

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property

Organization

International Bureau

(43) International Publication Date

23 September 2021 (23.09.2021)



(10) International Publication Number

WO 2021/185959 A1

(51) International Patent Classification:

A61K 31/352 (2006.01) G01N 33/74 (2006.01)

A61K 31/575 (2006.01) A61P 35/04 (2006.01)

A61K 31/7048 (2006.01) G01N 33/574 (2006.01)

A61K 45/06 (2006.01)

(21) International Application Number:

PCT/EP2021/056931

(22) International Filing Date:

18 March 2021 (18.03.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

20305287.3 19 March 2020 (19.03.2020) EP

(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **CENTRE LEON BERARD** [FR/FR]; Bâtiment Cheney D 28, rue Laennec, 69008 LYON (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, Rue Michel Ange, 75016 Paris (FR). **UNIVERSITÉ CLAUDE BERNARD - LYON 1** [FR/FR]; 43 Boulevard du 11 novembre 1918, 69100 Villeurbanne (FR).

(72) Inventors: **BONNELYE, Edith**; FACULTE DE MEDECINE RTH LAENNEC, 7 RUE GUILLAUME PARADIN, 69372 LYON Cedex 08 (FR). **MARIE, Julien**; U1052- CENTRE LEON BERARD 28 RUE LAENNEC - BAT CHENEY D, 69373 Lyon - CEDEX 08 (FR). **CLEZARDIN, Philippe**; U1033 - UFR DE MEDECINE LYON-EST 7-11 RUE GUILLAUME PARADIN - DOMAINE LAENNEC, 69372 Lyon - Cedex 08 (FR).

(74) Agent: **INSERM TRANSFERT**; 7 rue Watt, 75013 Paris (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,

HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: ESTROGEN-RELATED RECEPTOR ALPHA AGONISTS FOR THE TREATMENT AND THE PROGNOSIS OF BONE METASTASES

(57) Abstract: Bone is the most common metastatic site for breast cancer. The estrogen-related receptor alpha (ERR α) has been implicated in breast cancer cell dissemination to the bone from the primary tumor. However, its role after tumor cell anchorage in the bone microenvironment remains elusive. Here, the inventors reveal that ERR α inhibits the progression of bone metastases of breast cancer cells by increasing the immune activity of the bone microenvironment. Over-expressing ERR α in breast cancer bone metastases induced the expression of the chemokines CCL17 and CCL20 and repressed the production of transforming growth factor beta 3 (TGF- β 3). Subsequently, CD8⁺ T lymphocytes recruited to the bone metastases escaped TGF- β signaling#control were endowed with exacerbated cytotoxic features and metastases were strongly decreased. The clinical relevance of the findings in mice was confirmed in over 248 breast cancer patients. Thus, the inventors propose to consider ERR α expression, as a biomarker predictive of BM response to immunotherapies and /or as a good prognosis marker in BM progression once established, opening the path towards to the clinical use of ERR α #agonist to relieve patients with ERR α positive BM after primary tumor resection.



WO 2021/185959 A1

ESTROGEN-RELATED RECEPTOR ALPHA AGONISTS FOR THE TREATMENT AND
THE PROGNOSIS OF BONE METASTASES

5 **FIELD OF THE INVENTION:**

The present invention is in the field of medicine, in particular oncology and immunology.

BACKGROUND OF THE INVENTION:

10 Bone metastases (BM) are a frequent complication of cancer, occurring in up to 70 percent of patients with advanced breast cancer (BCa), and are associated with both high morbidity and elevated mortality (1)(2)(3). The progression of bone metastases relies on the ability of the malignant cell colonizing the bone and to modify bone micro-environment allowing the release of bone-stored factors including transforming growth factor (TGF- β), bone
15 morphogenetic protein (BMP) or insulin growth factor (IGF), which in turn stimulate bone metastases progression (2)(4). However treatments which mainly involved anti-resorptive agents of the bone failed to improve the overall survival of cancer patients even though it inhibited osteoclasts resorptive activity (3), implying that other mechanisms than the activation of osteoclasts by tumor cells are involved in modulating bone metastases growth. The immune
20 cells present in the bone, and particularly activated CD8⁺ T lymphocytes can repress the progression of BCa osteolytic bone metastases (5)(6)(7)(8). However, whether bone metastases can influence the activation of immune cells present in the bone and by which mechanisms is totally unknown.

 The estrogen-related receptor alpha (ERR α , or NR3B1 according to the Nuclear
25 Receptors Nomenclature Committee, 1999) is over expressed in 55% of breast tumors (9)(10). Though ERR α shares structural similarities with the estrogen receptors α/β , it does not bind estrogens and no natural ligand has yet been found (11), though several molecules can either increase or decrease ERR α activity, such as the inverse-agonists XCT790 or C29 (12)(13). ERR α is mainly involved in the adaptive bioenergetics response (11). In cancer, beside
30 angiogenesis, ERR α is strongly linked to tumor cell-invasion (14)(15). Notably, ERR α -positive tumors are associated with more invasive breast cancers and a higher risk of recurrence (9)(14). The over-expression of ERR α in BCa promotes tumor growth in the mammary gland and BCa metastatic dissemination to the bone (16). However, the role of ERR α in bone metastases outcome once they are anchorage in the bone microenvironment remains elusive.

SUMMARY OF THE INVENTION:

As defined by the claims, the present invention relates to estrogen-related receptor alpha (ERR α) agonists for the treatment of bone metastases.

DETAILED DESCRIPTION OF THE INVENTION:

Bone is the most common metastatic site for breast cancer. The estrogen-related receptor alpha (ERR α) has been implicated in breast cancer cell dissemination to the bone from the primary tumor. However, its role after tumor cell anchorage in the bone microenvironment remains elusive. Here, the inventors reveal that ERR α inhibits the progression of bone metastases of breast cancer cells by increasing the immune activity of the bone microenvironment. Over-expressing ERR α in breast cancer bone metastases induced the expression of the chemokines CCL17 and CCL20 and repressed the production of transforming growth factor beta 3 (TGF- β 3). Subsequently, CD8⁺ T lymphocytes recruited to the bone metastases escaped TGF- β signaling control were endowed with exacerbated cytotoxic features and metastases were strongly decreased. The clinical relevance of the findings in mice was confirmed in over 248 breast cancer patients. Thus, this study reveals an unexpected role for ERR α in the bone microenvironment immune properties that contributes to decreasing metastatic growth.

Methods of treatment:

The first object of the present invention thus relates to a method of treating bone metastases in a patient in need thereof comprising administering a therapeutically effective amount of an ERR α agonist.

As used herein, the term “bone metastasis” refers to metastatic bone disease, or cancer metastases that results from primary tumor invasion to bone. Invasion of the bone compartment by cancer cells causes imbalance between osteoclasts and osteoblasts, and leads to osteolytic bone metastasis. Bone metastasis may be present in multiple cancers including the vast majority of late-stage breast cancer patients. Bone metastasis may result in severe bone loss, debilitating fractures, and other life-threatening complications. In particular, the method of the present invention is particularly suitable for the treatment of breast cancer bone metastases.

Thus, the present invention also relates to a method of treating breast cancer bone metastases in a patient in need thereof comprising administering a therapeutically effective amount of an ERR α agonist.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a patient having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a patient beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., pain, disease manifestation, etc.]).

The second object of the present invention relates to a method of increasing the amount of tumor infiltrating cytotoxic T lymphocytes cells in bone metastases comprising administering to the patient a therapeutically effective amount of an $ERR\alpha$ agonist.

As used herein, the term "cytotoxic T lymphocyte" or "CTL" has its general meaning in the art and refers to a subset of T cells which express CD8 on their surface. CD8 antigens are members of the immunoglobulin supergene family and are associative recognition elements in major histocompatibility complex class I-restricted interactions. They are MHC class I-restricted, and function as cytotoxic T cells. Cytotoxic T lymphocytes are also called, CD8+ T cells, T-killer cells, cytolytic T cells, or killer T cells. As used herein, the term "tumor-

infiltrating cytotoxic T lymphocyte” refers to the pool of cytotoxic T lymphocytes of the patient that have left the blood stream and have migrated into the sites of metastases. For example, the ERR α agonist of the present invention has the ability to increase the amount of tumor-infiltrating cytotoxic T lymphocytes cells by more than about 10%, preferably with at least
5 about 15%, at least about 20%, at least about 25%, or more.

The inventors demonstrated that stimulating ERR α activation increase the amount of tumor infiltrating cytotoxic T lymphocytes cells in bone metastases.

As used herein, the term “ERR α ” has its general meaning in the art and refers to the human estrogen receptor-related receptor α protein.

10 As used herein, the term “ERR α agonist” has its general meaning in the art, and refers to a compound (natural or not) which has the capability of increasing the activity of ERR α . Typically, said compound increases the transcription from promoters containing ERR α binding sites. Typically, said agonist is a small organic molecule or a biological molecule (e.g. peptides, lipid, aptamer...).

15 Dietary plant products such as apigenine, daidzin, genistein, piceatannol, resveratrol, and rutacarpine, had been reported as ERR α agonists (Teng et al, 2017, Suetsugi et al, 2003). More recently flavones were described as ERR α agonist (Lynch et al, 2018).

Also, cholesterol that was identified by affinity chromatography of tissue lipidomes, using ligand binding domain of ERR α , is described as an efficient ERR α agonist (Wei and al.,
20 2016 that may be used in the field of cancer treatment (Casaburi and al., 2018, Silvente-Poirot et al, 2018).

The ERR α agonist may also be compounds, i.e bisphenol A, a series of pyrido (1,2-alpha) pyrimidin-4, statins (atorvastatin, cervastatin, fluvastatin et lovastatin) and axitinib (tyrosine kinase inhibitor) which also improve the receptor transcriptional activity as described
25 in Peng and al. (2011), Teng et al. (2014), Lynch et al. (2018). In order to identify an ERR α agonist and inverse-agonists, affinity chromatography with ERR α -ligand binding domain or High-Throughput Screening can be performed (Wei et al, 2016, Teng et al, 2017, Lynch et al, 2018, Lynch et al, 2019). ERR α agonists and inverse-agonists may be identified by cells line containing stable ERR α reporters (multiple hormone response element MHRE) cloned into
30 reporter vector that contains both green fluorescent protein (GFP) and Luc marker reporters in cells (ex: HEK293T) that expressed endogenous ERR α (Teng et al, 2017, Lynch et al, 2018). Cells are plated in tissue culture white assay plate. Compounds can be then tested on the plate followed by luciferin. Luminescence intensity of the assay plate can be then quantified using plate reader. If the potential agonist binds to the promoter of ERR α gene to activates

transcription, a bioluminescent signal is emitted (Teng et al, 2017, Lynch et al, 2018). Those results may be confirmed with a crystallographic reconstitution, well known in the state of art.

In some embodiments, the $ERR\alpha$ agonist is administered to the patient in combination with immune checkpoint blockade therapy.

5 As used herein, the “immune checkpoint blockade therapy” relates to a therapy that consists in administering the patient with at least one immune checkpoint inhibitor. As used herein, the term “immune checkpoint inhibitor” has its general meaning in the art and refers to any compound inhibiting the function of an immune inhibitory checkpoint protein. As used herein the term “immune checkpoint protein” has its general meaning in the art and refers to a
10 molecule that is expressed by T cells in that either turn up a signal (stimulatory checkpoint molecules) or turn down a signal (inhibitory checkpoint molecules). Examples of inhibitory checkpoint molecules include A2AR, B7-H3, B7-H4, BTLA, CTLA-4, CD277, IDO, KIR, PD-1, LAG-3, TIM-3 and VISTA. Inhibition includes reduction of function and full blockade. Preferred immune checkpoint inhibitors are antibodies that specifically recognize immune
15 checkpoint proteins. A number of immune checkpoint inhibitors are known and in analogy of these known immune checkpoint protein inhibitors, alternative immune checkpoint inhibitors may be developed in the (near) future. The immune checkpoint inhibitors include peptides, antibodies, nucleic acid molecules and small molecules. Examples of immune checkpoint inhibitors includes PD-1 antagonist, PD-L1 antagonist, PD-L2 antagonist CTLA-4 antagonist,
20 VISTA antagonist, TIM-3 antagonist, LAG-3 antagonist, IDO antagonist, KIR2D antagonist, A2AR antagonist, B7-H3 antagonist, B7-H4 antagonist, and BTLA antagonist. In some embodiments, the immune checkpoint inhibitor is selected from the group consisting of Ipilimumab, Nivolumab, Pembrolizumab, Atezolizuma, Avelumab, Durvalumab and Cemiplimab.

