finely divided solid which is mixed with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and is compacted to the desired shape and size. The powders and tablets can contain up to 99% of the active ingredient. The suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidone, low melting point waxes and ion exchange resins.

The invention contemplates different combinations of the two components and different combinations of administration regimes that will be determined by the practitioner according to the circumstances.

In another aspect, the invention relates to a composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent for use as a medicament.

In another aspect, the invention relates to a composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent for use in the treatment and/or prevention of metastasis in a subject suffering from cancer. In a particular embodiment, the cancer is breast cancer and the metastasis is lung metastasis. Alternatively, the invention relates to the use of a composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent in the manufacture of a medicament for the treatment and/or prevention of metastasis in a subject suffering from cancer. Alternatively, the invention relates to a method for the treatment and/or prevention of metastasis in a subject suffering from cancer.

The following examples serve to illustrate the invention and must not be considered as limiting the scope thereof.

**EXAMPLES**

**1. EXPERIMENTAL PROCEDURE**

*Cell lines*
MDA-MB-435 cells (435-P) supplied by Dr Fabra (IDIBELL) in 1992 and their metastatic variants established from primary cultures of lung (435-L), lymph node (435-N) and bone (435-B) metastases, were maintained under standard conditions and have been described in Mendez O et al. Clin Exp Metastasis 2005;22(4):297-307. Although controversial, it has recently been demonstrated that MDA-MB-435 cells are a useful breast cancer model and express both epithelial and melanocytic markers (Chambers AF et al. Cancer Res 2009;69(13):5292-3). In some experiments, breast cancer bone metastatic cell lines MDA-MB-231 and MDA-MB-231-P originally obtained from the European Type Culture Collection (ECACC 92020424) in 2007 were used and were maintained in accordance with ECACC guidelines for less than 4 months before use in these experiments. B02 cell line has been established from bone metastases caused by 231-P. This subclone of 231-P has been selected after six in vivo passages in nude mice using a heart injection model and is characterized by its unique predilection for bone metastasis. The characteristics of luciferase-expressing MDA-B02 cells have been described elsewhere (Peyruchaud O et al. J Biol Chem 2003; 278:45826-32). Cells were cultured under standard conditions.

**Cell viability assay**

The MTT tetrazolium assay was used as described elsewhere (Mosmann T. J Immunol Methods 1983;65:55-63). Briefly, 5 x 10^3 cells/well were incubated for 48 h in 96-well plates. The cells were serum-starved for 24 h and exposed for a further 24 h to 0-4 mM H_2O_2 (Sigma, Saint Louis, MO). Cell viability was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) at a concentration of 5 mg/ml. After 3 h incubation at 37°C, 100 μl of DMSO (Sigma) was added before measuring the optical density at 540 nm. The results were expressed as the percentage of cell viability (absorbance of stimulated cells/absorbance of control cells). For experiments involving cell nutrient deprivation, cells were seeded in complete medium for 24 hours, then cultured for a further 24, 48, 72 or 96 hours in Dulbecco’s Modified Eagle Medium with no serum, glucose or pyruvate (DMEM/Glc-/Pyr-, Invitrogen, San Diego, CA). Cell viability was measured as described above.

**Intracellular ROS measurement**
ROS generation in cells was assessed as described elsewhere (Vanden Hoek TL et al. J Mol Cell Cardiol 1997; 29:2571-83) using two probes: 2,7-dichlorofluorescein diacetate (DCFH$_2$-DA, Molecular Probes) and dihydroethidium (DHE, Sigma-Aldrich). These probes react specifically with H$_2$O$_2$ to induce the highly fluorescent DCF or ethidium (Et). Cells were incubated with 7 µM DCFH$_2$-DA in HBSS (Invitrogen) for 40 min or with 2 µM DHE in HBSS for 30 min at 37°C, according to the manufacturer's instructions, with or without H$_2$O$_2$ (0.4-4 mM). Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, Biogen, Spain) at 485-520 or 530-562 nm (DCF and Et, respectively) to assess the fluorescence intensity relative to protein concentration. All experiments were performed at least in triplicate.

**Cell transfections and constructs**

Retroviral transduction was used to label 435-L$_3$ lung metastatic cells. Vector preparation and packaging of viral particles was performed as described previously (Roman I et al. Biomaterials 2007;28:2718-28). A cell population that uniformly expressed the highest levels of eGFP (435-L$_3$-eGFP-CMV/Luc) was selected by FACS (MoFlo, Cytomation, Dako, Denmark).

**Stable PRDX2 knockdown in 435 lung metastatic cells**

Short hairpin RNA (shRNA) and corresponding scrambled sequences directed at PRDX2 target sites based on the human transcript (GI:33 188450) were designed using the online siRNA Target Designer software (Promega Corporation, Madison, WI). Pairwise oligonucleotides for shRNA-PRDX2, 5'-GAT GAC AGC AAG GAA TAT T-3' (SEQ ID NO:1) (shPRDX2#1, target nucleotide site 712-730) and 5'-GCT GGC TAA CGG AAG TGA-3' (SEQ ID NO:12) (shPRDX2#2, target nucleotide site 748-766), as well as a scrambled (sclb) control sequence (5-GGA AGT CAA CGA GTT AAA T-3') (SEQ ID NO:13) were cloned into the psiSTRIKE puromycin vector containing the U6 promoter (Promega). Plasmids produced by the Qiagen MaxiPrep protocol (Qiagen Inc., Valencia, CA) were tested for purity on a NanoDrop Spectrophotometer (Wilmington, USA). Sequence analysis confirmed the sequence integrity of the PRDX2 shRNA plasmids. Plasmids were transfected into 435-L$_3$ peGFP-CMV/Luc cells using Lipofectamine 2000 reagent. Cells were cultured for 2
weeks in the presence of neomycin G418 (750 µg/mL) to isolate 435-L3/shPRDX2 (shPRDX2) and 435-L3/scbl (scbl) clones. PRDX2 knockdown in transfectants was assessed by real-time RT-PCR and Western blot analysis.

