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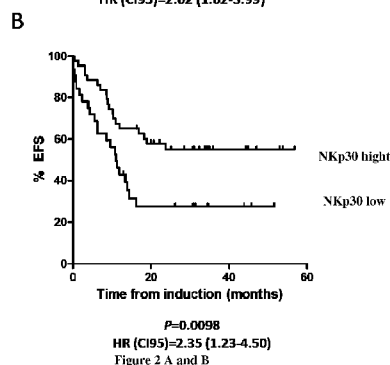
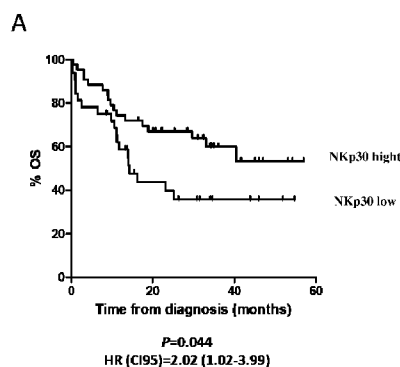
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(57) Abstract: The present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML). The therapeutic management of patients, classified as intermediate in the ELN classification is difficult. By following studies on a cohort of 202 patients, the inventors show that NKp30 could be useful for reclassifying 40% patients with intermediate prognosis in the group of adverse prognosis. This biomarker can thus rendered the patients eligible to appropriate post remission therapy (i.e. chemotherapy and allograft). Thus, the present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising the step of determining in a sample (e.g. fresh total peripheral blood samples or peripheral blood mononuclear cells) the expression level of NKp30. Typically the level is determined by flow cytometry.

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BIOMARKER FOR OUTCOME IN AML PATIENTS

FIELD OF THE INVENTION:

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The invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

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BACKGROUND OF THE INVENTION:

Patient stratification at diagnosis for acute myeloid leukemia (AML) is crucial for clinical decision making regarding post remission therapy. To date, patient stratification is based on cytogenetic and molecular classifications [Grimwade D et al., 1998 and Slovak ML et al., 2000]. As proposed by the European Leukemia Net (ELN) genetic classification based on FLT3/CEBP α /NPM1 mutational status further refines patient stratification, but clinical uncertainty remains with an unclassifiable group of patients with intermediate prognosis [Dohner H et al, 2010 and Stelljes M et al, 2013].

New molecular markers have been shown to impact prognosis and may be included in future revision of ELN classification [Dohner H et al, 2010; Grimwade D et al, 2010 and Patel JP et al, 2012] However, molecular markers do not account for the entire prognostic heterogeneity of AML and new markers are warranted. In this context, accurate estimation of the risk of relapse at diagnosis or after complete remission in patients with intermediate prognosis is essential for physicians in order to evaluate the potential benefits of intensive chemotherapy and allogeneic stem cell transplantation (allo-SCT).

Among candidate biomarkers, immune parameters are currently extensively evaluated in immunomonitoring studies. Among immune effectors implicated in immune surveillance in AML, Natural Killer (NK) cells are of particular importance. NK cells are key components of the innate immunity and substantially contribute to the antitumor immune responses, in

particular in the context of AML. NK cells are crucial immune effectors and play a key role in tumor rejection, with direct effect on tumor cells as well as an important role in regulation of the adaptive immune response through the cross-talk with antigen presenting cells. NK cells prevent emergence of transformed cells, and are involved in response to chemotherapy and radiotherapy. In AML, their direct effect on the tumor burden is illustrated by the success of hematopoietic stem cell transplantation with KIR-HLA mismatch in hematologic malignancies. NK cell anti-tumor activity is triggered by NK activating receptors, including Natural Cytotoxic Receptors (NCR) such as NKp30. Decay in anti-tumor activity of NK cells, related to defective activating NK receptor expression has been extensively described in many cancers. In particular, it has been previously shown that NCR expression at diagnosis is a potential discriminant biomarker in AML and in solid tumors. Hence, our group previously reported that low NKp30 expression on NK cells was significantly associated with reduced OS in AML.

SUMMARY OF THE INVENTION:

In this study, the inventors provide a formal validation of NKp30 prognostic value, as well as prospective evaluation and retrospective validation of this prognostic value in the subgroup of patients with intermediate cytogenetic risk and classified as intermediate in the ELN classification. More, they show that NKp30 recovery after complete remission is a better predictor of clinical outcome than NKp30 at diagnosis.

Thus, the invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

DETAILED DESCRIPTION OF THE INVENTION:

A first aspect of the invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample

obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

In one embodiment, the invention relates to a method for predicting the overall survival (OS) of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

In another embodiment, the invention relates to a method for predicting the event-free survival (EFS) of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

A second aspect of the invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) after complete remission comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

In one embodiment, the invention relates to a method for predicting predicting the overall survival (OS) of a patient suffering from acute myeloid leukemia (AML) after complete remission comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined

reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

5 In another embodiment, the invention relates to a method for predicting the event-free survival (EFS) of a patient suffering from acute myeloid leukemia (AML) after complete remission comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at
10 step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.

As used herein, the term “Overall survival (OS)” denotes the time from diagnosis of a disease such as AML (according to the invention) until death from any cause. The overall survival rate is often stated as a two-year survival rate (notably for AML), which is the
15 percentage of people in a study or treatment group who are alive two years after their diagnosis or the start of treatment.

As used herein, the term “event-free survival” (EFS) denotes the time after primary treatment for a cancer and relapse or death, whatever occurs first.

As used herein and according to the invention, the term “survival time” regroups the
20 terms OS and EFS.

As used herein, the term “Good Prognosis” denotes a patient with significantly enhanced probability of survival after treatment.

As used herein, the term “a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification”
25 denotes a patient with AML and classified according two international classification: the cytogenetic classification and the ELN classification (see for example the website located at leukemia-net.org). These two classifications enable to classify patients with AML into four groups: favorable, intermediate-I, intermediate-II, adverse. According to this classification, patient will be treated with chemotherapy alone (favorable prognosis) or with allogeneic stem
30 cell transplantation (allo-SCT) in first complete remission (adverse prognosis) (see for example Grimwade D et al., 1998; Slovak ML et al., 2000; Dohner H et al, 2010 and Stelljes M et al, 2013).

Patients with intermediate prognosis are difficult to manage and that's why, the inventors worked on this new powerful biomarker, NKp30. Thanks to this biomarker, up to 40% patients with intermediate prognosis can be re-classified in the group of adverse prognosis, and can be treated with the appropriate post remission therapy (i.e. chemotherapeutic compound and allo-SCT).

According to invention, when the patient receives allo-SCT, the hematopoietic stem cells come from a donor related or not to the recipient but of the same species.

As used herein and according to all aspects of the invention, the term "NKp30" denotes a receptor of the natural cytotoxicity receptors (NCRs) family. NKp30 is a triggering receptor expressed on the plasmatic membrane of NK cells, also known as CD337 or NCR3.

As used herein and according to all aspects of the invention, the term "sample" denotes, blood, peripheral-blood, serum, plasma or purified NK cells.

In one embodiment, the sample is fresh or frozen.

As used herein, the term "patient" refers to an individual with symptoms of AML.

Measuring the expression level of NKp30 can be done by measuring the gene expression level of NKp30 or by measuring the level of the protein NKp30 and can be performed by a variety of techniques well known in the art.

Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) 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 Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be

advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A “detectable label” is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N-[3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, antl1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole

(DAPI); 5',5''dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride);

5 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE),

10 fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and

15 derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin;

20 rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof.

25 Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782,

30 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM

(obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649, 138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., Science 281 :20132016, 1998; Chan et al., Science 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

Additional labels include, for example, radioisotopes (such as ^3H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd^{3+} , and liposomes.

Detectable labels that can be used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the

redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

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 Pirlkel 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, 1988. CISH is described in, e.g., Tanner et al., Am. J. 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.