25 As used the terms “combination” and “combination therapy” are interchangeable and refer to treatments comprising the administration of at least two compounds administered simultaneously, separately or sequentially. As used herein the term “co-administering” as used herein means a process whereby the combination of at least two compounds is administered to the same patient. The at least two compounds may be administered simultaneously, at
30 essentially the same time, or sequentially. The at least two compounds can be administered separately by means of different vehicles or composition. The at least two compounds can also be administered in the same vehicle or composition (e.g. pharmaceutical composition). The at least two compounds may be administered one or more times and the number of administrations of each component of the combination may be the same or different. The combination therapy

may provide “synergy” and prove “synergistic”, i.e., the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately.

As used herein, the term "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of the active agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the active agent to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of drug are outweighed by the therapeutically beneficial effects. The efficient dosages and dosage regimens for the active agent depend on the disease or condition to be treated and may be determined by the persons skilled in the art. A physician having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician could start doses of active agent employed in the pharmaceutical composition at levels lower than that required achieving the desired therapeutic effect and gradually increasing the dosage until the desired effect is achieved. In general, a suitable dose of a composition of the present invention will be that amount of the compound, which is the lowest dose effective to produce a therapeutic effect according to a particular dosage regimen. Such an effective dose will generally depend upon the factors described above. An exemplary, non-limiting range for a therapeutically effective amount of an inhibitor of the present invention is about 0.1-100 mg/kg, such as about 0.1-50 mg/kg, for example about 0.1-20 mg/kg, such as about 0.1-10 mg/kg, for instance about 0.5, about such as 0.3, about 1, about 3 mg/kg, about 5 mg/kg or about 8 mg/kg. An exemplary, non-limiting range for a therapeutically effective amount of a inhibitor of the present invention is 0.02-100 mg/kg, such as about 0.02-30 mg/kg, such as about 0.05-10 mg/kg or 0.1-3 mg/kg, for example about 0.5-2 mg/kg. Administration may e.g. be intravenous, intramuscular, intraperitoneal, or subcutaneous, and for instance administered proximal to the site of the target. Dosage regimens in the above methods of treatment and uses are adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In some embodiments, the efficacy of the treatment is monitored during the therapy, e.g. at predefined points in time. In some embodiments, the efficacy may be monitored by visualization of the disease area, or by other diagnostic methods described further herein, e.g. by performing one or more PET-CT scans. If desired, an effective daily dose of a

pharmaceutical composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

According to the present invention, the active agent (i.e. ERR α agonist) is administered
5 to the patient in the form of a pharmaceutical composition which comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers that may be used in these compositions include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water,
10 salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene- block polymers, polyethylene glycol and wool fat. For use in administration to a patient, the
15 composition will be formulated for administration to the patient. The compositions of the present invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. Sterile
20 injectable forms of the compositions of this invention may be aqueous or an oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable
25 vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil
30 or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents that are commonly used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers

which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation. The compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include, e.g., lactose. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added. Alternatively, the compositions of this invention may be administered in the form of suppositories for rectal administration. These can be prepared by mixing the agent with a suitable non-irritating excipient that is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax and polyethylene glycols. The compositions of this invention may also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, including diseases of the eye, the skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas or organs. For topical applications, the compositions may be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively, the compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissolved in one or more pharmaceutically acceptable carriers. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. Topical application for the lower intestinal tract can be effected in a rectal suppository formulation (see above) or in a suitable enema formulation. Patches may also be used. The compositions of this invention may also be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents.

Methods of prognosis:

A further object of the present invention relates to a method for predicting the survival time of a patient suffering from bone metastases comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient wherein said level correlates with the survival time of the patient.

In a particular embodiment, the patient suffers from breast cancer bone metastases. Thus, the present invention also relates to a method for predicting the survival time of a patient suffering from breast cancer bone metastases comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient wherein said level correlates with the survival time of the patient.

The method of the present invention is particularly suitable for predicting the duration of the overall survival (OS), progression-free survival (PFS) and/or the disease-free survival (DFS) of the cancer patient. Those of skill in the art will recognize that OS survival time is generally based on and expressed as the percentage of people who survive a certain type of cancer for a specific amount of time. Cancer statistics often use an overall five-year survival rate. In general, OS rates do not specify whether cancer survivors are still undergoing treatment at five years or if they've become cancer-free (achieved remission). DFS gives more specific information and is the number of people with a particular cancer who achieve remission. Also, progression-free survival (PFS) rates (the number of people who still have cancer, but their disease does not progress) includes people who may have had some success with treatment, but the cancer has not disappeared completely. As used herein, the expression "short survival time" indicates that the patient will have a survival time that will be lower than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a short survival time, it is meant that the patient will have a "poor prognosis". Inversely, the expression "long survival time" indicates that the patient will have a survival time that will be higher than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a long survival time, it is meant that the patient will have a "good prognosis".

As used herein, the term "tumor tissue sample" means any tissue tumor sample derived from the patient. Said tissue sample is obtained for the purpose of the in vitro evaluation. In some embodiment, the tumor sample may results from a biopsy performed in a primary tumor tissue sample. In some embodiments, the tumor sample may result from a biopsy performed in metastatic sample. The tumor tissue sample can be subjected to a variety of well-known post-collection preparative and storage techniques (e.g., fixation, storage, freezing, etc.) prior to

determining the expression of $ERR\alpha$ of interest. Typically the tumor tissue sample is fixed in formalin and embedded in a rigid fixative, such as paraffin (wax) or epoxy, which is placed in a mould and later hardened to produce a block which is readily cut. Thin slices of material can be then prepared using a microtome, placed on a glass slide and submitted e.g. to immunohistochemistry (IHC) (using an IHC automate such as BenchMark® XT or Autostainer 5 Dako, for obtaining stained slides). The tumour tissue sample can be used in microarrays, called as tissue microarrays (TMAs). TMA consist of paraffin blocks in which up to 1000 separate tissue cores are assembled in array fashion to allow multiplex histological analysis. This technology allows rapid visualization of molecular targets in tissue specimens at a time, either 10 at the DNA, RNA or protein level. TMA technology is described in WO2004000992, US8068988, Olli et al 2001 Human Molecular Genetics, Tzankov et al 2005, Elsevier; Kononen et al 1198; Nature Medicine.

In some embodiments, the expression level of $ERR\alpha$ is determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. 15 For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the subject) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR). Other methods of 20 Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA). In some embodiments, the level is determined by DNA chip analysis.

Probes may also be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) 25 and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH). Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pir1kel et al., Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al., Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al., Proc. Natl. Acad. Sci. 85:9664-9668, 30 1988. CISH is described in, e.g., Tanner et al., Am. .1. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

In some embodiments, the nCounter® Analysis system may be used to detect intrinsic gene expression. The basis of the nCounter® Analysis system is the unique code assigned to each nucleic acid target to be assayed (International Patent Application Publication No. WO

08/124847, U.S. Patent No. 8,415,102 and Geiss et al. Nature Biotechnology. 2008. 26(3): 317-325; the contents of which are each incorporated herein by reference in their entireties

Expression level of a gene may be expressed as absolute level or normalized level. Typically, levels are normalized by correcting the absolute level of a gene by comparing its expression to the expression of a gene that is not a relevant for determining the cancer stage of the subject, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. This normalization allows the comparison of the level in one sample, e.g., a subject sample, to another sample, or between samples from different sources.

In some embodiments, the expression level of $ERR\alpha$ is determined by determining the quantity of the $ERR\alpha$ protein. Methods for quantifying protein of interest are well known in the art and typically involve immunohistochemistry. Immunohistochemistry typically includes the following steps i) fixing the tumor tissue sample with formalin, ii) embedding said tumor tissue sample in paraffin, iii) cutting said tumor tissue sample into sections for staining, iv) incubating said sections with the binding partner specific for $ERR\alpha$, v) rinsing said sections, vi) incubating said section with a secondary antibody typically biotinylated and vii) revealing the antigen-antibody complex typically with avidin-biotin-peroxidase complex. Accordingly, the tumor tissue sample is firstly incubated with the binding partners having for $ERR\alpha$. After washing, the labelled antibodies that are bound to $ERR\alpha$ are revealed by the appropriate technique, depending of the kind of label is borne by the labelled antibody, e.g. radioactive, fluorescent or enzyme label. Multiple labelling can be performed simultaneously. Alternatively, the method of the present invention may use a secondary antibody coupled to an amplification system (to intensify staining signal) and enzymatic molecules. Such coupled secondary antibodies are commercially available, e.g. from Dako, EnVision system. Counterstaining may be used, e.g. Hematoxylin & Eosin, DAPI, Hoechst. Other staining methods may be accomplished using any suitable method or system as would be apparent to one of skill in the art, including automated, semi-automated or manual systems.

In some embodiments, the resulting stained specimens are each imaged using a system for viewing the detectable signal and acquiring an image, such as a digital image of the staining. Methods for image acquisition are well known to one of skill in the art. For example, once the sample has been stained, any optical or non-optical imaging device can be used to detect the stain or biomarker label, such as, for example, upright or inverted optical microscopes, scanning confocal microscopes, cameras, scanning or tunneling electron microscopes, scanning probe microscopes and imaging infrared detectors. In some examples, the image can be captured

digitally. Various automated sample processing, scanning and analysis systems suitable for use with IHC are available in the art. Such systems can include automated staining and microscopic scanning, computerized image analysis, serial section comparison (to control for variation in the orientation and size of a sample), digital report generation, and archiving and tracking of samples (such as slides on which tissue sections are placed).

Thus, in some embodiments, the method of the present invention comprises the steps consisting in i) providing one or more immunostained slices of tissue section obtained by an automated slide-staining system by using a binding partner capable of selectively interacting with $ERR\alpha$ (e.g. an antibody as above described), ii) proceeding to digitalisation of the slides of step i) by high resolution scan capture, iii) detecting the slice of tissue section on the digital picture iv) providing a size reference grid with uniformly distributed units having a same surface, said grid being adapted to the size of the tissue section to be analyzed, and v) detecting, quantifying and measuring intensity or the absolute number of stained cells in each unit.

In some embodiments, the method herein disclosed comprises the steps of i) determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will have a long survival time when the level determined at step i) is higher than the predetermined reference value or concluding that the patient will have a short survival time when the level determined at step i) is lower than the predetermined reference value.

In some embodiments, the predetermined reference value is a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skill in the art. For example, retrospective measurement of expression level of $ERR\alpha$ in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression of $ERR\alpha$ in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured expression levels of the gene(s) in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of

ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area is under the curve (AUC), the higher is the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, the predetermined reference value is determined by carrying out a method comprising the steps of a) providing a collection of samples; b) providing, for each sample provided at step a), information relating to the actual clinical outcome for the corresponding subject (i.e. the duration of the survival); c) providing a serial of arbitrary quantification values; d) determining the expression of $ERR\alpha$ for each sample contained in the collection provided at step a); e) classifying said samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising samples that exhibit a quantification value for said level that is higher than the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of samples are obtained for the said specific quantification value, wherein the samples of each group are separately enumerated; f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the subjects from which samples contained in the first and second groups defined at step f) derive; g)

reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested; h) setting the said predetermined reference value as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been calculated at step g).

5 For example the expression of $ERR\alpha$ has been assessed for 100 samples of 100 subjects. The 100 samples are ranked according to the expression of $ERR\alpha$. Sample 1 has the highest level and sample 100 has the lowest level. A first grouping provides two subsets: on one side sample Nr1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on
10 one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding subject, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated. The predetermined reference value is then selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other
15 terms, the expression of $ERR\alpha$ corresponding to the boundary between both subsets for which the p value is minimum is considered as the predetermined reference value. Assessments using the median value have already been published for $ERR\alpha$, as in Fradet et al, 2011 on a cohort of 250 patients and in Vargas et al, 2019 on 100 patients.