Nine hundred nanograms of total RNA from transfected cells was reverse transcribed with oligo-dT primer and M-MLV reverse transcriptase (Promega Corporation). PCR was performed using SYBR Green I dye in a LightCycler 480 Instrument (Roche Biochemicals, Germany). Experimental protocols for quantitative real-time PCRs were optimized for each primer reaction, as described previously (Mendez O et al. Clin Exp Metastasis 2005;22(4):297-307). The following primers were used: PRDX2, 5'-GTG TCC TTC GCC AGA TCA C-3' (SEQ ID NO: 14) and 5'-ACG TTG GCC TTA ATC GTG T-3' (SEQ ID NO:15); PRDX3, 5'-GTT GTC GCA GTC TCA GTG G-3' (SEQ ID NO:16) and 5'-GAC GCT CAA ATG CTT GAT G-3 (SEQ ID NO:17). Cyclophilin served as a reference for evaluating the expression level of other genes (cyclophilin: 5'-CTT TGA GCT GTT TGC AG-3' (SEQ ID NO: 18), 5'-CAC CAC ATG CTT GCC ATC C-3') (SEQ ID NO: 19).

Overexpression of PRDX2 in MDA-B02 bone metastatic cells.

A cDNA fragment encoding PRDX2 (transcript variant 1, GI:33188450) was isolated by RT-PCR using sense 5'-GAA CGC TAG CGC CAC CAT GCC CTC CGG TAA CGC-3' (SEQ ID NO:20) and antisense 5'-GAA CGA TAT CGC CAG CCT AAT TGT GTT TGG-3' (SEQ ID NO:21) primers. The PCR-amplified fragment was digested with Nhel and EcoRV and cloned into the bidirectional pBI-L vector (Clontech, Hampshire, UK). Stable B02-GFP/tTA cells were then cotransfected with the pBiL/PRDX2 construct and a plasmid conferring puromycin resistance (Promega) so that they would express PRDX2 and luciferase. Clones were selected after growing the cells for 2 weeks in the presence of puromycin (2 µg/mL). A transfectant overexpressing PRDX2 (B02/PRDX2, clone #88) and a control cell line expressing similar levels of luciferase (B02/GFP) were used in the present study. Transfectants were routinely cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Bio-Media, Boussens, France) and 1% (v/v) penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO2 humidified incubator. PRDX2
expression was assessed by Western blotting. Alternatively, luciferase activity was used as a surrogate marker for PRDX2 expression in B02 transfectants.

**In vivo mouse models, bioluminescence and PET imaging**

Six-week-old athymic Nude Balb/c female mice were used with the approval of the animal care committee. For the tumorigenesis experiments cells (10^6 cells in 50 μl serum-free medium) were inoculated i.m.f.p. (into the mammary fat pad) into anesthetized nude mice (Zhang RD et al. Invasion Metastasis 1991;11(4):204-15). Metastasis was induced by injecting 10^6 cells in 150 μl HBSS into the tail vein. In both cases, mice were controlled periodically during the experiment until symptoms of metastasis appeared, and metastasis development was monitored by non-invasive bioluminescence imaging (Peyruchaud O et al. cited ad supra). At the end of the protocols, anesthetized mice were sacrificed by cervical dislocation and organs of interest were collected for further analysis. Bone metastasis experiments were conducted as described previously (Peyruchaud O et al. J Bone Miner Res 2001;16(1):2027-34). Briefly, B02 transfectants overexpressing PRDX2 (B02/PRDX2, clone #88) or B02/GFP control cells (10^5 cells in 100 μl PBS) were inoculated intracardiacally into anesthetized nude mice. Radiographs of anesthetized mice were taken at scheduled intervals with the use of MIN-R2000 film (Kodak, Rochester, NY) using a cabinet x-ray system (MX-20, Faxitron X-Ray Corporation, Wheeling, IL). Osteolytic lesions were identified on radiographs as demarcated radiolucent lesions in the bone and the extent of bone destruction per animal was expressed in square millimeters, as described previously (Peyruchaud O et al. cited ad supra).

In vivo optical imaging of nude mice engrafted with 435 cells was performed as described previously (El Hilali N et al. Clin Cancer Res 2005;11:1253-58). The quantification and analysis of the photons recorded in the images was performed using Wasabi image analysis software (Hamamatsu photonics). The number of photons was expressed as photon counts (PHCs) and data were expressed as the number of PHCs versus the number of grafted cells. For the image quantification, the average number of PHCs/pixel of a selected area of interest was calculated, using Wasabi image analysis software (average PHCs/pixel).
Positron Emission Tomography (PET) were performed with the ClearPET commercially available system with a spatial resolution at the center of 1.3 mm. The phoswich method maintains a spatial resolution of 1.4 mm at 1 cm from the center. Mice were injected with 400 μCi of 2-deoxy-2[¹⁸F]fluoro-D-glucose ([¹⁸F]FDG) or sodium [¹⁸F]fluoride ion (Na[¹⁸F]) via the tail vein. Whole-body scans were acquired 15 min and 1 hour after tail vein injection, with the long axis of the mouse parallel to the long axis of the scanner.

**Protein expression in cells and tissues**

The specific antibodies used were: anti-PRDX1 to -6, anti-PRDX-S03 (LabFrontier, Seoul, Korea), anti-AOPl, anti-a-tubulin, anti-P-actin (Sigma), anti-PCNA (Santa Cruz, Santa Cruz, CA), anti-CD-31/PECAM-1 (BD Pharmingen, San Jose, CA) and anti-caspase-3 (Cell Signaling, Boston, MA). For the immunoblot analysis, cells from exponential cultures were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) and protein concentrations were determined using the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). To detect the overoxidized form of PRDX (PRDX-SO3), cells were stimulated with 3 mM H₂O₂ in complete medium for 24 hours prior to cell lysis. Fifty micrograms of protein were loaded in 12% SDS-PAGE gels and blotted onto PVDF membranes (Immobilon-P, Millipore Corporation, Bedford, MA), blocked with non-fat dried milk (5%) in PBS with 0.01% Tween-20. A semi-quantitative densitometric analysis using Quantity One software (BioRad Laboratories, Hercules, CA, USA) was carried out to assess the band densities.

For the immunofluorescence staining, cells on coverslips were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. They were then permeabilized and blocked with 0.2% Triton X-100 and 20% FBS in PBS for 1 h at room temperature. Fluorescence images obtained with a TCS SL laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) were processed with multicolor image analysis software (Leica). The means of three random areas with at least 20 cells each were scored for each sample.