5 Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-
10 limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, coumarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by
15 contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can
20 be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As
25 indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 01 17153.

It will be appreciated by those of skill in the art that by appropriately selecting labelled
30 probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can be labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target

sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 nm). Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are “specific” to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be

about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled
5 hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

Expression level of a gene may be expressed as absolute expression level or normalized
10 expression level. Typically, expression levels are normalized by correcting the absolute expression 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 patient, 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, TFRC, GAPDH, GUSB, TBP and
15 ABL1. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

Predetermined reference values used for comparison may comprise “cut-off” or “threshold” values that may be determined as described herein. Each reference (“cut-off”) value for NKp30 expression may be predetermined by carrying out a method comprising the steps of

20 a) providing a collection of samples from patients suffering of AML (after diagnosis of AML or after complete remission);

b) determining the expression level of NKp30 for each sample contained in the collection provided at step a);

c) ranking the tissue samples (particularly NK cells) according to said expression level

25 d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,

e) providing, for each sample provided at step a), information relating to the actual clinical outcome for the corresponding cancer patient (i.e. the duration of the event free survival (EFS) or the overall survival (OS) or both);

30 f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;

g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets

h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of NKp30 has been assessed for 100 AML samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression level. A first grouping provides two subsets: on one side sample Nr 1 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 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 AML patient, 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 reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference value. It should be noted that the reference value is not necessarily the median value of expression levels.

In routine work, the reference value (cut-off value) may be used in the present method to discriminate AML samples and therefore the corresponding patients.

Kaplan–Meier curves of percentage of survival as a function of time are commonly used to measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

The man skilled in the art also understands that the same technique of assessment of the expression level of a gene should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a gene of a patient subjected to the method of the invention.

Such predetermined reference values of expression level may be determined for any gene defined above.

According to the invention, the level of the protein NKp30 may also be measured and can be performed by a variety of techniques well known in the art.

Typically protein concentration may be measured for example by capillary electrophoresis-mass spectroscopy technique (CE-MS) or ELISA performed on the sample.

Detection of protein concentration in the sample may also be performed by measuring the level of the protein NKp30. In the present application, the “level of protein” or the “protein level expression” means the quantity or concentration of said protein. In another embodiment, the “level of protein” means the level of NKp30 protein fragments. In still another embodiment, the “level of protein” means the quantitative measurement of the protein NKp30 expression relative to a negative control.

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with proteins present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, capillary electrophoresis-mass spectroscopy technique (CE-MS).etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected using methods well known in the art.

Methods of the invention may comprise a step consisting of comparing the proteins and fragments concentration in circulating cells with a control value. As used herein, "concentration of protein" refers to an amount or a concentration of a transcription product, for instance the protein NKp30. Typically, a level of a protein can be expressed as nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for example. Alternatively, relative units can be employed to describe a concentration. In a particular embodiment, "concentration of proteins" may refer to fragments of the protein NKp30. Thus, in a particular embodiment, fragment of NKp30 protein may also be measured.

In a particular embodiment, the detection of the level of NKp30 can be performed by flow cytometry. When this method is used, the method consists of determining the mean fluorescence intensity (MFI) ratio (NKp30 MFI / isotype control MFI, referred to as rMFI) expressed on NK cells. According to the invention and the flow cytometry method, when the rMFI is high or bright, the level of NKp30 express on NK cells is high and thus the expression level of NKp30 is high and when the rMFI is low or dull, the level of NKp30 express on NK cells is low and thus the expression level of NKp30 is low.

Thus, according to the invention when the expression level of NKp30 is high the prognosis of the patient suffering from AML is good and when the expression level of NKp30 is low the prognosis of the patient suffering from AML is bad.

In another embodiment, the extracellular part of the NKp30 protein is detected.

In a further embodiment of the invention, methods of the invention comprise measuring the expression level of at least one further biomarker or prognostic score.

The term "biomarker", as used herein, refers generally to a cytogenetic marker, a molecule, the expression of which in a sample from a patient can be detected by standard methods in the art (as well as those disclosed herein), and is predictive or denotes a condition of the subject from which it was obtained.

Various validated prognostic biomarkers or prognostic scores may be combined to NKp30 in order to improve methods of the invention and especially some parameters such as the specificity (see for example Cornelissen et al. 2012).

For example, the other biomarkers may be selected from the group of AML biomarkers consisting of cytogenetics markers (like t(8;21), t(15;17), inv(16) see for example Grimwade et al., 2010 or Byrd et al., 2002), lactate dehydrogenase (see for example Haferlach et al 2003), FLT3, NPM1, CEBP α (see for example Schnittger et al., 2002, Dohner et al., 2010). The

prognostic scores that may be combined to NKp30 may be for example the disease risk index (DRI) (Armand et al 2012).

5 A further object of the invention relates to kits for performing the methods of the invention, wherein said kits comprise means for measuring the expression level of NKp30 in the sample obtained from the patient.

The kits may include probes, primers macroarrays or microarrays as above described. For example, the kit may comprise a set of probes as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, probes may be unlabelled and the ingredients for
10 labelling may be included in the kit in separate containers. The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards. Alternatively the kit of the invention may comprise amplification primers that may be pre-labelled or may contain an affinity purification or attachment moiety. The kit may further
15 comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

The present invention also relates to NKp30 as a biomarker for outcome of AML in a patient classified as intermediate in the cytogenetic classification and in the ELN classification.

20 A third aspect of the invention relates to a chemotherapeutic compound and/or an allogeneic stem cell (allo-SCT) for allo-SCT transplantation for use in the treatment of AML in a patient with a bad prognosis as described above.

In another aspect, the invention relates to i) a chemotherapeutic compound, and ii) an allo-SCT, as a combined preparation for simultaneous, separate or sequential use in the
25 treatment of AML in patient with a bad prognosis as described above.

In another embodiment, the invention relates to a method for treating AML in a patient with a bad prognosis as described above comprising administering to said subject in need thereof a chemotherapeutic compound or a allogeneic stem cell transplantation.

According to the invention, chemotherapeutic compounds may be selected in the group
30 consisting in: fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, campathecins, bleomycin, doxorubicin,

idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, epimbiem, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustine and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatinib mesylate, 5 hexamethylnelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrosophostins, protease inhibitors, inhibitors herbimycin A, genistein, erlotinib, temozolomide and vandetanib. In one embodiment, additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, 10 DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, anthracyclines, MDR inhibitors and Ca²⁺ ATPase inhibitors.

In another embodiment, further therapeutic active agent can be administered to the 15 patient. This active agent can be a hematopoietic colony-stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to filgrastim, sargramostim, molgramostim and epoietin alpha.

In a particular embodiment, the chemotherapeutic compound is the cytarabine or the anthracycline.

20 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 subjects at risk of contracting the disease or suspected to have contracted the disease as well as subjects 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 subject having 25 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 subject 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 30 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 subject 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 subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at 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., disease manifestation, etc.]).

A fourth aspect of the invention relates to a therapeutic composition comprising a chemotherapeutic compound and/or allogenic stem cells for allo-SCT according to the invention for use in the treatment of AML in patient with a bad prognosis as described above.

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Particularly, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids
5 for oral administration; time release capsules; and any other form currently can be used.

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.

10

FIGURES:

Figure 1: NKp30 expression on peripheral NK cells at diagnosis predicts clinical outcome.

15 Kaplan Meier estimates of overall survival (A) and event-free survival (B) by NKp30 expression at diagnosis (IPC prospective cohort). CI95: 95% confidence interval; HR, hazard ratio.

Figure 2: NKp30 expression stratifies patients with intermediate cytogenetic prognosis.