It should be noted that the predetermined reference value is not necessarily the median
20 value of expression levels of the gene. Thus in some embodiments, the predetermined reference value thus allows discrimination between a poor and a good prognosis for a subject. Practically, high statistical significance values (e.g. low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the invention, instead of using a definite
25 predetermined reference value, a range of values is provided. Therefore, a minimal statistical significance value (minimal threshold of significance, e.g. maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values
30 includes a "cut-off" value as described above. For example, according to this specific embodiment of a "cut-off" value, the outcome can be determined by comparing the expression of $ERR\alpha$ with the range of values, which are identified. In some embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum p value

which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. For example, a subject may be assessed by comparing values obtained by measuring the expression of $ERR\alpha$, where values higher than 5 reveal a good prognosis and values less than 5 reveal a poor prognosis. In some embodiments, a subject may be assessed by comparing values obtained by measuring the expression of $ERR\alpha$ and comparing the values on a scale, where values above the range of 4-6 indicate a good prognosis and values below the range of 4-6 indicate a poor prognosis, with values falling within the range of 4-6 indicating an intermediate occurrence (or prognosis).

The method of the present invention is also suitable for determining whether a patient suffering from bone metastases is eligible for a treatment with an immune checkpoint blockade therapy.

Thus a further object of the present invention relates to a method for determining whether a patient suffering from bone metastases will achieve a response with an immune checkpoint blockade therapy comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, wherein said expression indicates whether the patient will achieve or not a response.

In a more particular embodiment, the patient suffers from breast cancer bone metastases. Thus, the present invention also relates to a method for determining whether a patient suffering from breast cancer bone metastases will achieve a response with an immune checkpoint blockade therapy comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, wherein said expression indicates whether the patient will achieve or not a response.

The method for determining whether a patient suffering from bone metastases will achieve a response with an immune checkpoint blockade therapy comprising i) determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will achieve a response when the level determined at step i) is higher than the predetermined reference value.

The method is thus particularly suitable for discriminating responder from non-responder. As used herein the term "responder" in the context of the present disclosure refers to a patient that will achieve a response, i.e. a patient where bone metastases are eradicated, reduced or improved. According to the invention, the responders have an objective response and therefore the term does not encompass patients having stabilized bone metastases such that

the disease is not progressing after the immune checkpoint blockade therapy. A non-responder or refractory patient includes patients for whom bone metastases do not show reduction or improvement after the immune checkpoint blockade therapy. According to the invention the term “non-responder” also includes patients having stabilized bone metastases. Typically, the characterization of the patient as a responder or non-responder can be performed by reference to a standard or a training set. The standard may be the profile of a patient who is known to be a responder or non-responder or alternatively may be a numerical value. Such predetermined standards may be provided in any suitable form, such as a printed list or diagram, computer software program, or other media. When it is concluded that the patient is a non-responder, the physician could take the decision to stop the immune checkpoint blockade therapy to avoid any further adverse sides effects.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Fig. 1: Inhibition of BM development by $ERR\alpha$ overexpression in BCa

(A-B), 4T1-CT, 4T1- $ERR\alpha$ or 4T1-CT(af2) and 4T1- $ERR\alpha$ AF2 cells were inoculated into BALB/c mice. 13-15 days post-inoculation, osteolytic lesions were analyzed. Graphs illustrate the mean size (mm^2) $\pm$ SEM of osteolytic lesions (n=10 mice per group, Mann-Whitney, osteolysis: $P < 0.0001$: 4T1- $ERR\alpha$ versus CT) and (n=9, Mann-Whitney, osteolysis: $P < 0.0006$: $ERR\alpha$ AF2 versus CT(af2)). (C-D) Graphs illustrate the mean of the % of Bone Volume/Tissues Volume $\pm$ SEM (n=10, Mann-Whitney, BV/TV: $P < 0.0001$ for 4T1- $ERR\alpha$ versus CT and n=9, Mann-Whitney, BV/TV: $P < 0.0006$ for 4T1- $ERR\alpha$ AF2 versus CT(af2)). (E-F) Graphs illustrate the mean of % of Tumor Burden /Soft Tissues Volume $\pm$ SEM (n=10, Mann-Whitney, TB/STV: $P < 0.0001$ for 4T1- $ERR\alpha$ versus CT and n=9, Mann-Whitney, TB/STV: $P < 0.0002$ for 4T1- $ERR\alpha$ AF2 versus CT(af2)).

Figure 2: Overexpression of $ERR\alpha$ in BCa cells promotes $CD8^+$ T cytotoxic function.

Cell suspensions were prepared from metastatic legs (bone; A) or lungs (B) of mice (n = 4) inoculated with 4T1-CT or 4T1- $ERR\alpha$ cells. (A-B) Graphs representing the percentage of T cells ($CD3^+$), B cells ($CD19^+$), and $CD4/CD8$ T cells in the bone (A; mean $\pm$ SD; n = 4; Student *t* test; **, $P < 0.001$; ***, $P < 0.0001$) and in the lungs (B). Graphs illustrating the

production of FasL, LAMP-1, granzyme A and B, and IFN γ in CD8⁺ T cells: Graphs demonstrate the percentage of cells (C) and their absolute numbers (D).

Figure 3: Ccl17 and Ccl20 upregulation by ERR α in breast cancer cells.

(A) 4T1-ERR α , 4T1-CT, or 4T1-ERR α AF2 and 4T1-CT(af2) cells were analyzed [n = 2; ANOVA, P < 0.0001 for Ccl17 and Ccl20; unpaired *t* test, P = 0.0007 (Ccl17), P = 0.0005 (Ccl20) (4T1-ERR α), and P = 0.0004 (Ccl20) 4T1-ERR α AF2 versus 4T1-CT]. (B-C) 4T1-ERR α and 4T1-ERR α AF2 cells were cultured for 24 hours with the inverse agonist C29 (5 mmol/L; unpaired *t* test, P = 0.0005 and P = 0.0034 for Ccl17 and Ccl20, respectively).

Figure 4: Overexpression of ERR α in breast cancer inhibits Tgf β 3 expression and affects TGF β signaling in CD8⁺ T cells.

(A) 4T1-ERR α , 4T1-ERR α AF2, 4T1-CT, and 4T1-CT(af2) cells were analyzed [ANOVA, P < 0.0001 and unpaired *t* test, P < 0.0001 4T1-ERR α versus 4T1-CT, P = 0.0009 for 4T1-ERR α AF2 versus 4T1-CT(af2)]. (B) 4T1-ERR α cells were cultured for 24 hours with the inverse agonist XCT-790 (1 μ mol/L; unpaired *t* test, P = 0.0007). Data are plotted as mean $\pm$ SEM.

EXAMPLE:

Material & Methods

Cell lines

The mouse triple negative breast (TNBC) cancer cell line 4T1 (year 2012) (ATCC lot: 58603185-CRL-2539) and human luminal MCF7 (year 2012) (ATCC-HTB-22 Lot: **86012803**) were obtained from the American Type Culture Collection. MDA-MB-231/BO2-FRT (BO2) BCa cells, a subpopulation of the human MDA-MB-231 BCa line (TNBC) was selected for their high efficiency to metastasize to bone (27). TNBC cell lines and the luminal cell line were cultured in DMEM or RPMI-1640 (Life-Technologies) medium, respectively, supplemented with 10% fetal bovine serum (FBS, Perbio) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO₂ incubator. Mouse and human ESRRA cDNA (ERR α) and the dominant-negative co-activator domain AF2 (AF2) mutant were described previously (16)(17). Briefly, pSR α -ERR α WT and pEcmv-ERR α AF2 or respective empty vectors (CT) constructs were transfected into parental 4T1 cells and cultured for 4 weeks in puromycin (2 μ g/mL) (Life-Technologies). Three independent clones were obtained from pSR α -ERR α WT transfection (4T1-ERR α) and from pEcmv-ERR α AF2 transfection (4T1-ERR α AF2). Two independent clones were obtained from empty vectors transfection respectively pSR α -4T1-CT (4T1-CT) and pEcmv-4T1-CT (4T1-CTaf2). For MCF7 clones, a mix containing 1.5 μ g Retroviral pLPCX-Human-ERR α WT,

pLPCX-HumanERR α AF2 or empty vector and 0.5 μ g pCMV-VSV-G envelope vector (Cell-Biolabs) were used previously (16). 4T1-ERR α and 4T1-ERR α AF2 (pool of 3 clones each) cells were treated for 24 hours with the ERR α -inverse-agonists XCT790 (Sigma) or C29 (AGVdiscovery, France) at 1 μ M and 5 μ M, respectively, as described (13)(16)(28). DMSO was used as a vehicle (Veh).

Animal studies

6-week-old BALB/c female mice were purchased from Janvier (France) and housed in a SPF facility (ALECS platform (Faculté de Médecine Laennec, Lyon, France). BM experiments were performed by inoculating intra-arterially either 4T1-CT (pool of 2 clones) in parallel with 4T1-ERR α (pool of 3 clones), or 4T1-CT(af2) (pool of 2 clones) in parallel with 4T1-ERR α AF2 (pool of 3 clones) cell lines (5×10^5 cells in 100 μ L of PBS). Radiographs (LifeRay HM Plus, Ferrania) of animals were taken at 15 days after inoculation using X-ray (MX-20; Faxitron X-ray Corporation). The extent of bone destruction for each animal was expressed in mm². Animals were sacrificed and hind limbs were then collected for histology and histomorphometric analysis. Tibiae were scanned using microcomputed tomography (Skyscan1076, Skyscan, Belgium) with an 8.8 voxel size and an X-ray tube (50 kV; 80 mA) with 0.5 μ m aluminum filter and three-dimensional reconstructions were performed with a dedicated visualization software (NRecon&CTVox, and Skyscan) (17). Bone Volume/Tissue Volume: (%BV/TV) were carried out with CTAn (version 1.9, Skyscan) and CTVol (version 2.0, Skyscan) software. Dissected bones were then processed for histological (Goldner's Trichrome solution staining) and histomorphometric analyses (tumor burden-to-soft tissue volume (%TB/STV)) (17). Depletion of CD8⁺ T cells was performed by intra-peritoneal injection of anti-CD8 β (BioXCell, clone Lyt3.2; BE0223). 387.5 μ g per mouse were injected 4 times every two days, from day 10 after metastasis injection ie when osteolytic lesions start to be detectable.

Ethics statement

Mice were handled according to the French Ministerial Decree No.87-848 of 19 October 1987. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the Université-Lyon1 (France) (ethic committee CEEA-55 Comité d'Ethique en Expérimentation Animale-DR2014-44-DR2015-28).

Human sample meta-genomic analysis

Correlation analysis were performed using published datasets downloaded from the Gene-Expression-Omnibus including primary tumor, no-metastases, Visceral+bone or only BM (GSE12276-GSE2034-GSE2603) (n=248) (18)(19)(20). Z-scores were calculated

on normalized data of each dataset by subtracting the population mean from individual expression values for each gene and then dividing the difference by the population standard deviation.

Sign arrays

5 The expression levels of several chemokines known to influence T cell chemo-attraction were obtained by qPCR Sign Arrays (Cytokines Array and Inflammation Array). Indeed, two qPCR Sign Arrays: Cytokines Array (AnyGenes, CT1M1) (CliniSciences) and Inflammation Array (AnyGenes, IF1M1) (CliniSciences) were used to quantify expression of cytokines, chemokines and growth factors. Total RNA was extracted from 4T1-CT and 4T1-ERR α cells
10 and 2 μ g were reverse-transcribed as previously described (16). Real-Time PCR was performed according to the manufacturer's instructions. Two heat maps were generated using the heatmap.2 function in the gplots library of R (version 3.5.1). Only regulations that were reproducible between the two arrays are presented.

Protein-protein interaction network reconstruction and analysis.