Immunohistochemistry was carried out on six-micrometer-thick paraffin sections. These sections were treated with specific antibodies and incubated overnight at
4°C. The appropriate secondary biotinylated antibody (Vector Laboratories, Burlingame, CA) was used before hematoxylin (Sigma) counterstaining of slides. Slides were visualized in an Olympus BX60 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and images were taken and analyzed using a digital camera and Spot 4.2 software (Diagnostic Instruments, Inc., Sterling Heights, MI). To quantify the microvessel area, we calculated the ratio of the CD31-stained area to the total area (µm²) in the central part of the corresponding tumor sections (x20). The analysis was performed by light microscopy. Tumor fields were digitalized using a color video camera (Color View, Soft Imaging System & Olympus Cell image analysis software) to generate histological images. An average ratio of 5 tumors per experimental group was determined.

Tissue Microarrays (TMAs) and immunohistochemistry (IHC)

TMAs were prepared from three representative areas of the tumor which were carefully selected from hematoxylin-eosin-stained sections of 104-donor blocks (S.B. and S.H.). Core cylinders of 2 mm diameter were punched from each of them with a skin-biopsy punch and deposited into recipient paraffin blocks using a specific arraying device (Beecher Instruments, Sun Prairie, WI) as described elsewhere (Fernandez, P.L. et al. Virchows Arch, 438: 591-594, 2001). Three-m sections of the resulting microarray block were made and used for IHC analysis after being transferred to glass slides.

Anti-PRDX2 antibody (LabFrontier, Seoul, Korea) was used at 1/1000. Staining optimization, evaluation parameters and analyses were established by two pathologists (P.F. and S.B.) who where blinded to the clinical status. Antigens were retrieved by heating in a pressure cooker for seven minutes in the appropriate buffer. Primary antibodies were diluted in Dako RealTM Antibody Diluent Buffer (Dakocytomation): Tris buffer, pH 7.2, 15 mM NaN₃. LSAB+System-HRP (Dakocytomation) was used, including the biotinylated anti-rabbit, anti-mouse and antigoat immunoglobulins in PBS; streptavidin conjugated to HRP in PBS; and liquid 3-3’ Diaminobenzidine in chromogen solution.

Statistical Analysis.
The unpaired Student's t-test and one-way ANOVA were used to analyze in vitro experiments, and the results were presented as mean ± SEM. Analyses were performed using Stat View v5.0 software. For the in vivo experiments, parametric and non-parametric tests were applied to assess the observed differences in tumor volume at a specific time. For this purpose, the Student's t-test and the Wilcoxon test were used. Furthermore, to evaluate the effect of the differences over time we fitted linear mixed models (Pinheiro JC et al. Mixed-Effects Models in S and S-PLUS. Springer. (2000)), which took into account the random effects due to within-mice variation, given the repeated measures data structure in the experimental design. Estimated parameters and their variability were used to solve specific contrasts based on likelihood estimation. Thus, it could be assessed whether the difference was consistent throughout the period, and adjust the effect of time itself in the model. All analyses were performed using the statistical package R (R Development Core Team R: A language and environment for statistical computing. Foundation for Statistical Computing. Vienna, Austria. 2009). Statistical significance was set at $P < 0.05$.

**ERp57 protein knockdown.**

Stealth RNAi oligonucleotide duplexes targeting the open reading frame sequences of ERp57 as well as a non-targeting Stealth RNAi negative control (medium GC content) were obtained from Invitrogen (Paisley, UK). The following sequence was used: 5'-GAAGCUAUAUCC AAAGAAA-3' (SEQ ID NO:26). The RNA duplexes were introduced into B02 cells using Lipofectamine 2000 (Invitrogen) as a transfection agent. A total of 15x10^4 cells were seeded in six-well plates and transfected after 24 h (at 50-60% confluence). Protein knockdown was assessed 24, 48 and 72 h after transfection by semi-quantitative PCR and Western blot analysis.

**Stable ERp57 protein knockdown.**

ERp57 short hairpin RNA (shRNA) was generated using the siSTRIKE U6 Hairpin Cloning System (Promega, Madison, WI). The coding sequence of ERp57 was analyzed for sites of siRNA targeting using Promega's siRNA Target Designer and the following potential sequence was selected: shERp57 (5'-GAGCTT ACTGC ATGTTT AT-3') (SEQ ID NO:25). This corresponds to nucleotides
2378-2396 of the ERp57 transcript (GL67083697). Short hairpin primers were designed around these sequences and annealed before their ligation into the PstI site of the psiSTRIKE Neomycin vector, following the manufacturer's directions. In a similar fashion, control constructs were produced using scrambled versions of the shERp57 target sequence indicated above (5'-GGACCCTTAATGGTTTTT-3') (SEQ ID NO:27). Both the ERp57 targeted and the control nucleotide sequences were tested against the Genbank database to prevent undesired interaction with other mRNA transcripts. Plasmids were produced via the Qiagen MaxiPrep protocol (Qiagen, Inc., Valencia, CA), and purity and concentration were confirmed by analysis on a NanoDrop Spectrophotometer. B02 cells were transfected first with a retroviral vector peGFP-CMV/Luc expressing GFP and luciferase and afterwards with the shERp57 or sh control respectively, using Lipofectamine 2000 (Invitrogen) as a transfection agent. PCR and Western blot were used to validate the knockdown of ERp57 using the amplification of Neo as a positive control of transfection.

RNA extraction, reverse transcription and PCR.

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Purified RNA was reverse transcribed using 300 ng of RNA in a final volume of 20 µL. PCR was performed to evaluate the extent of ERp57 knockdown using cyclophilin as a housekeeping gene. Hae III was used as a marker. RT products from the carcinoma cell lines were analyzed for the expression of 10 target genes (HLA class I heavy chain, HLA A, B and C specific locus, β2m, TAP1, TAP2, tapasin, LMP2 and LMP7) by quantitative real-time PCR. cDNA synthesis was performed with the RNA Reverse Transcription System (Promega Corporation, Madison, WI), following the manufacturer's instructions. These PCR reactions were carried out in a Light Cycler using a DNA Master Probes Kit (Roche Diagnostics, Manheim, Germany). We used commercial kits (Roche Diagnostics and Search LC, GmbH Heidelberg) to test the housekeeping gene G6PDH and HPRT amplification.