Kaplan Meier estimates of overall survival (A) and event-free survival (B) by NKp30
20 expression at diagnosis in patients with intermediate prognosis (subgroup analysis of the IPC prospective cohort, N=76). Results were confirmed in an independent multicenter cohort of patients with intermediate prognosis (GOELAMS validation cohort, N=86) on both overall survival (C) and event-free survival (D). CI95: 95% confidence interval; HR, hazard ratio.

Figure 3: NKp30 expression stratifies patients with intermediate ELN.

25 Kaplan Meier estimates of overall survival (A) and event-free survival (B) by NKp30 expression at diagnosis in patients with intermediate ELN (pooled subgroup analysis of the IPC prospective cohort and the GOELAMS validation cohort, N=113). CI95: 95% confidence interval; ELN, European Leukemia Net cytogenetic classification; HR, hazard ratio.

Figure 4: Threshold determination for NKp30 expression on NK cells (IPC prospective
30 cohort). (A) Distribution histograms of NKp30 mean fluorescence intensity (MFI) ratio (NKp30 MFI / isotype control MFI) in patients with AML at diagnosis. The curves are estimates of population density distribution. (B) The volcano plot shows log of P value for overall survival according to threshold for NKp30 expression at diagnosis. The dashed line represents the threshold used in the rest of the study.

Figure 5: NKp30 recovery after CR is a better predictor of clinical outcome than NKp30 at diagnosis. (A) The kinetics of NKp30 expression was assessed after CR (day 30, 60 and 90 after the last induction chemotherapy) in the IPC prospective cohort. Thirty-nine patients were tested for NKp30 expression at day 30, 28 patients at day 60 and 23 patients at day 90. (B) Kinetics of NKp30 expression according to clinical outcome at 2 years. Kaplan Meier estimates for overall survival (C) and event-free survival (D) by NKp30 expression at day 30 after induction chemotherapy (N=39). CI95: confidence interval 95%; Diag: diagnosis; CR: complete remission; HR, hazard ratio; HV: healthy volunteers; NS: non significant.

Table 1: Baseline patients characteristics – IPC prospective cohort

Allo-SCT: allogeneic stem cell transplantation; Auto-SCT: autologous stem cell transplantation; BM: bone marrow; CR : complete remission ; FAB: French-American-British classification; M : male ; F : female; ITD: internal tandem duplication; MDS: myelodysplastic syndrome; NA : not available; Nb: number; t-AML: therapy-related AML; s-AML; secondary AML; OS: overall survival; EFS: event-free survival.

Characteristic		All	NKp30 low	NKp30 high
Patients, no.	N (%)	116 (100)	46 (39.7)	70 (60.3)
Age at diagnosis	Mean (SD)	47.1 (10.6)	47.5 (11.5)	46.8 (10.0)
Sex ratio, M/F		1.07	1.42	0.89
FAB category	N (%)			
M0		1 (0.9)	0 (0)	1 (1.4)
M1		15 (12.9)	6 (13.0)	9 (12.9)
M2		20 (17.2)	7 (15.2)	13 (18.6)
M3		0	0	0
M4		39 (33.6)	15 (32.6)	24 (34.3)
M5		29 (25.0)	14 (30.4)	15 (21.4)
M6		8 (6.9)	1 (2.2)	7 (10.0)
M7		0 (0)	0 (0)	0 (0)
Unclassified		4 (3.4)	3 (6.5)	1 (1.4)
Status at diagnosis	N (%)			
de novo		97 (83.6)	38 (82.6)	59 (84.3)
s-AML				
MDS		16 (13.8)	7 (15.2)	9 (12.9)
t-AML		3 (2.6)	1 (2.2)	2 (2.9)
White blood cell (10^9 cells/L)	Median (SD)	13 (66.7)	24.8 (84.9)	12 (48.6)
Cytogenetic prognosis	N (%)			
Favorable		21 (18.9)	9 (19.6)	12 (17.1)

Intermediate		76 (65.5)	33 (71.7)	43 (61.4)
Adverse		19 (16.4)	4 (8.7)	15 (21.4)
Mutations in intermediate cytogenetics group	N (%)			
Done		72	30	42
FLT3 ITD^{mut}		22/72 (30.6)	9/30 (30.0)	13/42 (31.0)
NPM1^{mut}		31/72 (43.1)	13/30 (43.3)	18/42 (42.9)
CEBPα^{mut} / FLT3^{wt} NPM1^{wt}		6/35 (17.1)	2/15 (13.3)	4/20 (20.0)
NPM1^{mut} / FLT3^{wt}		14/50 (28.0)	5/21 (23.8)	9/29 (31.0)
ELN	N (%)			
Favorable		41 (35.3)	16 (34.8)	25 (35.7)
Intermediate		56 (48.3)	26 (56.5)	30 (42.9)
Intermediate-I		32 (27.6)	12 (26.1)	20 (28.6)
Intermediate-II		24 (20.7)	14 (30.4)	10 (14.3)
Adverse		19 (16.4)	4 (8.7)	15 (21.4)
Blasts (blood) at diagnosis	Mean (SD)	46.5 (32.8)	52.5 (34.0)	42.6 (31.5)
Blasts (BM) at diagnosis	Mean (SD)	66.4 (22.9)	69.9 (20.2)	64.1 (24.4)
Response at d15	N (%)	67 (57.8)	23 (50.0)	44 (62.9)
No response at d15		26 (22.4)	12 (26.1)	14 (20.0)
NA (Not evaluable or induction death)		23 (19.8)	11 (23.9)	12 (17.1)
Post induction CR	N (%)	98 (84.5)	36 (78.3)	62 (88.6)
No post induction CR		18 (15.51)	10 (21.7)	8 (11.4)
Induction death		8 (6.9)	6 (13.0)	2 (2.8)
No CR achieved		10 (8.6)	4 (8.7)	6 (8.6)
Nb of induction for CR	N (%)			
1		73 (62.9)	25 (54.3)	48 (68.6)
2		23 (19.8)	9 (19.6)	14 (20.0)
3		2 (1.7)	2 (4.3)	0 (0)
Consolidation	N (%)			
Chemotherapy		47 (40.5)	18 (39.1)	29 (41.4)
Auto-SCT		13 (11.2)	3 (6.5)	10 (14.3)
Allo-SCT		56 (48.3)	25 (54.3)	31 (44.3)
2years post diagnosis death rate	N (%)	42/98 (42.9)	22/42 (52.4)	20/56 (35.7)
Median OS (months)		Undefined	13.4	Undefined
Median EFS (months)		29.9	25.1	Undefined

Table 2: Baseline patients characteristics – GOELAMS validation cohort

Allo-SCT: allogeneic stem cell transplantation; Auto-SCT: autologous stem cell transplantation; BM: bone marrow; CR : complete remission ; FAB: French-American-British classification; M : male ; F : female; ITD: internal tandem duplication; MDS: myelodysplastic syndrome; NA : not available; Nb: number; t-AML: therapy-related AML; s-AML; secondary AML; OS: overall survival; EFS: event-free survival.