15 The protein-protein interaction network with BIOGRID (release 3.4.160) from Homo sapiens with PSICQUIC (Proteomics-Standard-Initiative-Common-QUery-InterfaCe) retrieval (10242018) and Cytoscape environment was used (21). A BIOGRID (<https://thebiogrid.org/>)-based custom approach was used to define a protein interactome of the following proteins: ESRRA-CCL17- CCL20-OPG-NRIP1-SRC1-SRC2-SRC3-PGC1A-PGC1B-CCR4 and
20 CCR6. The resulting interactome encompasses 911 proteins (hereby defined as "Extended Network of ESRRA, CCL17, CCL20"). To determine the connectors between CCL17, CCL20 and ESRRA, a custom approach combining shortest path and connectivity degree analysis was applied to determine a "Minimal Network of ESRRA, CCL17, CCL20" (containing 101 proteins) acknowledging connections that may support ESRRA signaling (22). We overlaid and
25 extracted information from the Gene-Ontology-consortium to pinpoint proteins that are already known to be involved in the immune system process, as well as T and B cell homeostasis (GO-IDs: 0002376, 0043029, 0001782) to create "Minimal Network specific to immune response to tumor" (containing 52 proteins). To determine the connectors between ESRRA and CCL17, ESRRA and CCL20, a shortest path was applied to the "Minimal Network of ESRRA, CCL17
30 and CCL20". For refereeing purposes, the generated network maps were up-loaded on NDEx (<http://www.ndexbio.org/#/>) with a temporary access reachable for the extended network or the minimal network (23)(24)(25). This temporary link will be replaced by a permanent one on the NDEx website and indexed with granted DOIs.

Ex vivo cell preparation

For hind limbs, muscles were removed and bones were sliced, then incubated at 37°C with a 1/10 solution of collagenase hyaluronidase (Stem cell) for 2 hours. Bones were then mechanically disrupted with a syringe plunger, filtered and cells were collected. For lung metastases (LM), lungs were crushed with a syringe plunger on a filter (100 µm) (BD Bioscience) and cells were collected. Cells released from lungs and bones were incubated at 37°C in the presence of DMEM (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Perbio/Thermo Scientific) and 6-thioguanine (Sigma A4882) (10 µg/mL) for 2 and 4 weeks, respectively. The cells were then counted after being stained using Crystal Violet (RAL diagnostic 317980).

Flow Cytometry

Cells from spleen and lungs, obtained after mechanical disruption, and flushed bone marrow cells were pre-incubated with anti-CD16/32 (93 clone, Biolegend) and stained for surface marker for 30 minutes at 4°C with the following antibodies: anti-CD45 (30-F11 clone, BD or eBiosciences), anti-CD3e (145-2C11 clone, BD), anti-CD4 (GK1.5 clone, BD), anti-CD8 (53-6.7 clone, BD or eBiosciences), anti-CD19 (1D3 clone, BD), anti-CD11b (M1/70 clone, eBiosciences), anti-CD11c (N418 clone, eBiosciences), anti-CCR4 (2G12 clone Biolegend) anti-CCR6 (29-2217 clone Biolegend), anti-Ly6C (AL21 clone, BD), anti-Ly6G (1A8 clone, BD), anti-F4/80 (BM8 clone, Biolegend), anti-CD107a (LAMP-1)(1D4B clone, BD), anti-FasL (MFL3 clone, eBiosciences). For cytokine production, cells were first incubated for 4 hours with PMA (P1585-1MG, Sigma), Ionomycin (I0634-1MG, Sigma) and Brefeldin A (00-4506-51, Life Technologies). Intracellular staining was performed with the Transcription Factor Staining Buffer Set (00-5523-00, eBiosciences), according to manufacturer's recommendations. The following antibodies were used: anti-Foxp3 (R16-715 clone, BD), anti-IFN-γ (XMG1.2 clone, BD), anti-GzA (GzA-3G8.5 clone, eBiosciences), anti-GzB (GB11 clone, Invitrogen), anti-Ki67 (11F6 clone Biolegend), anti-pSMAD2/3 (D27F4 clone, Cell Signalling) coupled with anti-rabbit A488. CD8⁺ T cell depletion was checked by flow cytometry on metastatic bone marrow and spleen using anti-CD8α (53-6.7 e-Biosciences). Data were acquired on a LSR-II (BD Biosciences) and analyzed with the FlowJo software version X.

Determination of ERRα binding sites

The analysis of ERRα binding sites on TCA AGGTCA promoter regions was performed using the GTRD ("Gene Transcription Regulation Database") that includes ChIP-seq data (<http://gtrd.biouml.org/>)(26).

Chromatine immunoprecipitation (ChIP)

ChIP assays were performed as previously described from MDA-MB231-B02-CT and -ERR α cells (Fradet et al, 2010) using either a monoclonal rabbit anti-ERR α (13826)(Cell-Signaling) or a control rabbit IgG(2729) antibody (Cell-Signaling). The immune-precipitated
5 genomic DNA was purified using NucleoSpin Clean-up columns (Macherey-Nagel, Germany) and analyzed by qPCR. Quantification of ChIP enrichment was calculated relative to input values. Distal and proximal elements of ERR α gene were used as negative and positive controls respectively (Deblois et al. 2016).

Real time RT-PCR

10 Total RNAs were extracted with Trizol-reagent (Life-Technologies) and 2 μ g were reverse-transcribed using qScriptTM cDNA SuperMix (Quanta-Biosciences). Real-time PCR was performed on a Mastercycler-ep-Realplex (Eppendorf) with primers specific to human and mouse genes using Quantifast-SYBR-Green (Life-Technologies) according to the manufacturer's instructions. The ribosomal protein RPL32 (L32) gene was used as a
15 housekeeping gene for quantification and relative results expressed as fold differences equal to $2^{-\Delta\Delta C_t}$.

Histology-Immunocytochemistry

Tibia bearing metastases as well as lungs were fixed in 4% PFA (paraformaldehyde) (Antigenfix Diapath P0014), embedded in paraffin (Histowax Histolab 00403) then cut (5 μ m
20 sections) on a microtome (Microm HM 350S). Immunocytochemical analyses were performed by incubating tissue sections overnight with goat polyclonal antibody ERR α (V-19, Santa Cruz) (1/40), rabbit polyclonal anti-human/mouse CCL17 (PA5-34515, ThermoFisher) (1/100), rabbit polyclonal anti-mouse CCL20 (ab139585, Abcam) (1/100), rabbit polyclonal anti-human/mouse activated TGF- β 3 (ab15537, Abcam) (1/100). Sections were then incubated with
25 HRP-conjugated anti-mouse (K4000, Dako) and anti-rabbit (K4002, Dako) according to the manufacturer's recommendations or anti-goat (sc2020, Santa Cruz)(1/300) antibodies for 1 hour and were detected using 3,3'-diaminobenzidine (K3467, Dako) according to the manufacturer's instructions. Counterstaining was performed using Mayer's hematoxylin (Merck) according the supplier's protocol. Lungs sections were made at three different depths
30 for each mouse and stained with H&E. Metastasis counting was performed in double blind.

TUNEL assay

Bone sections were deparaffinized and rehydrated followed by permeabilization with 0.2% triton (T9284, Sigma) and digestion with proteinase K (1 μ g/mL) (K182001, ThermoFisher). For positive control, sections were incubated with DNase I at 1mg/mL (Sigma,

11284932001). Sections were then incubated with biotin-16-dUTP (Sigma, 11093070910) and TUNEL enzyme (Sigma, 11767305001) in deoxynucleotidyltransferase buffer (Tris-HCl 125mM (Euromedex, EU0011), sodium cacodylate 200mM (Sigma, C0250), BSA 6mM (Sigma, A7906), CoCl₂ 1mM (Sigma, 15862-1ml-F)) at 37°C for 60 minutes in a humid atmosphere. Sections were washed in stop buffer (300mM NaCl (Sigma, S3014), 30mM NaC₆H₅O₇-sodium citrate (**Sigma, 71406**)) and blocked with 2% BSA (Sigma, A7906). Sections were then labelled with streptavidin-phycoerythrin (PE) (eBiosciences, 12-4317-87) and DAPI (Euromedex, 1050-A) and mounted with Fluoromount (Sigma, F4680-25ml) (upright microscope zeiss axioimager (sip 60549)).

10 ***Real time RT-PCR***

Total RNAs were extracted with Trizol-reagent (Life-Technologies) and 2µg were reverse-transcribed using qScript™ cDNA SuperMix (Quanta-Biosciences). Real-time PCR was performed on a Mastercycler-ep-Realplex (Eppendorf) with primers specific to human and mouse genes using Quantifast-SYBR-Green (Life-Technologies) according to the manufacturer's instructions. The ribosomal protein L32 gene was used as a housekeeping gene for quantification and relative results expressed as fold differences equal to $2^{-\Delta\Delta C_t}$.

15 ***Statistical analyses***

Data were analyzed statistically using either the non-parametric Mann-Whitney U test or unpaired t-test for in vivo studies (n = 10 mice for each group (bioStaTGV), unblinded studies). In vivo data on bone were confirmed (n = 3) on smaller groups (n = 4). In vitro assays were repeated at least twice and performed on triplicate samples. Data were analyzed using ANOVA and paired Student t-test to assess the differences between groups. All data are presented as means ± SEM with similar variances between groups. Correlation scores for meta-analysis were calculated using the Pearson-correlation-coefficient. Statistical significance was determined by GraphPad Prism v5.02 using the two-sided Student t-test. All statistical analyses were performed using the GraphPad Prism software (San Diego, USA). P-values less than 0.05 were considered statistically significant.

25 **Results:**

30 ***ERRα expression in BCa cells inhibits metastases growth in bones***

In order to assess the role of ERRα in BCa cells after tumor cell-anchorage in the bone microenvironment, BALB/c mice were intra-arterially injected with first a pool of three independent 4T1 tumor cell clones over-expressing-ERRα (4T1-ERRα), and a pool of two 4T1 tumor cell clones transfected with empty vector controls (4T1-CT) (16). Remarkably, fifteen

days later, radiographic analysis revealed that animals bearing 4T1-ERR α tumors had osteolytic lesions that were 70% smaller than those of mice bearing 4T1-CT tumors (9.39 ± 2.6 vs 3.08 ± 1.45 mm²) (**Fig. 1A**). The inhibitory effect of ERR α on BCa cell growth was associated with mild bone destruction (**Fig. 1C**). Histological and histomorphometric analyses also demonstrated the limitation of BM progression when BCa cells over-expressed ERR α (**Fig. 1E**). In clear contrast, when clones expressing a dominant-negative form (4T1-ERR α AF2) with their respective controls clones 4T1-CTaf2 were injected, we found a 60% increase in osteolytic lesions in animals bearing ERR α AF2 tumors compared to control (4T1-CTaf2) mice (3.82 ± 1.99 vs 9.27 ± 2.064 mm²) leading to their earlier sacrifice prior 4T1-CT(af2) BM reach the percentage of osteolysis observed in 4T1-CT (**Fig. 1B**). Concomitantly, increased bone destruction and tumor burden were observed (**Fig. 1D, 1F**). Given that 4T1 cells also colonize the lung (29), we analyzed the effects of ERR α expression on the development of LM. As opposed to the bone, both numbers of LM and numbers of BCa colonies extracted from the lungs were independent of the expression levels of ERR α in 4T1 cells (**data not shown**). Of note, the ERR α over-expression in LM was observed in animals bearing 4T1-ERR α tumors compared to control groups (**data not shown**). Taken together, this first set of data reveals that the over-expression of ERR α in BCa cells prevents their growth in the bone, and suggests that ERR α expression in BCa may affect the bone microenvironment to prevent BM progression.

ERR α expression in BCa cells increases T cell anti-tumor response in the bone

Given the importance of the immune response in the control of tumor growth, in particular metastases (5)(6)(7)(8), we next analyzed the effects of the expression of ERR α by BCa cells on the bone immune system. It is worth noting that no significant effect was observed on innate cells including dendritic cells, macrophages, with the exception of slight decrease in neutrophils (15%) in the bone colonized by 4T1-ERR α compared to 4T1-CT cells. However, we found that metastatic legs of animals bearing 4T1-ERR α tumors contained 5 times more T cells than those colonized with 4T1-CT cells (**Fig. 2A**). Moreover, in line with the larger 4T1-CT BM observed, which developed at the expense of the bone marrow that is largely depicted to sustain all stages of B cell medullary development (7)(8), the CD19⁺ compartment was 4-5 times lower in mice bearing 4T1-CT BM compared to 4T1-ERR α BM (**Fig. 2A**). Of note, T cell enrichment was neither observed in the lungs where 4T1-ERR α cells were also anchored (**Fig. 2B**), nor in the none invaded lymphoid organs such as the spleen, implying that the expression of ERR α in BCa cells affected the T cell homeostasis after their anchorage in the bone. Interestingly, the increase in T cell proportion after 4T1-ERR α cell bone settlement was largely restricted to the CD8⁺T lymphocyte compartment, with a 3.5-fold increase in their

number and percentage in bones bearing 4T1-ERR α tumors compared to bone colonized with 4T1-CT cells (**Fig. 2A**), whereas the proportion of CD4⁺T cells in the bone, including that of Foxp3⁺ regulatory T (Treg) cells was unaffected (data not shown).