EXAMPLE 1: Expression patterns and subcellular distribution of peroxiredoxins in lung metastatic MDA-MB-435 breast cancer cells.
Human peroxiredoxin expression (PRDXs 1-6) was first examined in parental 435-P cells and their metastatic variants to validate their differential expression, according to previously reported data (Espana L et al. Am J Pathol 2005; 167: 1125-37). It was found that PRDX2 and PRDX3 levels were higher in lung metastatic variants (435-L₂, 435-L₃ and 435-L₂₃) than in 435-P cells and lymph node (435-N) or bone (435-B) metastatic variants (Fig. 1a, left panel). The more aggressive 435-L₃ cell line showed the highest overall level of peroxiredoxin expression compared to parental cells, as shown in Table 1:

<table>
<thead>
<tr>
<th></th>
<th>435-N</th>
<th>435-B</th>
<th>435-L₂</th>
<th>435-L₃</th>
</tr>
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<tr>
<td>PRDX1</td>
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<td>0.9</td>
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<tr>
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<td>1.5</td>
<td>1.7</td>
<td>3.7</td>
</tr>
<tr>
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<td>1.2</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
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<td>1.8</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
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<td>1</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>PRDX6</td>
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<td>1</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
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<td>4.6</td>
<td>6.4</td>
<td>7.9</td>
<td>12.1</td>
</tr>
</tbody>
</table>

Table 1. Band density of peroxiredoxins in metastatic variants compared with parental cells. Density in parental cells was set to 1. Total band density in parental cells = 6.

Furthermore, confocal laser scanning microscopy was used to investigate the compartmental subcellular distribution of PRDX2 and PRDX3. PRDX3 was localized in the mitochondria, whereas PRDX2 was cytosolic (Fig. 1A, right panel). Moreover, PRDX2 translocated from the cytosol to the nucleus in response to an oxidant challenge with its substrate hydrogen peroxide (3 mM), while PRDX3 remained localized in the mitochondrial membrane (Fig. 1A, right panel). PRDX2 protein translocation under oxidative stress was not related to metastatic activity and occurred in both 435-P and 435-L₃ cells. Hence, PRDX2 and PRDX3 may exert their antioxidant activity in
different cellular compartments without redundancy to control intracellular reactive oxygen species.

**EXAMPLE 2:** Lung metastatic MDA-MB-435 breast cancer cells have increased oxidative stress resistance.

The oxidative stress response was analyzed in 435-P and lung metastatic variants 435-L₂ and 435-L₃ to investigate the relationship between PRDX expression and the cell's ability to resist oxidative stress. It was found that parental cells were more susceptible to oxidative stress than their lung metastatic variants (Fig. 1b). This was evident at a concentration of 2 mM H₂O₂ and even more marked at a concentration of 3 mM H₂O₂, at which the viability of 435-L₃ cells was twice as high as parental cells (60% vs. 30%, *P* <0.01). At this concentration, the viability of the less aggressive 435-L₂ cells was also still higher than that of the parental cells, although to a lesser extent (40% vs. 30%, *P* <0.01). This correlated well with the overall levels of peroxiredoxins detected in these cells. It was further determined the internal generation of ROS in 435 cells, using 2',7'-dichlorofluorescein diacetate (DCFH₂-DH) as a ROS scavenger (Fig. 1c). 435-L₃ cells produced lower amounts of ROS than 435-P cells in physiological conditions, and this effect was maintained under oxidative stress (*P* <0.05 for both conditions). The ratios of ROS production in basal vs. oxidative stress conditions were similar in both cell lines (435-P, 0.46; 435-L₃, 0.37), which indicates a similar increase in cellular stress levels. Furthermore, it was examined the oxidative state of PRDXs in basal conditions and under oxidative stress by measuring PRDX-SO₃, which is the irreversibly overoxidized form of PRDX. Figure 1d shows that under severe oxidative stress, inactive PRDX-SO₃ accumulated more in parental than in lung metastatic cells. This suggests that there was a higher degree of depletion of active PRDX in 435-P cells via inactivation of the peroxidase function and subsequent PRDX degradation (Chevallet M *et al.* J Biol Chem 2003; 278: 37146-53).

The use of a histone deacetylase inhibitor valproic acid, which causes an accumulation of ROS in transformed cells, confirmed that the ROS-generating agents counteracted PRDXs and prevented breast cancer lung metastasis (Figure 2).
EXAMPLE 3: Downregulation of PRDX2 sensitizes lung metastatic MDA-MB-435 breast cancer cells against ROS.

To investigate whether peroxiredoxins provide lung metastatic cells with more effective protection against oxidative stress, 435-L3 cells were transiently depleted of PRDX2 or PRDX3 using siRNA oligos (Fig. 3A). Knockdown of PRDX2 or PRDX3 sensitized cells against H_2O_2 and induced more stress than in nontransfected 435-L3 cells (Fig. 3B). Moreover, intracellular ROS levels increased in 435-L3 cells that were transiently depleted of PRDX2 and PRDX3 when cells were exposed to H_2O_2 (Fig. 3C).

Interestingly, cells depleted of PRDX2 had significantly higher basal levels of ROS than untransfected 435-L3 cells, 435-L3 cells transfected with a control oligo, and siPRDX3-transfected cells (Fig. 3C). This indicates that the ROS scavenger function of PRDX2 in lung metastatic cells was greater than that of PRDX3.

It was therefore decided to stably downregulate PRDX2 expression in 435-L3 cells to further investigate the consequences of elevated PRDX2 in lung metastatic breast cancer cells in vivo. Two plasmids (psiSTRIKE/shPRDX2p2 and -shPRDX2p2) targeting two distinct regions of the PRDX2 transcript and psiSTRIKE/scbl control constructs were transfected into luciferase expressing 435-L3 cells, which had been transduced with retroviral vector prEGFP-CMV-PLuc for eGFP and luciferase expression. Stable clones were selected with neomycin. PRDX2 expression was verified by Western blot analysis and qPCR (Fig. 4a). Plots of the amount of light produced by predetermined numbers of 435-L3/scbl cells vs. the number of cells were linear throughout the range of cells tested (R^2 = 0.9928). This shows that light measurements can be used to estimate cell numbers. A stably downregulated clone (shPRDX2 from psiSTRIKE/shPRDX2p2, clone #7) with low protein expression was chosen, as well as a pool of control clones transfected with the scrambled version of PRDX2 (scbl) for further characterization (Fig. 4a). shPRDX2 cells showed significantly higher sensitivity to H_2O_2-induced oxidative stress (Fig. 4b) than 435-L3 and scbl control cells (P <0.0005 for both cell lines). Production of internal ROS correlated well with PRDX2 levels in cells under basal and oxidative stress conditions (Fig. 4c). No significant differences in cell cycle characteristics were found under basal or oxidative stress conditions, as revealed by flow cytometry.
EXAMPLE 4: PRDX2 has a cause-effect role in lung metastasis.