Characteristic		All	NKp30 low	NKp30 high
Patients, no.	N	86	9 (10.5)	77 (89.5)
Age at diagnosis	Mean (SD)	46.3 (11.0)	48.6 (11.2)	46.0 (11.0)
Sex ratio, M/F		1.26	8	1.0
FAB category	N (%)			
M0		1 (1.2)	0 (0)	1 (1.3)
M1		13 (15.1)	2 (22.2)	11 (14.3)
M1/M2		2 (2.3)	1 (11.1)	1 (1.3)
M2		19 (22.1)	1 (11.1)	18 (23.4)
M3		0 (0)	0 (0)	0 (0)
M4		17 (19.8)	1 (11.1)	16 (20.8)
M4/M5		1 (1.2)	0 (0)	1 (1.3)
M5		21 (24.4)	2 (22.2)	19 (24.7)
M6		1 (1.2)	0 (0)	1 (1.3)
M7		0 (0)	0(0)	0 (0)
Unclassified		1 (1.2)	0 (0)	1 (1.3)
NA		10 (11.6)	2 (22.2)	8 (10.4)
Status at diagnosis de novo s-AML	N (%)	NA NA	NA NA	NA NA
White blood cell (10⁹ cells/L)	Median (SD)	32.3 (30.3)	79 (31.4)	31.0 (27.6)
Cytogenetic prognosis	N (%)			
Favorable		0 (0)	0 (0)	0 (0)
Intermediate		86 (100.0)	9 (100.0)	77 (100.0)
Adverse		0 (0)	0 (0)	0 (0)
Mutations in intermediate group	N (%)			
Done		86	9	77

FLT3 ITD^{mut}		31/86 (36.0)	3/9 (33.3)	28/77 (36.4)
NPM1^{mut}		44/86 (51.2)	2/9 (22.2)	42/77 (54.5)
CEBPα		10/86 (11.6)	0/9 (0)	11/77 (14.3)
NPM1^{mut}/FLT3^{wt}		26/55 (47.3)	2/6 (33.3)	24/49 (49.0)
ELN				
Favorable		29 (33.7)	2 (22.2)	27 (35.1)
Intermediate		57 (66.3)	7 (77.8)	50 (64.9)
Intermediate-I		36 (41.9)	3 (33.3)	33 (42.9)
Intermediate-II		17 (19.8)	3 (33.3)	14 (18.2)
NA		4 (4.7)	1 (11.1)	3 (3.9)
Adverse		0 (0)	0 (0)	0 (0)
Blasts (blood) at diagnosis	Mean (SD)	43.3 (33.0)	64.5 (29.5)	40.1 (32.7)
Blasts (BM) at diagnosis	Mean (SD)	61.9 (23.3)	72.1 (19.8)	60.8 (23.5)
Response at d15	N (%)		3 (33.3)	49
No response at d15		52 (60.5)	5 (55.5)	(63.6)
NA (Not evaluable or		32 (37.2)	1 (11.1)	27
induction death)		2 (2.3)		(35.1)
				1 (1.3)
Post induction CR	N (%)	72 (83.7)	6 (66.7)	66 (85.7)
No post induction CR		14 (16.3)	3 (33.3)	11
Induction death		5 (5.8)	1 (11.1)	(14.3)
No CR achieved		9 (10.5)	2 (22.2)	4 (5.2)
NA		1 (1.2)	0 (0)	7 (9.1)
				1 (1.3)
Nb of induction for CR	N (%)		4 (44.4)	48
1		52 (60.5)	2(22.2)	(62.3)
2		15 (17.4)	0 (0.0)	13
3		4 (4.7)		(16.9)
				4 (5.2)
Consolidation	N (%)			NA
Chemotherapy		NA	NA	NA
Auto-SCT		NA	NA	25
Allo-SCT		27 (31.4)	2 (22.2)	(32.5)

Median OS		56.0	22.7	56.6
Median PFS		24.7	10.4	44.3

Table 3: Cox regression, IPC prospective cohort

Multivariate Cox regression models were used to assess the predictive value of NKp30 expression while adjusting for the prognostic factors in the population (age at diagnosis, disease status, ELN, leukocytosis, and allogeneic stem cell transplantation as a time-dependent covariate).

	Multivariate HR for OS			Multivariate HR for EFS		
Variable	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Age at diagnosis						
<50	Reference			Reference		
≥50	0.89	0.47 to 1.67	0.71	1.01	0.56 to 1.81	0.96
Disease status						
De novo	Reference			Reference		
MDS/t-AML	1.48	0.69 to 3.14	0.31	1.41	0.69 to 2.84	0.34
ELN						
Favorable	Reference			Reference		
Intermediate	13.15	4.36 to 39.60	<0.001	6.61	3.01 to 14.53	<0.001
Adverse	10.16	2.75 to 37.40	0.005	9.23	3.33 to 25.55	<0.001
Leucocytosis at diagnosis						
<50 G/L	Reference			Reference		
≥50 G/L	0.95	0.47 to 1.90	0.88	1.41	0.76 to 2.62	0.28
Consolidation						
No allo-SCT	Reference			Reference		
Allo-SCT	0.48	0.21 to 1.06	0.07	0.20	0.09 to 0.44	<0.001
NKp30 MFI ratio						
High	Reference			Reference		
Low	2.26	1.19 to 4.27	0.012	2.38	1.33 to 4.24	0.003

EXAMPLE:**Material & Methods**Patients and study design

5 Baseline NKp30 expression on NK cells at diagnosis was assessed in a total of 202 patients. Two cohorts of patients were analyzed. The Paoli Calmettes Institute (IPC) prospective cohort included 116 patients with newly diagnosed non-acute promyelocytic leukemia (APL) AML admitted between November 2007 and November 2012, aged 18 to 65 years and treated with conventional 3+7 induction chemotherapy as previously described
10 [Devillier R et al., 2015]. The Groupe Ouest Est d'Etude des Leucémies Aiguës et autres Maladies du Sang (GOELAMS) validation cohort included 86 patients from the LAM2006IR prospective multicenter randomized trial, included between November 2007 and April 2012 (NCT00860639). All patients had previously untreated AML with intermediate cytogenetics. Patients received conventional 3+7 induction chemotherapy with or without the addition of
15 Gemtuzumab Ozogamicin. Patients with APL AML and patients above 66 years were not eligible for this study. All participants gave written informed consent in accordance with the Declaration of Helsinki. The entire research procedure was approved by the ethical review boards from the IPC and the GOELAMS.

Clinical samples

20 Fresh total peripheral blood samples (IPC prospective cohort) or peripheral blood mononuclear cells (PBMC) cryopreserved in 90%FCS /10%DMSO (GOELAMS validation cohort) were obtained from randomly selected patients at diagnosis before induction chemotherapy and from age-matched healthy volunteers (N=24) and analyzed by flow cytometry. For the GOELAMS validation cohort, handling, conditioning and storing of patients
25 samples were performed by the FILOtheque AML (N° BB-0033-00073), tumor bank of the FILO group, Cochin hospital, Paris. Analyses were performed in the Biopathology department and on the IPC Immunomonitoring platform. Assays were performed blinded to the study endpoint. Samples from the IPC prospective cohort and from the GOELAMS validation cohort were analyzed by independent experimenters.

Flow cytometry

30 A FACS Canto II (IPC prospective cohort) and a LSR Fortessa (GOELAMS validation cohort) (BD Biosciences, San Jose, CA), and FACS Diva Software (BD Biosciences) were used for flow cytometry. NK cells from whole blood EDTA or frozen PBMC were immunostained with fluorescein isothiocyanate (FITC)-conjugated or Phycoerythrin-Texas Red-x™ (ECD)-

conjugated anti-CD3, Phycoerythrin-Cyanine 7 (PC7)- or Allophycocyanin (APC) AF700–
conjugated anti-CD56, and APC- or Krome Orange™ (KO)–conjugated anti-CD45 antibodies.
Dead cells were excluded with LIVE/DEAD® Near IR dead cell stain kit (Thermo Fisher
Scientific Inc, Rockford, IL). NKp30 triggering receptor expression was measured with
5 Phycoerythrin (PE)-conjugated monoclonal antibodies. Isotype controls were mouse (PE)-
conjugated monoclonal antibodies. All antibodies used were kindly provided by Beckman-
Coulter, Marseille, France. Red blood cells were lysed with BD FACS Lysing solution (BD
Biosciences, San Jose, CA) before acquisition. The NKp30 mean fluorescence intensity (MFI)
ratio (NKp30 MFI / isotype control MFI, referred to as rMFI) was calculated for each patient.
10 NKp30 expression was assessed at diagnosis and after complete remission, at day 30, 60 and
90 after the last induction chemotherapy.