In order to further characterize the CD8⁺ T cells over-represented in the bone after 4T1-ERR α cell colonization, we then analyzed their ability to produce cytotoxic molecules and cytokines. Strikingly, in bone colonized with 4T1-ERR α cells, we found that CD8⁺ T cells expressed higher levels of FasL, Granzyme (Granz) A and B, in association with LAMP1 at their cell surface as well as IFN- γ whereas these molecules were barely detectable in CD8⁺ T cells from bones bearing 4T1-CT cells (**Fig. 2C,D**). In total agreement with this exacerbated cytotoxic program of CD8⁺ T in the presence of 4T1-ERR α BCa cells in the bone, we found that a large fraction of 4T1-ERR α BM underwent apoptosis compared to 4T1-CT cells (**data not shown**). Importantly, no exacerbated sign of cytotoxic activity was observed in the non-invaded spleen or in the metastatic lungs of mice bearing 4T1-ERR α cells. Importantly, the depletion of CD8⁺ T cells was sufficient (**data not shown**) to increase 4T1-ERR α BM progression (**data not shown**). This set of data suggests that the expression of ERR α in BCa cells influences the CD8⁺ T cell homeostasis and increases their anti-tumor cytotoxic program in the bone allowing the control of the tumor progression.

ERR α expression leads to high levels of CCL17 and CCL20 production in BCa cells

The aforementioned data strongly suggest that the expression of ERR α by BCa cells influences the bone microenvironment to promote an efficient anti-tumor response. Interestingly, we failed to find any difference in Ki67 staining between CD8⁺T cells evolving with either 4T1-CT or 4T1-ERR α BM, suggesting that ERR α expression affected T cell-recruitment to the bone rather than their proliferation in the bone. In order to address this hypothesis, we monitored the expression levels of several chemokines known to influence T cell chemo-attraction and found a 2- and 3-fold up-regulation of Ccl17 and Ccl20 in 4T1-ERR α cells, respectively (**Fig. 3A**). C29 or XCT-790 were sufficient to inhibit the over-expression of both Ccl17 and Ccl20, while no effect was observed in 4T1-ERR α AF2 cells ruling out any side effects (**Fig. 3B, C**), arguing in favour of a direct role for ERR α in the control of the expression of these two chemokines (12)(13). This idea was reinforced following the analysis of ChIP-seq data revealing binding site for ERR α in the promoter of Ccl17 and in Ccl20. Of note, the ability of ERR α to up-regulate Ccl17 and Ccl20 was also observed in other BCa cells including MCF7 and MDA-MB-231-B02 cells (16)(17). Interestingly, the up-regulation of CCL17 and CCL20, at both gene and protein levels due to ERR α over-expression was sustained in 4T1-ERR α BM (**data not shown**) but lost in LM.

Given that CCL17 and CCL20 were reported to attract the fraction of activated CD8⁺ T cells expressing CCR4 and CCR6 (30), we next monitored the expression of both chemokine receptors on CD8⁺ T cells from the bone of animals bearing BM. In total agreement with the ability of 4T1-ERR α cells to sustain their production of CCL17 and CCL20 in the bone, and the activated phenotype of CD8⁺ T cells (**data not shown**), we found that, in bone colonized by 4T1-ERR α , a large fraction of CD8⁺ T cells expressed either CCR4 or CCR6 or both (**data not shown**). Thus, BCa cells over-expressing ERR α are endowed with a unique ability to produce high amounts of CCL17 and CCL20 and efficiently recruit activated CD8⁺ T cells to the bone.

ERR α expression in BCa cells reduces their TGF- β 3 production and decreases TGF- β signaling in bone metastases

Since the cytotoxic program of CD8⁺T cells was largely exacerbated in legs bearing 4T1-ERR α cells, we next assessed the mechanisms by which ERR α over-expression in BCa cells increased their cytotoxic function in the bone. To this end, we further investigated the connection between CCL17-CCL20 and ERR α (ESRRA) by choosing a global approach combining bioinformatic analyses of protein interaction networks and transcriptional regulator databases (21). We created the “Minimal Network specific to immune response to tumor” (containing 52 proteins) (**data not shown**) and by shortest path analysis, we then identified two new ESRRA-CCL17 or ESRRA-CCL20-associated regulators: VCAM and TGF- β 3 (**data not shown**). We and others reported that TGF- β signalling in T cells inhibits the cytotoxic differentiation program of CD8⁺ T cells both in humans and mice (31)(32), we thus focused on this cytokine. The analysis of Tgf- β 3 expression revealed a 70% decrease in 4T1-ERR α compared to 4T1-CT cells (**Fig. 4A**). Ex vivo bone cultures confirmed that ERR α up-regulation in BCa cells negatively regulates Tgf- β 3 expression with a 55% decrease compared to control BCa BM (**data not shown**). Strikingly, immunohistological staining revealed that the production of TGF- β 3 was largely decreased in BCa BM over-expressing ERR α (**data not shown**). In agreement with the decrease of Tgf- β 3 expression in 4T1-ERR α , we observed an increase in TGF- β 3 levels in 4T1-ERR α AF2 BM (**data not shown**) and a 5-fold increase in Tgf- β 3 expression after treatment of 4T1-ERR α cells with the inverse agonist XCT-790 (**Fig. 4B**). Similar results were observed in MCF7 and MDA-MB-231-B02 human cell lines ruling out an effect restricted to mouse 4T1 cells. Altogether these results reveal that the over-expression of ERR α represses the expression of TGF- β 3 in BCa and could thus prevent the BCa BM from creating an immunosuppressive microenvironment provided by TGF- β signal activation in immune cells.

In order to unconditionally confirm that CD8⁺T cell evolving in 4T1-ERR α colonized bone escape TGF- β signaling control, we next analyzed CD8⁺T cells from metastatic legs for the phosphorylation of SMAD2/3 proteins which translates specifically the TGF- β signaling activation (33). Clearly, SMAD2/3 phosphorylation was 2-3 times lower compared to that of CD8⁺ T cells from 4T1-CT metastatic legs (**data not shown**). Thus, in addition to increasing CD8⁺T cell recruitment to the bone, ERR α over-expression in BCa cells impairs their ability to produce high amounts of TGF- β 3, decreasing TGF- β signaling in CD8⁺T cells, a key repressor of their cytotoxic activity and capacity to eliminate cancer cells.

Over-expression of ESRRA (Err α) in patient tumours is associated with high levels of CCL17 CCL20 and low levels of TGF- β expression

Finally, we addressed the relevance of our data obtained in mice to the human pathology. We performed a meta-analysis on 248 patients including luminal and triple-negative breast tumors (TNBC) split into four groups: all tumors (n = 248), patients without metastases (No Mets, n = 121), patients that had visceral and bone metastases (Visceral+Bone metastases, n = 53) and patients that had only bone metastases (Bone Only, n = 74). As in mice, the ESRRA (Err α) expression was positively correlated with that of CCL17 and CCL20 and inversely proportional to that of TGF- β in patients with metastases restricted to the bone (Bone Only) with luminal and TNBC tumors (**Table 1A,B**). Correlations were also identified in no-Mets, All, and Visceral +Bone groups of luminal or TNBC patients (**Table 1A,B**). Thus, this set of data from human sample analyses strongly suggests that, similarly to mice, the over-expression of ERR α in human BCa cells allows them to create an immune-efficient environment in the bone by increasing the production of chemokines capable of attracting activated T cells to the bone and decreasing the production of TGF- β essential for repressing the cytotoxic activity of T cells.

Discussion:

Cancer cells adapt to the microenvironment, shaped by their own doing, which in turn influence their fate. This interplay is particularly important for cells forming metastases, which leave their primary microenvironment to settled in a new, second one. Here, we revealed that the level of ERR α expression on BCa metastases promotes their ability to condition an efficient anti-tumor CD8⁺ T cell response selectively in the bone.

CD8⁺ T cells have been described as critical inhibitors of bone metastases. Indeed, in mice, the alteration of CD8⁺ T cell development after metastases implantation in the bone, or the deprivation of CD8⁺ T cells, were reported to increase tumor growth (5)(6). Osteoclasts

have been depicted to secrete chemokines that can attract CD8⁺T cells (34). However, the regulation of the BM burden by CD8⁺T cells seems totally independent of the osteoclast activity (6). Our study reveals that the cancer cells per se can influence both the recruitment and the cytotoxic activity of the CD8⁺T cells in the bone. Moreover, the ability of the BCa metastases to condition the immune response in the bone can be in part orchestrated by the levels of expression ERR α on the BCa and potentially to the sensitivity of metastases to the ERR α ligand(s). The selective effects of ERR α expression in BCa on the tumor burden of BM and anti-tumor response in the bone strongly suggest that unlike the lung, the bone could constitute a microenvironment with high levels of the ERR α ligand(s) that so far remain uncharacterized. Another alternative is that in the lung, but not the bone could be highly enriched in inhibitors of ERR α signaling or negative regulators of ERR α expression that remain to be identified. Thus, the ability of the metastases to induce or not a potent immune response maybe dictated by both the tumor per se and the microenvironment where it is anchored. In the case of BCa cells, we propose to place ERR α at the core of this interplay between metastases and their new microenvironment

In addition to increasing the recruitment of activated CD8⁺T cells to the bone, the over-expression of ERR α on BCa cells also decreased their ability to produce high amounts of TGF- β 3. Depletion experiments confirmed that CD8⁺T cells are key anti-tumor immune cells whose activation and recruitment are controlled by the levels of ERR α expression on BM. All forms of TGF- β have been reported as potent immune-regulators and share a common receptor (35). While TGF- β 1 is predominant in the immune system, TGF- β 3 is mainly produced by muscles, bones but also by various cancer cells (36). The repression of TGF- β 3 production in ERR α BCa cells, subsequently affects TGF- β signaling in CD8⁺T cells present in the bone. TGF- β signaling represses the expression of numerous transcription factors associated with cytotoxicity, as well as T-Bet a key inducer of IFN- γ (37). Therefore, over-expressed ERR α BCa cells that settle in the bone are unable to sustain an immunosuppressive microenvironment based on high levels of TGF- β signaling in T cells and repression of cytotoxic program and IFN- γ production. Interestingly IFN- γ also contributes to the suppression of BM. Indeed, IFN- γ has been reported to reduce both RANKL expression and osteoclast formation, counterbalancing the aberrant bone resorption which facilitates tumor growth (38). Concomitantly, inhibition of bone resorption also leads to the decrease in TGF- β release from the bone matrix (4), thus potentially contributing to amplifying the activation of T cells including their production of IFN- γ .

It is likely that effector CD8⁺T cells that reach the BM have previously been primed in the draining lymph nodes or by the spleen-presenting antigens from the primary tumor and/or the metastases. As in the primary tumor, the activated CD8⁺T cell population in contact with the BM is actually heterogeneous and composed of cells recently activated and activated memory cells. Interestingly, in both mice and humans, the fraction of CD8⁺T cells that expresses CCR6 and CCR4 has been depicted to rapidly mount an efficient response, corresponding to activated /memory T cells (30). Once implanted in the bone, we found that the BCa over-expressing ERR α have unique ability to sustain high expression of CCL17 and CCL20 and low expression of TGF- β 3, thus attracting the activated/memory CD8⁺T cells whose their anti-tumor cytotoxic function is magnified by the lack of repression by TGF- β signaling.

In conclusion, this study assigns an unsuspected role for ERR α expression in BCa on the bone immune system that conditions the BM growth outcome, providing the mechanistic basis for understanding how ERR α expression in BCa can impact the bone microenvironment and reduce BM growth. ERR α seems to appear at the core of this interplay between BCa metastases and their new environment, integrating signals from the microenvironment to develop an efficient anti-tumor response. Therefore, we propose to consider ERR α expression on BCa, as a biomarker predictive of BM response to immunotherapies and /or as a good prognosis marker in BM progression once established, opening the path towards to the clinical use of ERR α agonist to relieve patients with ERR α positive BM after primary tumor resection.