To examine whether downregulation of PRDX2 in lung metastatic cells affects their metastatic capacity and their propensity to grow in the lungs, i.m.f.p. orthotopic tumors were induced by inoculating nude mice (n = 7 per group) with a PRDX2 knockdown clone (shPRDX2, clone #7) or with control cells (scbl). Tumorigenesis and metastatic progression in animals was monitored periodically (Fig. 5A and B). Statistical analysis using a linear model algorithm to compare PRDX2 knockdown cells and the scbl control cells showed that, 40 days after cell inoculation, the tumors in mice inoculated with shPRDX2 cells tended to be smaller (82.8 ± 47.0 mm³) than the scbl control tumors (187.9 ± 97.5 mm³). However, these differences in tumor volume were not statistically significant (P = 0.165). Moreover, the metastatic progression to lungs after tumor induction, as quantified from the normalized photon flux (NPF) of cancer cells, differed between groups (Fig. 6A). By the end of the protocol at day 82 the NPF differences were significant between scbl and shPRDX2 groups (P <0.005). Indeed, although all of the mice developed primary tumors in both groups, the metastasis incidence in mice bearing shPRDX2 tumors was substantially different from that observed in control animals (incidence: 14% versus 86%, respectively) (Fig. 5B). To avoid bias in metastatic evolution due to differences in primary tumor size, the global incidence of lung metastasis in each group relative to the primary tumor size at the time of tumor exeresis was evaluated by calculating the NPF ratios of metastasis/tumor for each animal (Fig. 6B). The differences in metastatic capacity were statistically significant by the end of the experiment (P <0.005).

The ex vivo expression of PRDX2 in tumor samples from shPRDX2 tumor-bearing mice and control animals was further examined by IHC analysis. As expected, stable knockdown of PRDX2 persisted in breast tumors and in metastatic tissues, tumors and metastasis from mice bearing shPRDX2 cells expressing low levels of PRDX2 (Fig. 5C). Furthermore, there were no differences among tumors in PCNA staining as a marker of proliferation; expression was high in all breast tumors at the time of exeresis (Fig. 5C). In contrast, increased cell death as indicated by caspase-3 activation was evident in shPRDX2 tumors relative to controls. This could explain the
differences in tumor volume between shPRDX2 and scbl control mice. Hence, there may be a negative imbalance between cell proliferation and cell death in shPRDX2 net tumor growth as a consequence of PRDX2 downregulation (Fig. 5C), which favored cell death.

Moreover, the expression of endothelial marker CD31 was examined as an indicator of neovasculature in breast tumors, to measure tumor angiogenic activity as a possible cause of diminished metastatic activity in PRDX2 knockdown tumors (Fig. 5D). Although similar numbers of vessels were produced in all groups, a parametric Wilcoxon test revealed that tumors from shPRDX2 mice had a significantly lower value for vascular lumen than control tumors ($P < 0.05$). With regard to blood vessel structure in lung metastases, no vasculature was formed in the single case of shPRDX2 lung metastases, unlike metastases from control mice. Thus, PRDX2 knockdown leads to diminished tumor neovascularization, which can have consequences for metastasis activity.

PRDXs gene expression profiling was analyzed across a series of breast tumors, GSE2603.6 The total of PRDXs was significantly associated with lung metastatic progression ($P=0.0055$) but not with the rest of metastases ($P=0.077$), suggesting that PRDX2 function might be regulated at the protein level. Moreover, to demonstrate if PRDX2 is relevant to human cancer, its expression in breast cancer tissues from 104 patients with (27 patients had lung and 44 had bone metastases was further analyzed, including among them 10 patients with both and other metastasis) or without metastasis progression using tissue array technology (TMA). PRDX2 was considered to be positive when strong expression was detected, to avoid false positives, taking into account the known expression in a control tissue. Statistical analysis of the data showed significant association between lung metastasis progression and high expression of PRDX2 in breast carcinomas ($p<0.053$) (Figure 7). In addition, a correlation between bone metastasis and tumor expression of PRDX2 ($p=0.209$) was not found. Thus, PRDX2 in breast carcinomas might help in the selection of treatment strategies.

**EXAMPLE 5:** Downregulation of PRDX2 specifically inhibits the growth of metastatic cells in lungs.
Since the number of cells lodged in lungs was lower in shPRDX2-tumor bearing mice than in control animals 40 days after i.m.f.p. implantation, it was further explored which of the metastatic steps in lung colonization by PRDX2 knockdown cells could be a rate-limiting process. shPRDX2 and scbl control cells were then intravenously injected and monitored the homing of cells to the lungs over time. There was a time-dependent increase in the shPRDX2 and scbl cell burden in lungs. The number of scbl cells retained there was statistically significantly higher until the end of the experiment at day 55 ($P = 0.056$) than that observed in lungs of mice injected with shPRDX2 cells. This indicates that downregulation of PRDX2 inhibits the growth of cancer cells in lungs (Fig. 8A). Whole-body in vivo imaging of animals confirmed that the lung metastasis burden was substantially lower in mice inoculated with shPRDX2 cells than in control mice, whereas the burden of scbl and shPRDX2 cells in lymph nodes was similar (Fig. 8B). Furthermore, H&E staining and optical microscopy showed higher lung colonization in control mice (Fig. 8C) than in shPRDX2 mice. An analysis of GFP positive cells in ex vivo lung samples of mice injected with shPRDX2 and scbl cells confirmed the in vivo results. The lymph node PRDX2 expression was examined by IHC and found that it was low and similar in all samples. This suggests that there is different selection pressure on lymph node metastatic cell lodgment (Fig. 7C).