Threshold determination

Patients were classified into two groups, NKp30^{low} and NKp30^{high}, according to NKp30
rMFI. The dichotomy between NKp30^{low} and NKp30^{high} patients was based on dispersion
15 criteria of the IPC prospective cohort. Fig 4A displays inter-individual variability of NKp30
expression in AML patients. The distribution of NKp30 expression was a juxtaposition of three
Gaussian distributions. (d'Agostino and Pearson normality test and Kernel density estimation).
The intersection between the first and the second peak was NKp30 rMFI = 7.8 (Fig 4A). All
the possible thresholds were tested in the range of NKp30 expression for overall survival (OS)
20 (Fig 4B). The threshold based on dispersion criteria was discriminant for survival analyses. For
the rest of the study, patients from both cohorts were classified into 2 distinct subgroups
(NKp30^{high} and NKp30^{low} phenotype) for survival analyses according to this threshold.

For patients assessable for NKp30 expression after complete remission, a second
threshold was determined according to the methodology described above. The intersection
25 between the 2 Gaussian distributions was 18.6 (data not shown).

End Points and Statistical Analysis

Statistical analyses were carried out using SPSS (SPSS software, Chicago IL), Graph
Pad Prism (Graph Pad Software, San Diego, CA) and R software (www.r-project.org). The
limit of significance was set at P<0.05. The X², Fisher's exact test and t test were used to
30 compare baseline variables among patients with high or low NKp30 phenotype. For multiple
comparisons, a Kruskal-Wallis test was used followed by a Dunn's post-test. Overall survival
(OS) was defined as the time from diagnosis until death from any cause, and event-free survival
(EFS) as the time between induction and relapse or death, whatever occurred first. Patients
without an event were censored at the time of their last follow-up. Survival times were estimated

by Kaplan-Meier method and compared using the log-rank test. Cumulative incidence of relapse (CIR) was calculated using cumulative incidence estimates to accommodate competing risks. CIR were compared by Gray test. A multivariate Cox regression model was used to assess the predictive value of NKp30 expression while adjusting for other prognostic factors (age at diagnosis, disease status, ELN, leukocytosis, and allo-SCT as a time-dependent covariate). Subgroup analyses were defined a priori. This study conformed to the recommendations for tumor marker prognostic studies (REMARK).

Results

Baseline patient characteristics

The patient characteristics, stratified by NKp30 expression groups, are summarized in Table 1 (IPC prospective cohort) and Table 2 (GOELAMS validation cohort). In the IPC prospective cohort, 21 patients had favorable cytogenetics (18.9%), 76 had intermediate cytogenetics (65.5%) and 19 had unfavorable cytogenetics (16.4%). The mean age ($\pm$ SD) at induction was 47.1 years ($\pm$ 10.6). Median follow-up after diagnosis was 35.1 months. In the GOELAMS validation cohort, 100% of patients had intermediate cytogenetics. The mean age ($\pm$ SD) at induction was 46.3 years ($\pm$ 11.0). Median follow-up after diagnosis was 25.2 months. Cytogenetic classification and European Leukemia Net (ELN) genetic classification (FLT3/CEBP α /NPM1 mutational status) were routinely determined in the Biopathology departments of the centers involved in this study.

Baseline NKp30 expression on Natural Killer (NK) cells was assessed by flow cytometry. In the IPC prospective cohort, among the 116 patients, 70 (60.3%) had NKp30^{high} phenotype, and 46 (39.7%) had low NKp30^{low} phenotype (Table 1). In the GOELAMS validation cohort, among the 86 patients, 77 (89.5%) NKp30^{high} phenotype and 9 (10.5%) had NKp30^{low} phenotype. The frequency of patients with NKp30 high and low phenotype did not differ between age, cytogenetics or number of inductions.

NKp30 expression on peripheral NK cells at diagnosis predicts clinical outcome

In univariate analysis of the IPC prospective cohort, patients with NKp30^{high} phenotype at diagnosis had better OS (HR= 1.97; 95%CI = [1.12-3.48]; P=0.017) (Fig 1A) and EFS (HR=2.09; 95%CI = [1.19-3.64]; P=0.0095) (Fig 1B) than patients with NKp30^{low} phenotype, with better 3-year OS and EFS rates for the group with NKp30^{high} phenotype (67.5% vs 42.9% and 58.4% vs 35.1%, respectively). There was no significant difference in cumulative incidence of relapse (CIR) between patients with high and low NKp30 expression (P=0.109; HR=1.76; 95%CI = [0.88-3.50]) (data not shown). In the Cox model, high NKp30 expression was

significantly associated with improved OS and EFS, independent of other factors ($P=0.012$ and $P=0.003$, respectively) (Table 3).

NKp30 status stratifies patients with intermediate cytogenetic prognosis

An important challenge is to stratify AML patients with intermediate cytogenetic prognosis. In a subgroup analysis of patients with intermediate cytogenetic prognosis (IPC prospective cohort, $N=76$), the prognostic value of NKp30 remained significant in both OS (HR=2.02; 95%CI = [1.02-3.99]; $P=0.044$) and EFS (HR=2.35; 95%CI = [1.23-4.50]; $P=0.0098$) (Fig 2A and B, respectively), with better 3-year OS and EFS rates for the group with NKp30^{high} phenotype (60.4% vs 35.8% and 55.0% vs 27.5%, respectively). In order to validate this result, we studied the impact of NKp30 expression on clinical outcome on a retrospective multicenter cohort of 86 patients with intermediate prognosis (GOELAMS validation cohort). In this cohort, patients with low NKp30 expression had reduced OS (HR=3.57; 95%CI = [1.14-11.15]; $P=0.029$) and EFS (HR=6.87; 95%CI = [2.06-22.86]; $P=0.0079$) (Fig 2C and D, respectively), with better 3-year OS and EFS rates for the group with NKp30^{high} phenotype (58.2% vs 12.7% and 54.5% vs 0%, respectively).

Stratification at diagnosis is currently based on cytogenetic classification and completed with European Leukemia Net (ELN) genetic classification. In addition to cytogenetic classification, ELN classification further defines a group of patients with intermediate prognosis. We hypothesized that NKp30 status could refine this stratification. We assessed the prognostic value of NKp30 in patients with intermediate ELN. Here, we merged the 2 cohorts to reach relevant effectives ($N=113$). In these patients, NKp30 significantly risk-stratified patients, with lower OS (HR=2.18; 95%CI = [1.20-3.95]; $P=0.0097$) and EFS (HR=2.45; 95%CI = [1.37-4.39]; $P=0.0036$) in patients with low NKp30 expression (Fig 3A and B, respectively), and better 3-year OS and EFS rates for the group with NKp30^{high} phenotype (48.8% vs 20.7% and 46.0% vs 16.1%, respectively).

NKp30 recovery after CR is a better predictor of clinical outcome than NKp30 at diagnosis

NKp30 expression was assessed in 39 patients from the IPC prospective cohort in CR after chemotherapy. Induction chemotherapy resulted in significant increase of NKp30 expression at day 30 that was maintained at least until day 90 (Fig 5A). We then divided patients in two groups based on presence or absence of death 2 years after diagnosis. In the group of patients who do not die within 2 years, NKp30 expression significantly increased at day 30 (Fig 5B). This increase remained significant until day 60. By contrast, in the group of patients who die within 2 years after diagnosis, no significant increase was evidenced compared to diagnosis

(Fig 5B). We then analyzed survival stratified by NKp30 expression at day 30. NKp30 status after complete remission was a better predictor of clinical outcome than NKp30 status at diagnosis, with higher HR for OS (HR=3.26 (95%CI = [1.08-9.77], P=0.035) and EFS (HR=2.97 (95%CI = [1.11-7.98], P=0.031) (Fig 5C and D, respectively).

5

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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.