TABLES:

A)

Correlation with <i>ESRR</i>	All (N = 248) Luminal		No Mets (N = 121)		Visceral + Bone (N = 53)		Bone Only (N = 74)	
	R	P-value	R	P-value	R	P-value	R	P-value
<i>CCL17</i>	0.4933	< 0.0001	0.2725	0.0025	0.4918	0.0002	0.5547	< 0.0001
<i>CCL20</i>	0.3145	< 0.0001	0.2209	0.0149	0.2136	0.1246	0.3058	0.0081
<i>TGFβ3</i>	- 0.449	0.0001	-0.215	0.0176	- 0.448	0.0007	-0.428	0.0001

B)

Correlation <i>ESRRA</i> with	All (N = 110) Triple negative		No Mets (N = 50)		Visceral + Bone (N = 53)		Bone Only (N = 16)	
	R	P-value	R	P-value	R	P-value	R	P-value
<i>CCL17</i>	0.313	0.0009	0.096	0.5038	0.254	0.0663	0.648	0.0067
<i>CCL20</i>	0.272	0.0040	0.190	0.1855	0.332	0.0153	0.604	0.0132
<i>TGFβ3</i>	- 0.465	0.0001	-0.327	0.0201	- 0.438	0.0010	-0.705	0.0022

Table 1: Correlation in clinics in BCa patients. (A, B) Meta-analysis of public datasets (GSE12276, GSE2034 and GSE2603) (n = 248) revealed a positive correlation between the expression of *ESRRA* and *CCL17* and *CCL20* and a negative correlation with *Tgf-β3* expression levels in luminal (A) and triple-negative (B) breast tumors. Correlation scores were calculated using the Pearson correlation coefficient. P-values less than 0.05 were considered statistically significant.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

1. Kan C, Vargas G, Pape F, Clézardin P. Cancer Cell Colonisation in the Bone Microenvironment. Int J Mol Sci. 2016 Oct 4;
2. Croucher P, McDonald M, Martin T. Bone metastasis: the importance of the neighbourhood. Nat Rev Cancer. 2016 May 25; 373–86.
3. Handforth C, D'Oronzo S, Coleman R, Brown J. Cancer Treatment and Bone Health. Calcif Tissue Int. 2018 Feb; 251–64.
4. Esposito M, Guise TA, Kang Y. The Biology of Bone Metastasis. Cold Spring Harb Perspect Med. 2018 Jun 1;
5. Bidwell B, Slaney C, Withana N, Forster S, Cao Y, Loi S, et al. Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape. Nat Med. 2012; 1224–31.
6. Zhang K, Kim S, Cremasco V, Hirbe A, Collins L, Piwnica-Worms D, et al. CD8⁺ T cells regulate bone tumor burden independent of osteoclast resorption. Cancer Res. 2011 Jul 15; 4799–808.

7. Mori G, D'Amelio P, Faccio R, Brunetti G. Bone-immune cell crosstalk: bone diseases. *J Immunol Res*. 2015;

8. Pacifici R, Weitzmann M. Immunobiology and bone. In: *Primer on the metabolic bone diseases and disorders of mineral metabolism*. ninth. 2019. p. 992–1003.

5 9. Ariazi EA, Clark GM, Mertz JE. Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. *Cancer Res*. 2002 Nov 15; 62(22):6510–8.

10. Deblois G GV. Oestrogen-related receptors in breast cancer: control of cellular metabolism and beyond. *Nat Rev Cancer*. 2013; 13(1):27–36.

10 11. Audet-Walsh E, Giguère V. The multiple universes of estrogen-related receptor α and γ in metabolic control and related diseases. *Acta Pharmacol Sin*. 2015 Jan; 36(1):51–61.

12. Patch RJ, Searle LL, Kim AJ, De D, Zhu X, Askari HB, et al. Identification of diaryl ether-based ligands for estrogen-related receptor alpha as potential antidiabetic agents. *Journal of medicinal chemistry*. 2011 Feb 10;54(3):788–808.

15 13. Willy PJ, Murray IR, Qian J, Busch BB, Stevens WC Jr, Martin R, et al. Regulation of PPARgamma coactivator 1alpha (PGC-1alpha) signaling by an estrogen-related receptor alpha (ERRalpha) ligand. *Proc Natl Acad Sci U S A*. 2004 Jun 15; 101(24):8912–7.

14. Tam I, Giguère G. There and back again: The journey of the estrogen-related receptors in the cancer realm. *J Steroid Biochem Mol Biol*. 2016 Mar;13–9.

20 15. Dwyer MA, Joseph JD, Wade HE, Eaton ML, Kunder RS, Kazmin D, et al. WNT11 expression is induced by estrogen-related receptor alpha and beta-catenin and acts in an autocrine manner to increase cancer cell migration. *Cancer Res*. 2010 Nov 15; 70(22):9298–308.

25 16. Vargas G, Bouchet M, Bouazza L, reboul P, Boyault C, Gervais M, et al. ERR α promotes breast cancer cell dissemination to bone by increasing RANK expression in primary breast tumors. *Oncogene*. 2018 Nov 26;

17. Fradet A, Sorel H, Bouazza L, Goehrig D, Depalle B, Bellahcene A, et al. Dual function of ERRalpha in breast cancer and bone metastasis formation: implication of VEGF and osteoprotegerin. *Cancer research*. 2011 Sep 1; 71(17):5728–38.

30 18. Bos P, Zhang X, Nadal C, Shu W, Gomis R, Nguyen D, et al. Genes that mediate breast cancer metastasis to the brain. *Nature*. 2009 Jun 18; 1005–9.

19. Wang Y, Klijn J, Zhang Y, Sieuwerts A, Look M, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet*. 2005 Feb 19; 671–9.

20. Minn A, Gupta G, Siegel P, Bos P, Shu W, Giri D, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005 Jul 28; 518–24.

21. Stark C, Breitkreutz B, Reguly T, Boucher L, Breitkreutz A, Tyers M. BioGRID: a general repository for interaction datasets. *Nucleic Acids Res*. 2006;

5 22. Scardoni G, Tosadori G, Pratap S, Spoto F, Laudanna C. Finding the shortest path with PesCa: a tool for network reconstruction. *F1000Res*. 2015 Aug 5;

23. Pratt D, Chen J, Welker D, Rivas R, Pillich R, Rynkov V, et al. NDEx, the Network Data Exchange. *Cell Systems*. 2015; 302–5.

24. Pillich R, Chen J, Rynkov V, Pratt W, Pratt D. NDEx: A Community Resource
10 for Sharing and Publishing of Biological Networks.

25. Pratt D, Chen J, Pillich R, Rynkov V, Gary A, Demchak B, et al. NDEx 2.0: A Clearinghouse for Research on Cancer Pathways. *Cancer Res*. 2017 Nov 1;58-61.

26. Yevshin I, Sharipov L, Valeev T, Kel A, Kolpakov F. GTRD: a database of transcription factor binding sites identified by ChIP-seq experiments. *Nucleic Acids Res*. 2017;
15 D61–7.

27. Peyruchaud O, Winding B, Pecheur I, Serre CM, Delmas P, Clezardin P. Early detection of bone metastases in a murine model using fluorescent human breast cancer cells: application to the use of the bisphosphonate zoledronic acid in the treatment of osteolytic lesions. *Journal of bone and mineral research : the official journal of the American Society for*
20 *Bone and Mineral Research*. 2001 Nov; 16(11):2027–34.

28. Patch R, Searle L, Kim A, De D, Zhu X, Askari H, et al. Identification of diaryl ether-based ligands for estrogen-related receptor alpha as potential antidiabetic agents. *J Med Chem*. 2011 Feb 10; 788–808.

29. David M, Ribeiro J, Descotes F, Serre CM, Barbier M, Murone M, et al.
25 Targeting lysophosphatidic acid receptor type 1 with Debio 0719 inhibits spontaneous metastasis dissemination of breast cancer cells independently of cell proliferation and angiogenesis. *Int J Oncol*. 2012; 1133–41.

30. Ferguson A, Engelhard V. CD8 T cells activated in distinct lymphoid organs differentially express adhesion proteins and coexpress multiple chemokine receptors. *J*
30 *Immunol*. 2010 Apr 15; 4079–86.

31. He X, He X, Dave V, Zhang Y, Hua X, Nicolas E, et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature*. 2005 Feb 24; 826–33.

32. Marie J, Liggitt D, Rudensky A. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity*. 2006 Sep; 441–54.
33. Derynck R, Zhang Y. Smad-dependent and Smad-independent pathways in
5 TGF-beta family signalling. *Nature*. Oct 9; 425(6958):577–84.
34. Kiesel J, Buchwald Z, Aurora R. Cross-presentation by osteoclasts induces FoxP3 in CD8+ T cells. *J Immunol*. 2009 May 1; 5477–87.
35. Chen W, Ten Dijke P. Immunoregulation by members of the TGFβ superfamily. Chen W1, Ten Dijke P2. *Nat Rev Immunol*. 2016 Nov 25; 723–40.
- 10 36. Letterio J, Roberts A. Regulation of immune responses by TGF-beta. *Annu Rev Immunol*. 1998; 137–61.
37. Oh S, Mo L. TGF-β: guardian of T cell function. *J Immunol*. 2013 Oct 15;3973–
9.
38. Nagy E, Lei Y, Martínez-Martínez E, Body S, Schlotter F, Creager M, et al.
15 Interferon-γ Released by Activated CD8+ T Lymphocytes Impairs the Calcium Resorption Potential of Osteoclasts in Calcified Human Aortic Valves. *Am J Pathol*. 2017 Jun; 1413–25.

CLAIMS:

1. A method of treating bone metastases in a patient in need thereof comprising administering a therapeutically effective amount of an $ERR\alpha$ agonist.
2. A method of increasing the amount of tumor infiltrating cytotoxic T lymphocytes cells
5 in bone metastases comprising administering to the patient a therapeutically effective amount of an $ERR\alpha$ agonist.
3. The method of claim 1 or 2 wherein the patient suffers from breast cancer bone metastases.
4. The method of claim 1 wherein the $ERR\alpha$ agonist is administered to the patient in
10 combination with an immune checkpoint blockade inhibitor.
5. The method of claim 4 wherein the immune checkpoint inhibitor is selected from the group consisting of PD-1 antagonist, PD-L1 antagonist, PD-L2 antagonist CTLA-4 antagonist, VISTA antagonist, TIM-3 antagonist, LAG-3 antagonist, IDO antagonist, KIR2D antagonist, A2AR antagonist, B7-H3 antagonist, B7-H4 antagonist, and BTLA
15 antagonist.
6. The method of claim 4 wherein the immune checkpoint inhibitor is selected from the group consisting of Ipilimumab, Nivolumab, Pembrolizumab, Atezolizuma, Avelumab, Durvalumab and Cemiplimab.
7. A method for predicting the survival time of a patient suffering from bone metastases
20 comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient wherein said level correlates with the survival time of the patient.
8. The method of claim 7 that comprises the steps of i) determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii)
25 concluding that the patient will have a long survival time when the level determined at step i) is higher than the predetermined reference value or concluding that the patient will have a short survival time when the level determined at step i) is lower than the predetermined reference value.

34 -

9. The method of claim 7 or 8, wherein the patient suffers from breast cancer bone metastases.

5 10. A method for determining whether a patient suffering from bone metastases will achieve a response with an immune checkpoint blockade therapy comprising determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, wherein said expression indicates whether the patient will achieve or not a response

10 11. The method of claim 9 that comprises the steps of i) determining the expression level of $ERR\alpha$ in a tumor tissue sample obtained from the patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will achieve a response when the level determined at step i) is higher than the predetermined reference value.