The evolution of lung metastasis in controls limited the course of the experiments. Thus, by the time lung and lymph node metastasis had developed no signal compatible with bone metastasis was detected. If during metastasis formation cells that reach lungs are selected by their ability to remove ROS and PRXD2 is an exponent of this organe-specific phenotype, we wondered why PRXD2 also decreased in metastasis from hypoxic tissue like bone (Figure 1A). PRDX2 was stably overexpressed in the highly bone metastatic B02-GFP/tTA cell line (selected from six in vivo/in vitro passes), which expressed low levels of the protein (Figure 8A, left panel). Two clones (#10 and #88) overexpressing PRDX2 were obtained. Clone #88 was selected for further studies as the luciferase expression levels were similar to those expressed by the B02/GFP control cell line, thereby allowing direct comparison by bioluminescence imaging in bone metastasis experiments in vivo. Overexpression of PRDX2 in B02 cells (Figure 9a, left panel) was inversely correlated with intracellular ROS production
(Figure 9a, right panel); B02/PRDX2 cells produced less ROS than B02/GFP control cells in basal conditions (P<0.05) and under oxidative stress stimuli (P<0.005).

The effect of overexpression of PRDX2 on breast cancer bone metastasis formation was next examined. B02/PRDX2 cells (clone #88) and B02/GFP control cells were inoculated intracardiacally into nude mice (n = 6 per group). A radiographic analysis of hind legs at day 36 and day 40 after tumor cell inoculation showed that mice bearing B02/PRDX2 tumors had significantly smaller osteolytic lesions than mice injected with B02/GFP cells (Fig. 9b, left panel). The mean area of osteolytic lesions at day 40 was 5.1 ± 2.9 mm² and 15.8 ± 1.6 mm² for B02/PRDX2 and B02/GFP tumor-bearing animals, respectively (P <0.005). Bioluminescence imaging of mice revealed that the B02/PRDX2 tumor burden in animals was statistically significantly less than that observed in control mice injected with B02/GFP cells (P <0.05). This indicates that PRDX2 overexpression impaired B02 cell growth in bones (Fig. 9c).

The decreased in vivo skeletal growth of B02/PRDX2 cells, in contrast to the effect of PRDX2 in lung metastatic cells, suggested that PRDX2 functions as a subsidiary of host tissue microenvironment cross-talk.

Next, some experiments were performed to visualize in vivo the metabolic features of metastatic bone lesions using radiopharmaceuticals that target tumors cells with a high glycolytic metabolism ([18F]FDG) or bind to bone ([18F]NaF). The PET scan of animals did not show any accumulation of FDG in metastatic hind limbs (Fig. 9D, middle panel), despite the presence of BLI-positive tumor cells (Fig. 9d, left panel). In contrast, there was a far greater accumulation of NAF in hind limbs (Fig. 9d, right-hand panel), which confirms the presence of osteolytic lesions. Thus, B02 cells can grow very efficiently in a low-glucose bone microenvironment in vivo. In contrast, lung tissue rich in glucose was an inhospitable environment for B02 cell growth.

The chemical composition of metastatic variants was then studied by Raman microspectroscopy in order to assess the importance of the metabolic pressure of the target organ to select the metabolic phenotype of metastatic cells. The principal component analysis models using the spectral region 600-1700 cm⁻¹ differentiated the data set of lung metastatic 435-L3 cells from the bone metastatic 435-B cells. Loadings on PC3 containing the bands: 1003 cm⁻¹, trigonal ring breathing of the benzene ring (present in phenylalanine residue); 1443 cm⁻¹, methylene scissoring deformation and
1655 cm⁻¹, HC=CH stretch (cis conformation), compatible with fatty acids and protein; and loadings on PC4, bands in region 729 and 1617 cm⁻¹, compatible with NADH, were involved. Under glucose deprivation, 435-L₃ and 435-B metastatic cells were almost completely separated by the PC4 component, which increased in bone metastatic cells. Moreover, chemical hypoxia shifted each variant in opposite PC3 way: while 435-B increased PC3 value, the 435-L₃ cells decreased it. These results indicated that metastatic variants had different metabolic phenotype and can be separated by their different metabolic features.

**EXAMPLE 6: PRDX2 regulates the metabolic stress response of lung metastatic cells.**

To further explore the role of PRDX2 in the selection of cells according the bioavailability of glucose in the target organ, cells from both breast cancer models, 435-P (lung metastatic tropism) and 231 cells (bone metastatic tropism) and their metastatic variants were challenged through starving them from glucose (Figure 10a). 435-L₃ cells survived significantly more than 435-P cells (P<0.0005). As 435-P and 435-L₃ shPRDX2 had only 50% of viability, the data suggested that PRDX2 provided 435-L₃ cells with the ability to the preferential use of glucose. In addition, PRDX2-induced B02 cells more susceptible against glucose deprivation with regard to parental cells (P=0.016), suggesting that PRDX2 disturbed the metabolic switch of the bone glucose-independent cells. Moreover, 435-B cells (P=0.001) and B02 cells (P=0.003) were less affected by hypoglycemic conditions, with regard their respective control cells. The putative glucose dependence metabolism of lung metastatic cells was checked by measuring the lactate production of 435-L₃ and 435-B cells with regard to protein concentration: 435-L₃ had 20.88 mM and 435-B had 14.57 mM lactate/mg protein. These results suggested the glycolytic phenotype of lung metastatic cells and indicate that the overexpression of PRDX2 in lung metastatic cells might subtend the preferential use of glucose (Figure 10b).

PRXD2 contributes to lung microenvironment adaptation through its ROS scavenger function, which promotes angiogenesis. Indeed, lung metastatic cells might be able to utilize glucose for oxidative metabolism, as in well-oxygenated (aerobic) tumor regions (Semenza GL. J. Clin Invest 2008; 118: 3835-7), and consequently need
protection from free radicals and ROS through an extensive, complex and effective antioxidant defense system (Lehtonen ST et al. Int J Cancer 2004;111:514-21). In contrast, shPRXD2 cells accumulate internal ROS and have inefficient proangiogenic activity, which hinders metastasis progression (Fig. 10B). In bones, B02 cells have been selected from an anaerobic and low-glucose microenvironment, and have the ability to use glutamine and/or pyruvate as a fuel. In this context, PRDX2 may be able to reduce bone metastasis growth by distorting the metabolic benefit of B02 in such a microenvironment. This suggests that PRDX2 might regulate both the oxidative and metabolic stress response of metastatic cells. Thus, the authors of the invention hypothesized that for metastatic cells to grow in lungs, they require an excess of redox scavenging molecules so that they can adapt to a microenvironment with increased oxygen pressure (pO₂), and compensate for the high levels of ROS delivered by the use of glucose during oxidative metabolism. When low levels of peroxiredoxins do not allow efficient redox scavenging, metastatic cells might enter a quiescent state, thus avoiding the metabolic activity that could compromise their life.