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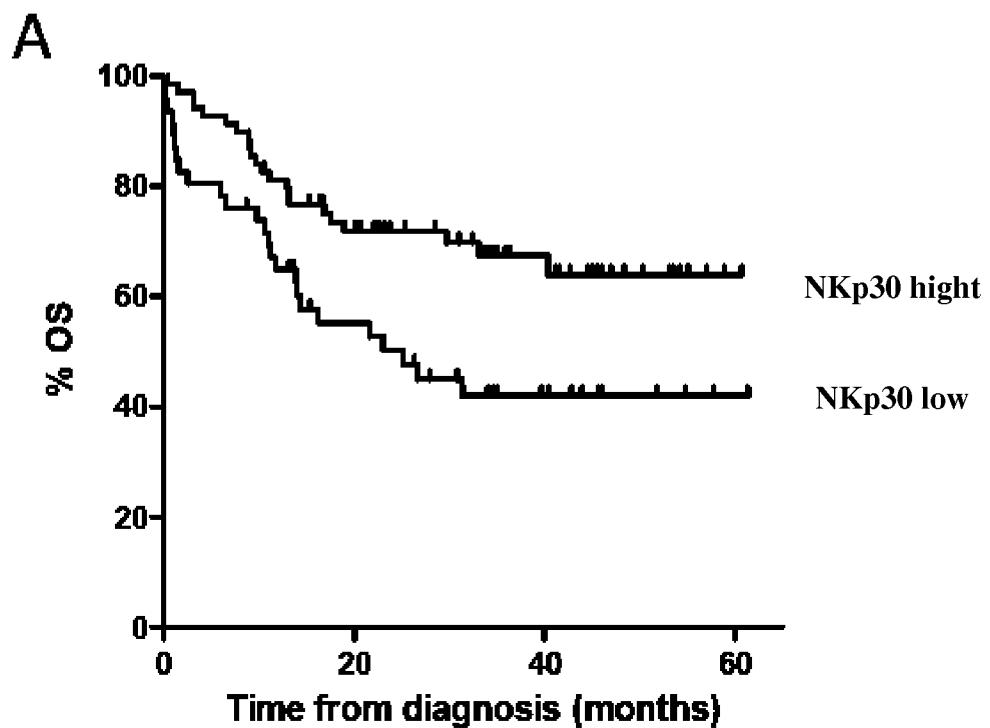
25 Slovak ML, Kopecky KJ, Cassileth PA, et al: Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood 96:4075-4083, 2000.

Stelljes M, Krug U, Beelen DW, et al: Allogeneic transplantation versus chemotherapy as postremission therapy for acute myeloid leukemia: a prospective matched pairs analysis.
30 Journal of Clinical Oncology:JCO. 2013.50. 5768, 2013.

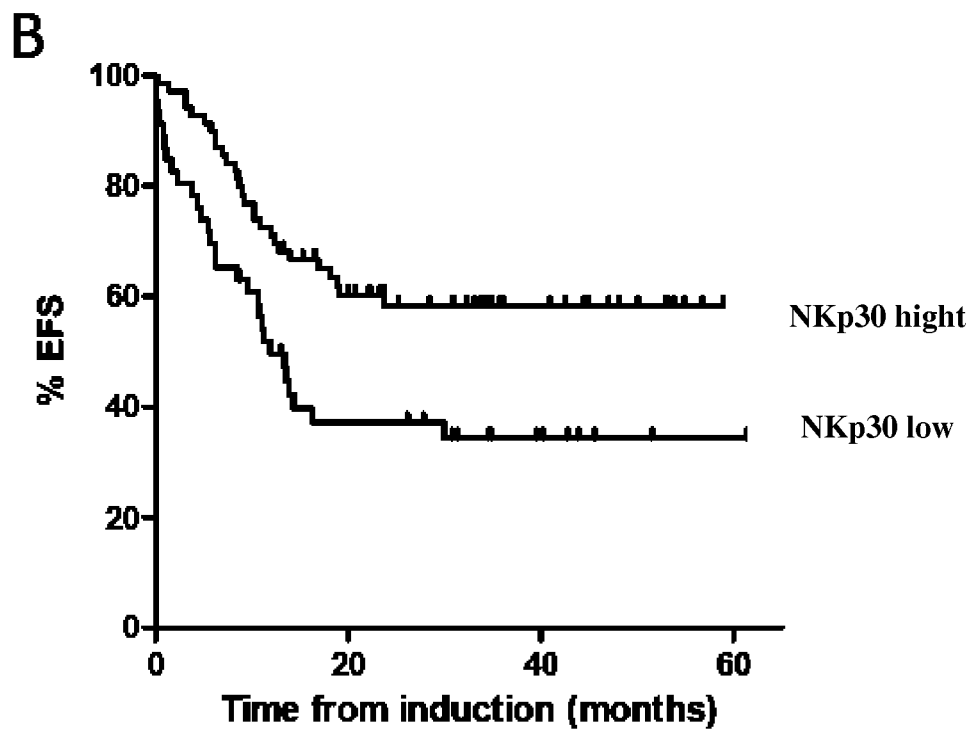
CLAIMS:

1. A method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and classified as intermediate in the cytogenetic classification and in the ELN classification comprising i) determining in a sample obtained from the patient the expression level of NKp30 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is higher than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is lower than its predetermined reference value.
2. A method according to claim 1 wherein the sample is blood, peripheral-blood, serum, plasma or purified NK cells.
3. A method according to claims 1 or 2 wherein the expression level of NKp30 is determined by flow cytometry.
4. NKp30 for use as a biomarker for outcome of AML in a patient classified as intermediate in the cytogenetic classification and in the ELN classification.
5. A chemotherapeutic compound and/or an allogeneic stem cell transplantation (allo-SCT) for use in the treatment of AML in a patient with a bad prognosis as described in claims 1 to 3.
6. A i) chemotherapeutic compound, and ii) an allo-SCT, as a combined preparation for simultaneous, separate or sequential use in the treatment of AML in patient with a bad prognosis as described in claims 1 to 3.

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 $P=0.017$

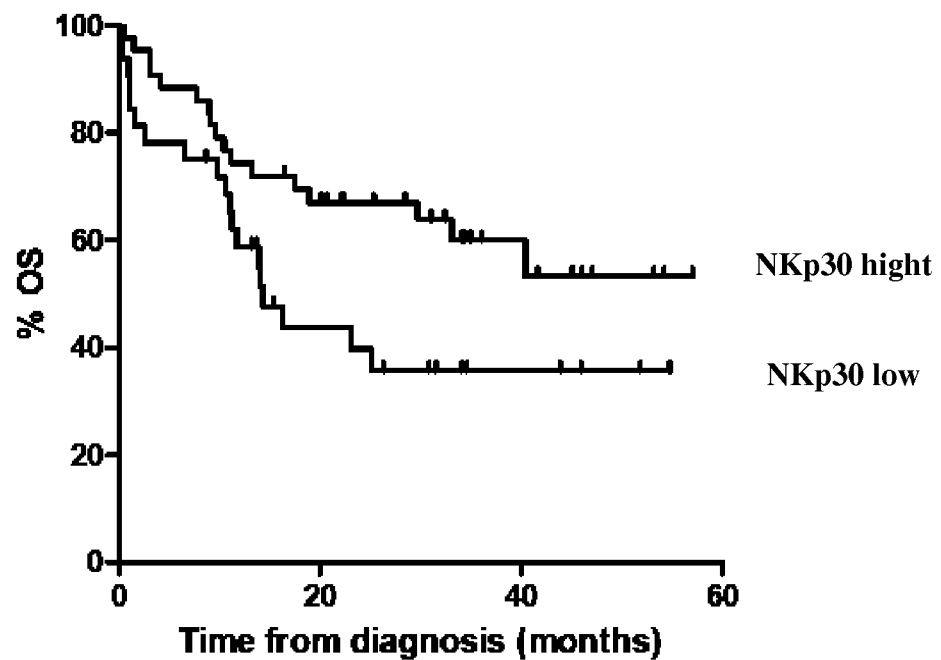
HR (CI95) = 1.97 (1.12-3.48)