12. The method of claim 10 or 11, wherein the patient suffers from breast cancer bone metastases.

15

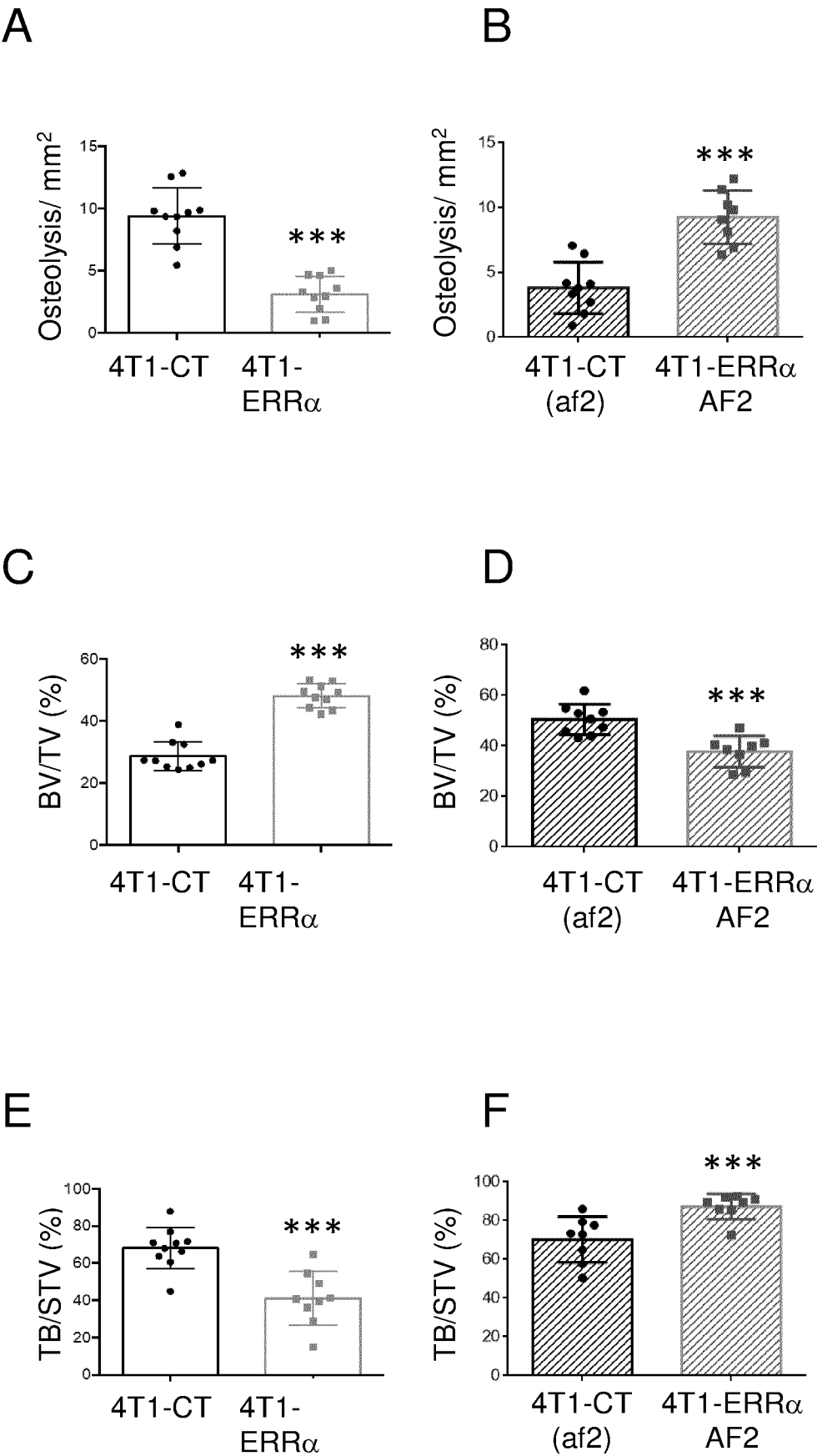


Figure 1

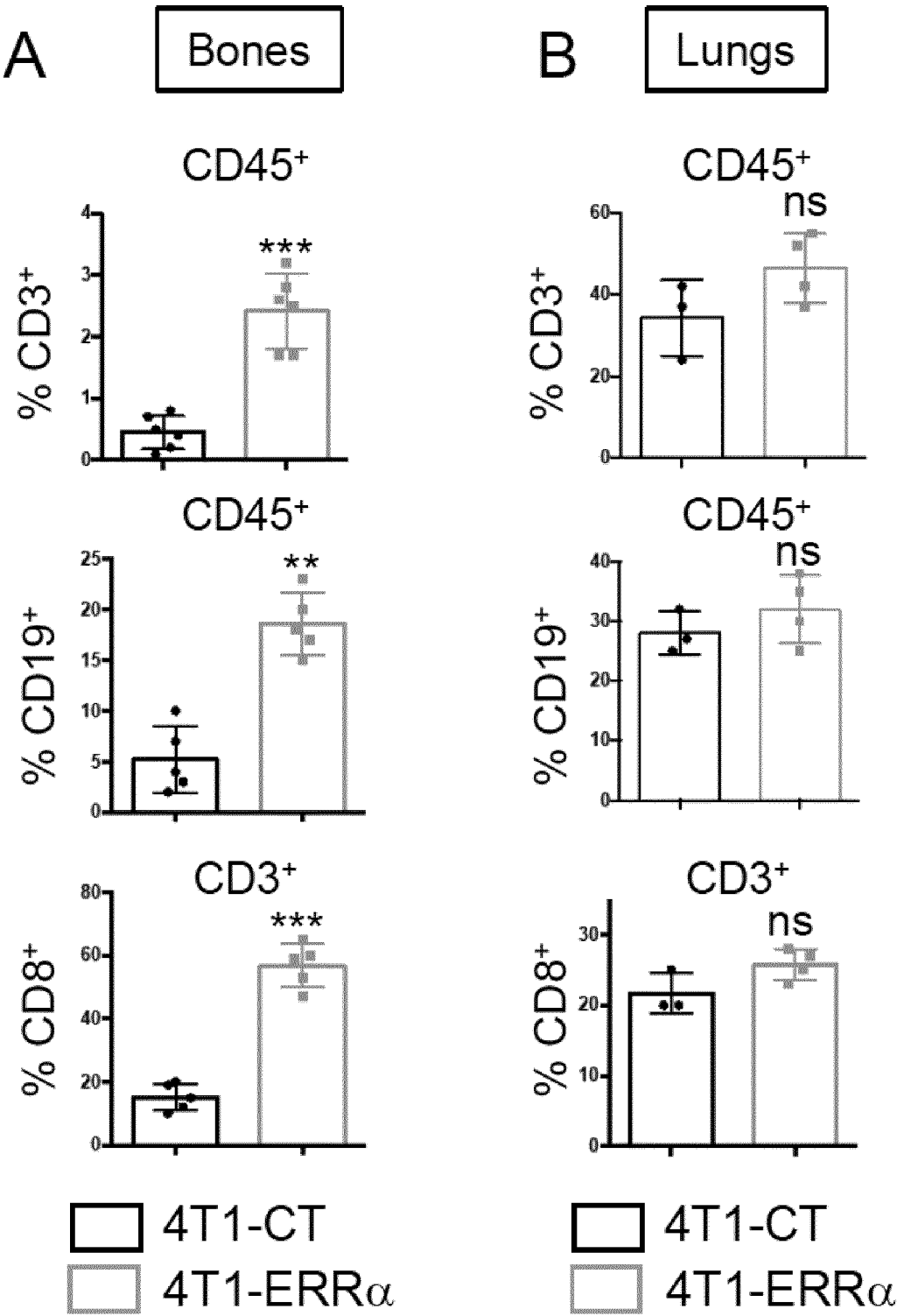


Figure 2A, B

3/6

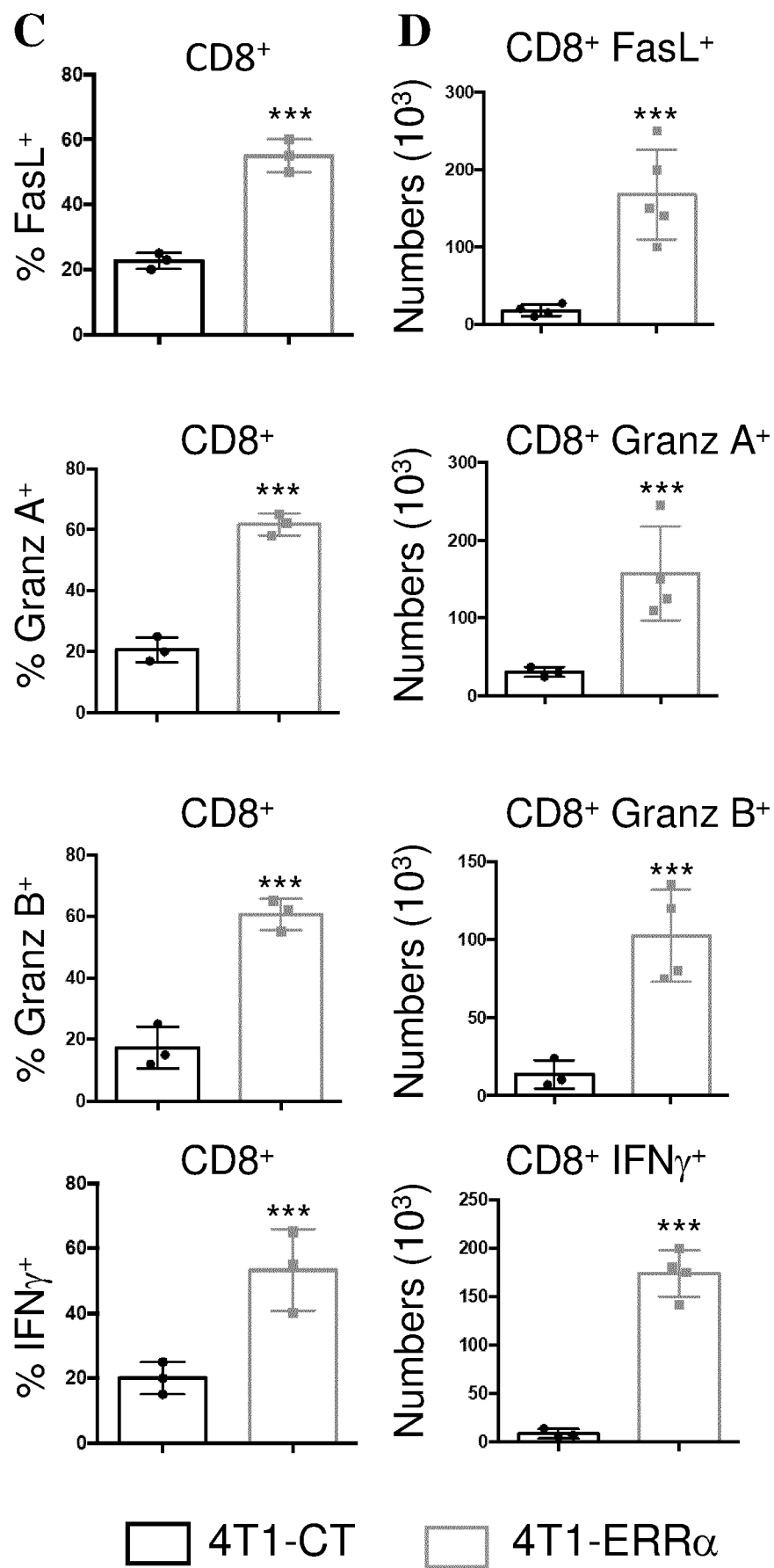


Figure 2C, D

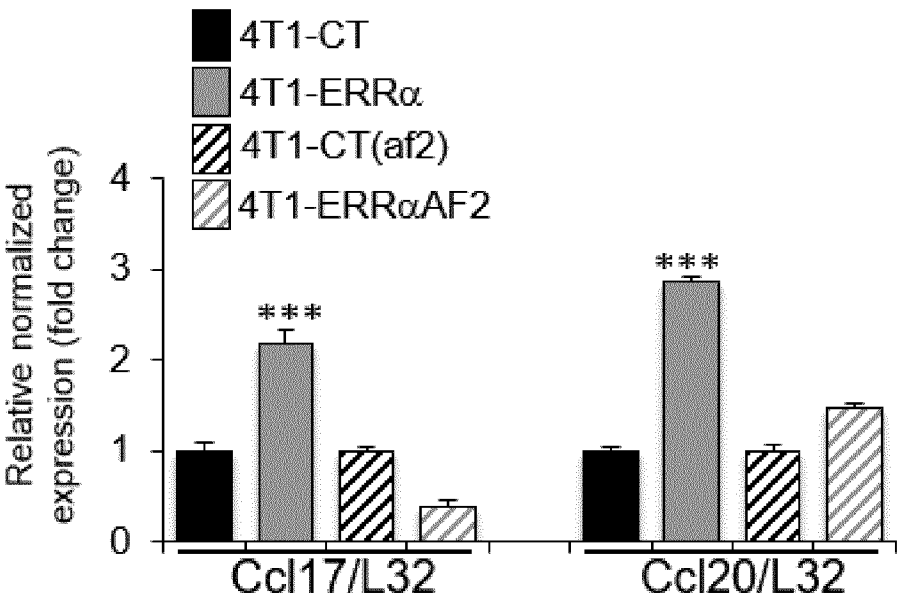


Figure 3A

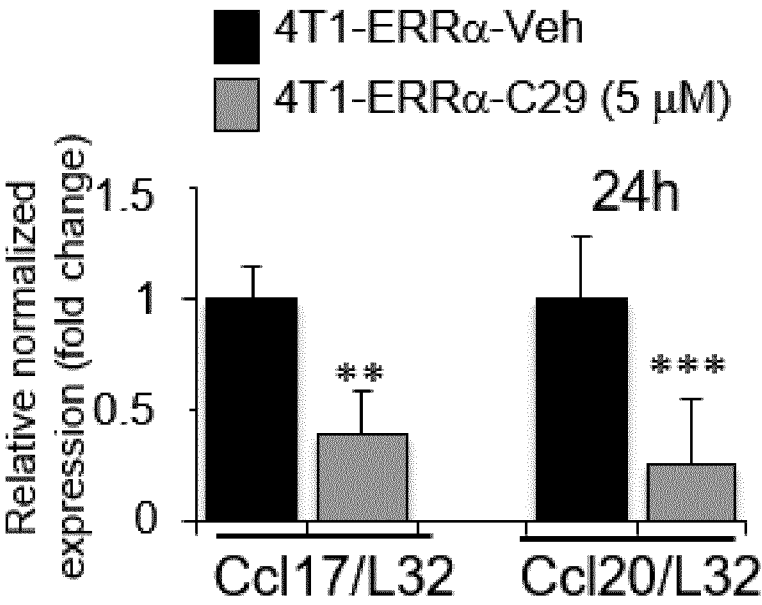


Figure 3B

5/6

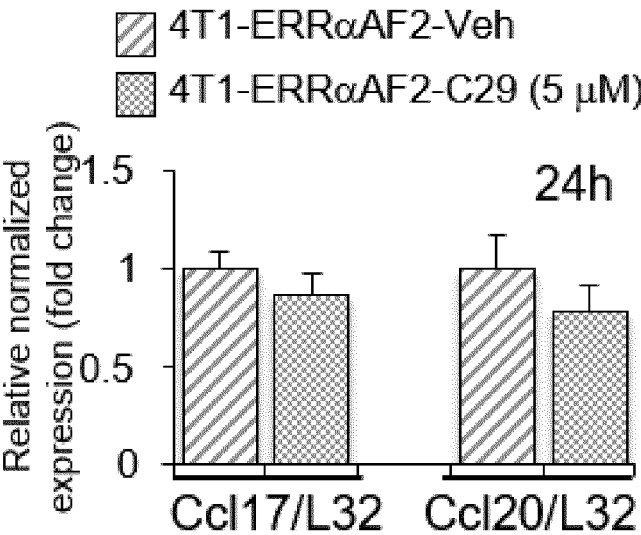


Figure 3C

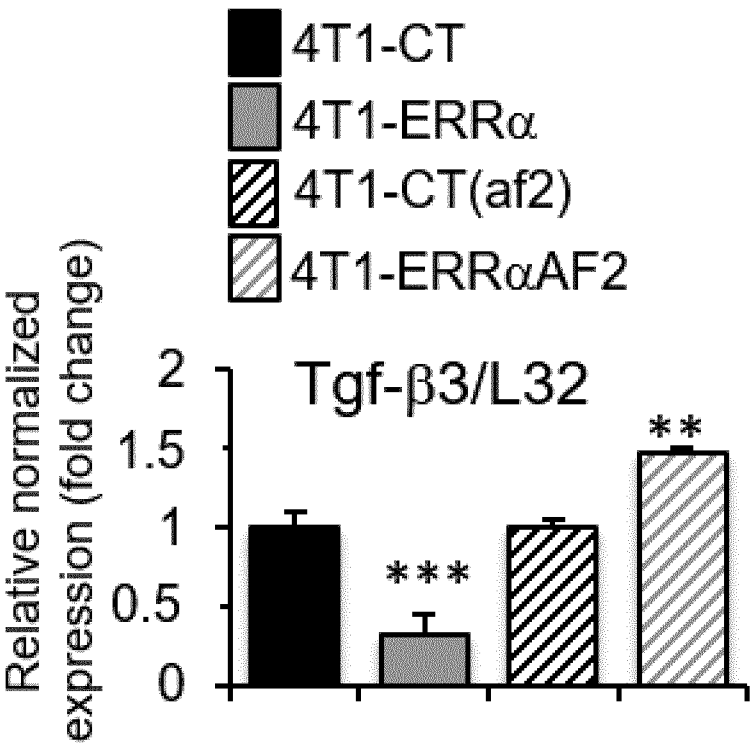


Figure 4A

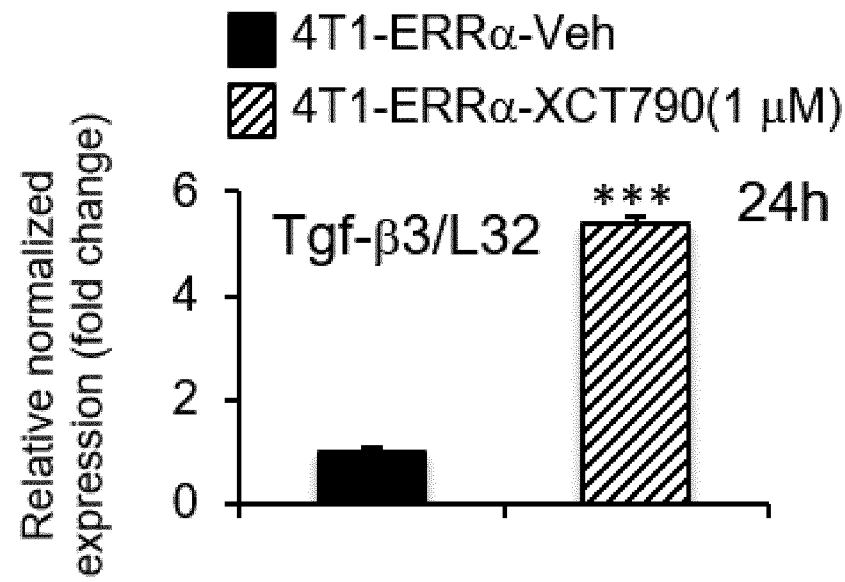


Figure 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/056931

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/352 A61K31/575 A61K31/7048 A61K45/06 G01N33/74
A61P35/04 G01N33/574
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/119505 A1 (AUBIN JANE E [CA] ET AL) 13 May 2010 (2010-05-13)	1-3
Y	claims 1,5,6	1-12
X	US 2009/258845 A1 (BURNETT BRUCE P [US] ET AL) 15 October 2009 (2009-10-15) paragraph [0057]; claims 1-29	1,2
X	WO 2012/044577 A1 (EXELIXIS INC [US]; SMITH DAVID [US]; HUSSAIN MAHA [US]) 5 April 2012 (2012-04-05) claims 12,18	1,2
X	WO 2012/104210 A1 (INST NAT SANTE RECH MED [FR]; UNIV CLAUDE BERNARD LYON [FR] ET AL.) 9 August 2012 (2012-08-09)	7-9
Y	page 9, line 24 - page 10, line 12; claims 10,11; example 4	1-12
	-/--	

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 June 2021

Date of mailing of the international search report

14/06/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Durrenberger, Anne

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/056931

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2018/214583 A1 (SCHOLZ ARNE [DE]) 2 August 2018 (2018-08-02) claims 2,16 -----	4-6
A	VARGAS G ET AL: "ERR[alpha] promotes breast cancer cell dissemination to bone by increasing RANK expression in primary breast tumors", ONCOGENE, NATURE PUBLISHING GROUP UK, LONDON, vol. 38, no. 7, 26 November 2018 (2018-11-26), pages 950-964, XP036702971, ISSN: 0950-9232, DOI: 10.1038/S41388-018-0579-3 [retrieved on 2018-11-26] cited in the application the whole document -----	1-12
A	A. FRADET ET AL: "Dual Function of ERR in Breast Cancer and Bone Metastasis Formation: Implication of VEGF and Osteoprotegerin", CANCER RESEARCH, vol. 71, no. 17, 1 September 2011 (2011-09-01), pages 5728-5738, XP055756314, US ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-11-1431 cited in the application the whole document -----	1-12