EXAMPLE 7: ERp57/GRP58 is a putative biomarker for liver metastasis in breast cancer

Sample collection

The Breast Cancer Committee of the Catalan Institute of Oncology (I.C.O.) and the Hospital Universitari de Bellvitge (HUB) supplied samples from patients diagnosed between 1988 and 2006. The series of 122 breast cancers included 71 consecutive primary ductal breast carcinomas at initial diagnosis from metastatic patients in treatment at the time of the study, with one or several organs affected and 51 patients with positive lymph nodes at surgery without metastatic progression after a minimum follow-up of five years. Three patients had brain as unique metastasis location and ten patients also had dissemination at bone (n=7) lung (n=6) and liver (n=4). A total of 48 tumors with bone metastasis, 23 with liver and 31 with lung metastasis were included.

Tissue Microarrays (TMAs) and immunohistochemistry (IHC)
TMAs were prepared from three representative areas of the tumor which were carefully selected from hematoxylin-eosin-stained sections of 122-donor blocks (S.B. and S.H.). Core cylinders of 2 mm diameter were punched from each tumor with a skin-biopsy punch and deposited into recipient paraffin blocks using a specific arraying device (Beecher Instruments, Sun Prairie, WI) as described elsewhere. 25 Three-μm sections of the resulting microarray block were cut and used for IHC analysis after being transferred to glass slides.

The experimental conditions were anti-ERp57/GRP58 (polyclonal antibody anti-SP5391CP peptide) at 1/1000 60min citrate buffer. The positive control tissue was pancreas. Staining optimization, evaluation parameters and analyses were established by two pathologists who where blinded to the clinical status.

Antigens were retrieved by heating in a pressure cooker for seven minutes in the appropriate buffer. Primary antibodies were diluted in Dako RealTM Antibody Diluent Buffer (Dakocytomation): Tris buffer, pH 7.2, 15 mM NaN3. LSAB+System-HRP (Dakocytomation) was used, including biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in PBS; streptavidin conjugated to HRP in PBS; and liquid 3-3’ diaminobenzidine in chromogen solution.

**Statistical analysis**

To evaluate the correlation of ERp57/GRP58 expression with liver metastasis, immunostained samples were graded on a three-category scale (negative, weak positive, and strong positive). The marker was catalogued as overexpressed in strong positive samples. The association of liver metastasis for each marker was tested using a 2-sided Fisher exact test and summarized by calculating the sensitivity among tumors that developed metastasis, and specificity among tumors without metastasis, for strong positive values. Positive and negative likelihood ratios were also calculated as integrated predictive indexes, as was the area under the ROC curve (aROC). Markers were assessed using a multivariate logistic regression model in a forward stepwise procedure to identify the best combination to discriminate liver metastasis. In all the analyses, associations were considered significant when \( p < 0.05 \).
Expression of ERp57/GRP58 in breast cancer primary tumors is associated with liver metastasis progression

Since ERp57/GRP58 was expressed in primary tumors, to estimate the probability of specific liver metastasis outcomes, we further analyzed them in a series of primary breast carcinomas using tissue array technology (TMA).

We considered a marker to be positive when strong expression was detected, to avoid false positives, taking into account the known expression in a control tissue (see Figure 1). Statistical analysis of the data showed significant associations between liver metastasis progression and high expression of ERp57/GRP58 (p<0.0001). A multivariate analysis based on stepwise logistic regression retained ERp57/GRP58 to discriminate liver metastasis. The aROC for this combination was 0.680 (see Figure 12).

The results show that ERp57/GRP58 is good candidate for biomarker of liver metastasis in breast cancer.

EXAMPLE 8: ERp57/GRP58 is a putative target for bone metastasis in breast cancer

ERp57 has a cause-effect role in bone metastasis

We decided to stably knockdown ERp57 expression in B02GFP/tTA with the siSTRIKE U6 Hairpin Cloning System to investigate the consequences of elevated ERp57 in bone metastasis in vivo. We chose stable shERp57B02 downregulated clones #21 and #32, and the scrambled version of ERp57 (scbl) for further characterization. Plots of the amount of light vs. the number of B02GFP/tTA cells were linear throughout the range of cells tested, R2 = 0.993. Moreover, ERp57 expression was verified by Western blot and IF analysis (Fig. 13A). To examine whether downregulation of ERp57 in bone metastatic cells affects their metastatic capacity and their propensity to grow in the bones, we induced bone metastasis by endovenous (e.v.) injection in NOD/SCID mice (n = 7 per group) of ERp57 knockdown clones, shB02 #21 and #32, or control cells (scbl). The diffuse photon accumulation over the entire animal body was monitored periodically by BLI. As expected, the first evidence of bioluminescence emission from bones, which is indicative of bone metastasis, was seen
at day 14 after e.v. injection of cancer cells in control animals. The follow-up of mice revealed that the tumor burden of shERp57B02C21 and shERp57B02C32 animals was statistically significantly less than that observed in control mice injected with shB02/GFPCT4 cells (P <0.01 in sh21, p<0.0001 in sh32 compared to ct4) from day 34 until the end of the experiment at day 60 (Fig. 13B, left panel). A radiographic analysis of hind legs at day 45 and day 60 after tumor cell inoculation showed that mice bearing shERp57B02C21 and shERp57B02C32 cells had significantly smaller osteolytic lesions than mice injected with shB02/GFPCT4 cells (Fig. 13B, right panel). Moreover, the metastasis incidence in mice bearing shERp57 cells was different from that observed in control animals: shERp57B02C21, 25% (1/4); shERp57B02C32 40% (2/5) and shB02/GFPCT4, 66.7% (4/6). Lung micro- or macrometastasis were found in all groups: shERp57B02C21, 100% (5/5); shERp57B02C32 80% (4/5) and shB02/GFPCT4, 66.7%> (4/6). These results indicated that ERp57 overexpression improved B02 cell growth in bones and had an organ specific cause-effect role in bone metastasis development.