 $P=0.0095$

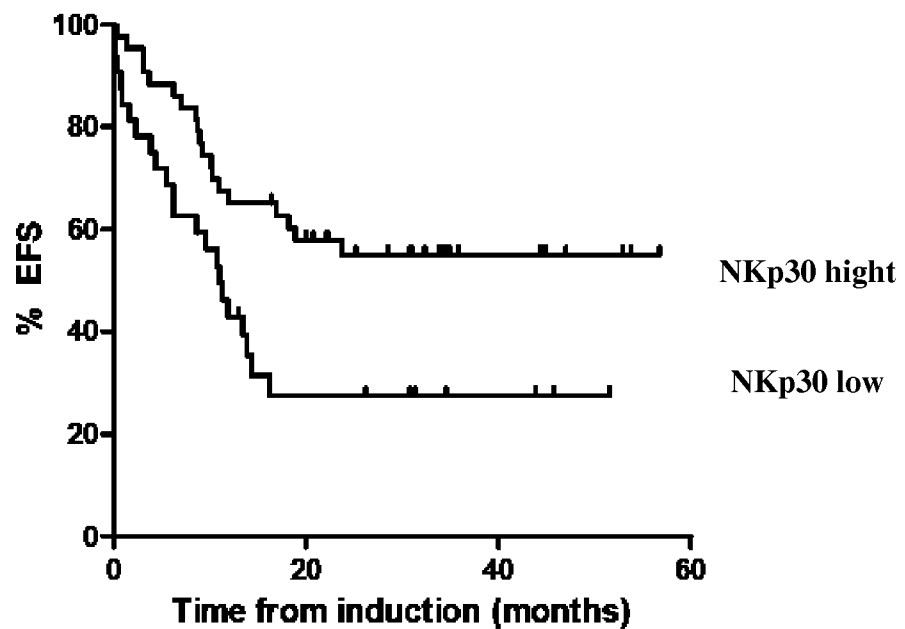
HR (CI95) = 2.09 (1.19-3.64)

Figure 1 A and B

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A $P=0.044$

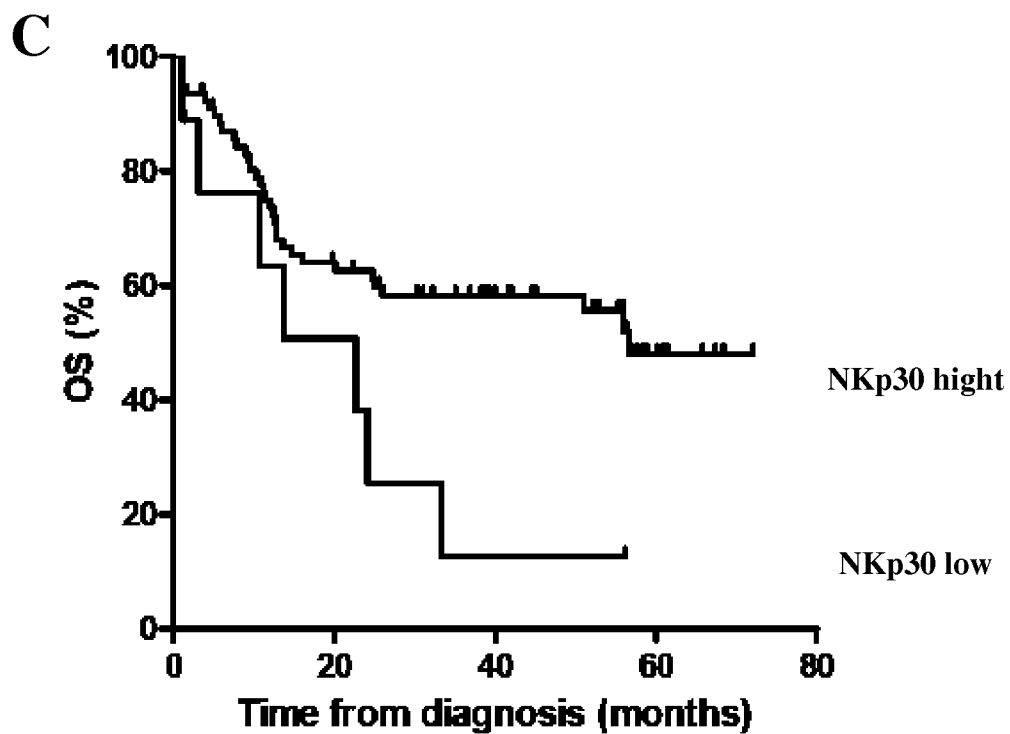
HR (CI95)=2.02 (1.02-3.99)

B $P=0.0098$

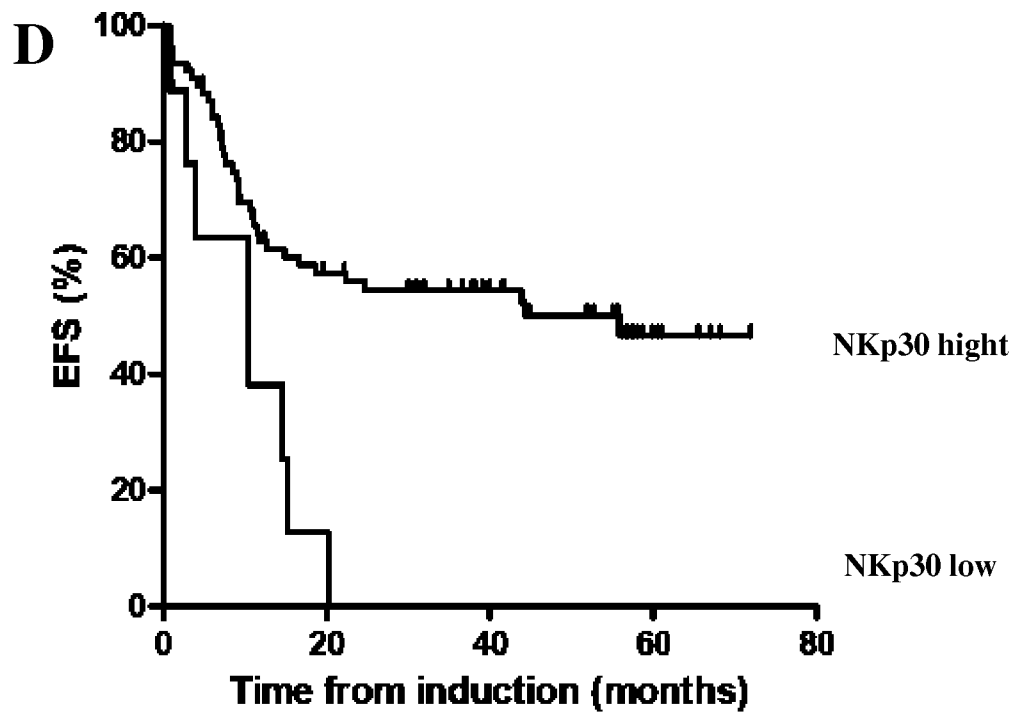
HR (CI95)=2.35 (1.23-4.50)