A	CLÉZARDIN P: "Pathophysiology of bone metastases", ONCOLOGIE, SPRINGER- VERLAG FRANCE, PARIS, FR, vol. 17, no. 3, 18 March 2015 (2015-03-18), pages 69-74, XP035477086, ISSN: 1292-3818, DOI: 10.1007/S10269-015-2489-4 [retrieved on 2015-03-18] page 71, right-hand column -----	1-12
A	US 2016/169896 A1 (BONNELYE EDITH [FR] ET AL) 16 June 2016 (2016-06-16) claims -----	1-12
A	US 2016/032400 A1 (GOMIS ROGER [ES] ET AL) 4 February 2016 (2016-02-04) claims -----	1-12
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/056931

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MANUEL SCIMECA ET AL: "Breast cancer metastasis to bone: From epithelial to mesenchymal transition to breast osteoblast-like cells", SEMINARS IN CANCER BIOLOGY., 1 February 2020 (2020-02-01), XP055756312, US ISSN: 1044-579X, DOI: 10.1016/j.semcancer.2020.01.004 the whole document -----	1-12
A	FONTANELLA CATERINA ET AL: "Skeletal metastases from breast cancer: pathogenesis of bone tropism and treatment strategy", PLOS BIOLOGY, RAPID SCIENCE PUBLISHERS, DORDRECHT, vol. 32, no. 8, 7 September 2015 (2015-09-07), pages 819-833, XP035904495, ISSN: 0262-0898, DOI: 10.1007/S10585-015-9743-0 [retrieved on 2015-09-07] page 822 -----	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/056931

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010119505 A1	13-05-2010	AU 6814100 A CA 2284103 A1 US 2006211049 A1 US 2010119505 A1 WO 0122988 A1	30-04-2001 30-03-2001 21-09-2006 13-05-2010 05-04-2001
US 2009258845 A1	15-10-2009	US 2009258845 A1 US 2013078318 A1 US 2015190365 A1	15-10-2009 28-03-2013 09-07-2015
WO 2012044577 A1	05-04-2012	AU 2011307309 A1 CA 2812744 A1 CN 103391773 A EP 2621483 A1 JP 2013537918 A US 2014057908 A1 WO 2012044577 A1	02-05-2013 05-04-2012 13-11-2013 07-08-2013 07-10-2013 27-02-2014 05-04-2012
WO 2012104210 A1	09-08-2012	EP 2670862 A1 US 2013316359 A1 WO 2012104210 A1	11-12-2013 28-11-2013 09-08-2012
US 2018214583 A1	02-08-2018	EP 3334465 A1 US 2018214583 A1 WO 2017025496 A1	20-06-2018 02-08-2018 16-02-2017
US 2016169896 A1	16-06-2016	EP 2909333 A1 US 2016169896 A1 WO 2014060477 A1	26-08-2015 16-06-2016 24-04-2014
US 2016032400 A1	04-02-2016	AU 2014229505 A1 BR 112015023783 A2 CA 2906394 A1 CN 105324491 A EP 2971113 A2 JP 6577873 B2 JP 2016516403 A KR 20150122731 A US 2016032400 A1 WO 2014140933 A2	03-09-2015 24-10-2017 18-09-2014 10-02-2016 20-01-2016 18-09-2019 09-06-2016 02-11-2015 04-02-2016 18-09-2014