We further examined the ex vivo expression of ERp57 in bone metastasis samples from sh21 and sh32 metastasis-bearing mice and control animals by IHC analysis. As expected, stable knockdown of ERp57 persisted in bone metastasis (Fig. 13C). Furthermore, there were PCNA differences among tumors indicating proliferation decrease in shERp57B02 metastasis with regard to controls (Fig. 13C). Increased cell death as indicated by caspase-3 activation was evident in shERp57B02 bone metastasis relative to controls.
CLAIMS

1. An *in vitro* method for determining the risk of developing lung metastasis in a subject suffering from breast cancer comprising determining the expression level of PRDX2 in a tumour sample of said subject, wherein if the expression level of said gene is increased with respect to a reference value, said subject has an increased risk of developing lung metastasis.

2. An *in vitro* method for designing a personalized therapy for a subject suffering from breast cancer comprising determining the expression level of PRDX2 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, then a therapy directed to prevent and/or treat lung metastasis is selected.

3. An *in vitro* method for determining the risk of developing liver metastasis in a subject suffering from breast cancer comprising determining the expression level of ERp57/GRP58 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, said subject has an increased predisposition of developing liver metastasis.

4. An *in vitro* method for designing a personalized therapy for a subject suffering from breast cancer comprising determining the expression level of ERp57/GRP58 in a tumour sample of said subject wherein if the expression level of said gene is increased with respect to a reference value, then a therapy directed to prevent and/or treat liver metastasis is selected.

5. Method according to any of claims 1 to 4, wherein the determination of the expression level comprises the determination of the mRNA level of said gene/s or a fragment thereof, the determination of the cDNA level or a fragment thereof or determining the protein level encoded by said gene/s or a variant thereof.
6. Method according to claim 5 wherein the determination of the expression level is carried out by quantitative PCR or by a DNA or RNA array.

7. Method according to claim 5 wherein the determination of the protein level is carried out by western blot, ELISA or a protein array.

8. A PRDX2 and/or PRDX3 inhibitor for use in the treatment and/or prevention of lung metastasis in breast cancer.

9. The inhibitor for use according to claim 8, wherein said inhibitor is selected from the group of (i) an interfering RNA specific for PRDX2 or PRDX3, (ii) a polynucleotide encoding an interfering RNA specific for PRDX2 or PRDX3, (iii) an antisense oligonucleotide specific for PRDX2 or PRDX3, (iv) a ribozyme or DNA enzyme specific for PRDX2 or PRDX3, and (v) an inhibitory antibody specific for PRDX2 or PRDX3.

10. The inhibitor for use according to claim 9, wherein the interfering RNA is a siRNA specific for PRDX2 or PRDX3.

11. The inhibitor for use according to claim 10 wherein the siRNA specific for PRDX2 is directed to positions 712-730 or positions 748-766 of the sequence identified as SEQ ID NO:1 and wherein the siRNA specific for PRDX3 is directed to positions 741 to 765 of the sequence identified as SEQ ID NO:7.


13. A ERp57/GRP58 inhibitor for use according to claim 12 wherein the metastasis is bone metastasis.

14. The ERp57/GRP58 inhibitor for use according to claims 12 or 13, wherein said inhibitor is selected from the group of (i) an interfering RNA specific for a
ERp57/GRP58, (ii) a polynucleotide encoding an interfering RNA specific for ERp57/GRP58, (iii) an antisense oligonucleotide specific for ERp57/GRP58, (iv) a ribozyme or DNA enzyme specific for ERp57/GRP58, and (v) an inhibitory antibody specific for ERp57/GRP58.

15. The ERp57/GRP58 inhibitor for use according to claim 14, wherein the interfering RNA is a siRNA specific for ERp57/GRP58.

16. The inhibitor for use according to claim 10 wherein the siRNA specific for ERp57/GRP58 is directed to positions 2378-2396 of the sequence identified as SEQ ID NO:24.

17. A ROS-generating agent for use in the treatment and/or prevention of lung metastasis in breast cancer.

18. The ROS-generating agent for use according to claim 17, wherein the cancer has increased levels of PRDX2 and/or PRDX3.

19. The ROS-generating agent for use according to claims 17 or 18, wherein said ROS-generating agent is selected from the group of acetaminophen, elesclomol, 2,2'-dithio-bis-ethane sulfonate compounds, vitamin C, glutamate, sulindac sulfide, buthionine-sulfoximine, nitrofurantoin, phorone, transition metals, uric acid, homocysteine, anthracycline, bleomycin and cisplatin.

20. A composition comprising a PRDX2 and/or PRDX3 inhibitor and a ROS-generating agent.

21. A composition according to claim 20 wherein the PRDX2 and/or PRDX3 inhibitor is selected from the group of (i) an interfering RNA specific for PRDX2 or PRDX3, (ii) a polynucleotide encoding an interfering RNA specific for PRDX2 or PRDX3, (iii) an antisense oligonucleotide specific for PRDX2 or PRDX3, (iv) a ribozyme or
DNA enzyme specific for PRDX2 or PRDX3, and (v) an inhibitory antibody specific for PRDX2 or PRDX3.

22. A composition according to claims 20 or 21 wherein the ROS-generating agent is selected from the group of acetaminophen, elesclomol, 2,2'-dithio- bis-ethane sulfonate compounds, vitamin C, glutamate, sulindac sulfide, buthionine-sulfoximine, nitrofurantoin, phorone, transition metals, uric acid, homocysteine, anthracycline, bleomycin and cisplatin.

23. A composition according to any of claims 20 to 22 for use as a medicament.

24. A composition according to any of claims 20 to 22 for use in the treatment and/or prevention of lung metastasis in breast cancer.
FIG. 1
FIG. 1 (cont.)
Vehicle  Treated

PRDX2 i.m.f. p.Tumors

H&E of lung metastasis

Tumor weigh  Metastasis

Treated  0.15±0.05 gr  2/7 micromet
Vehicle  0.25±0.12 gr  5/7 macromet

FIG. 2
FIG. 3
FIG. 4
FIG. 5
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**FIG. 5 (cont.)**
FIG. 9
FIG. 9 (cont.)
FIG. 10
Liver Metastasis

Lung, Bone and Brain Metastasis

No Metastasis

FIG. 11
FIG. 12

Area under the curve: 0.680
FIG. 13
FIG. 13 (cont.)