Figure 2 A and B

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 $P=0.029$

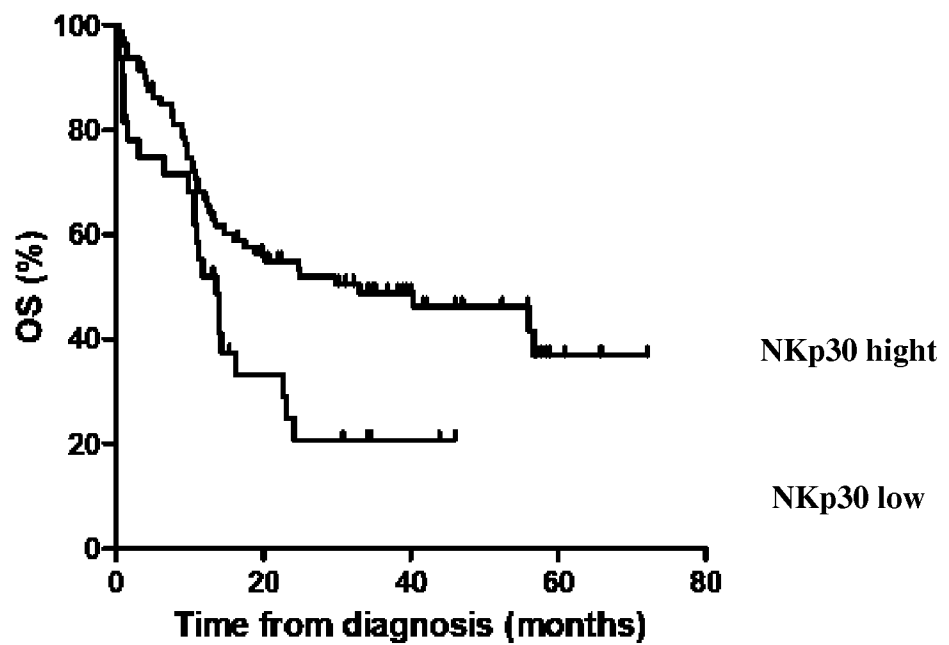
HR (CI95) =3.57 (1.14-11.15)

 $P=0.0079$

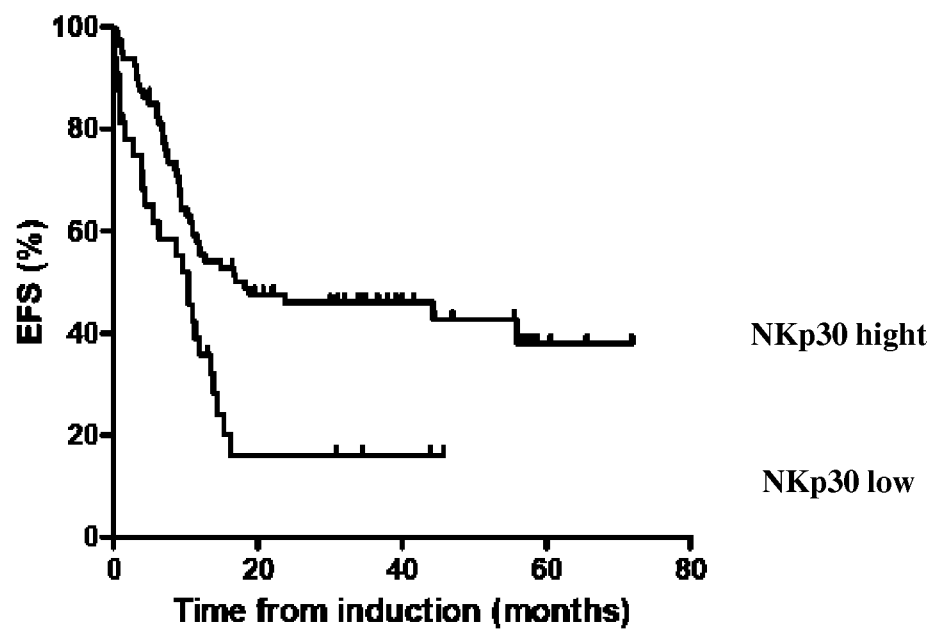
HR (CI95) =6.87 (2.06-22.86)

Figure 2 C and D

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A $P=0.0097$

HR (CI95) = 2.18 (1.20-3.95)

B $P=0.0036$

HR (CI95) = 2.45 (1.37-4.39)

Figure 3 A and B

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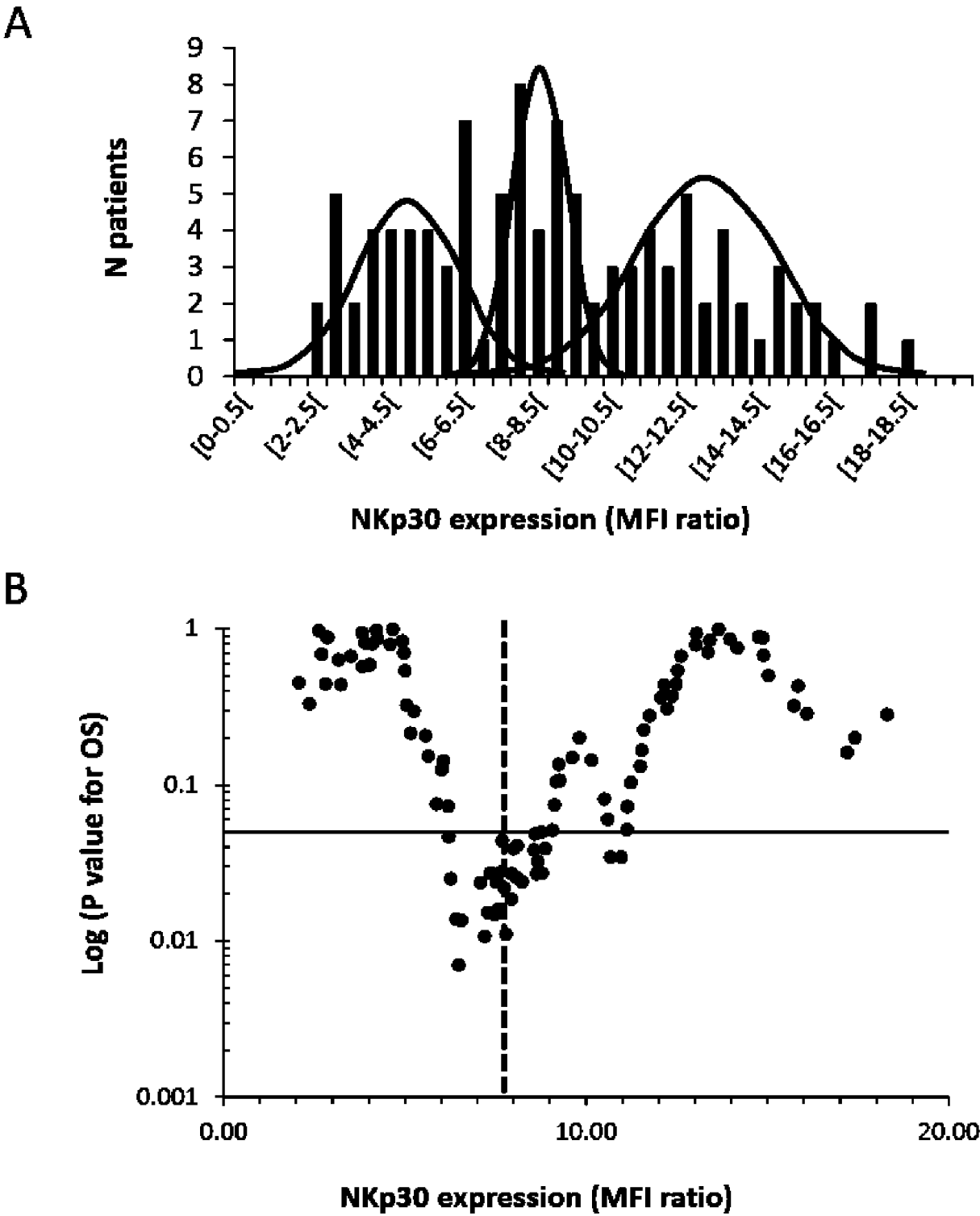


Figure 4 A and B

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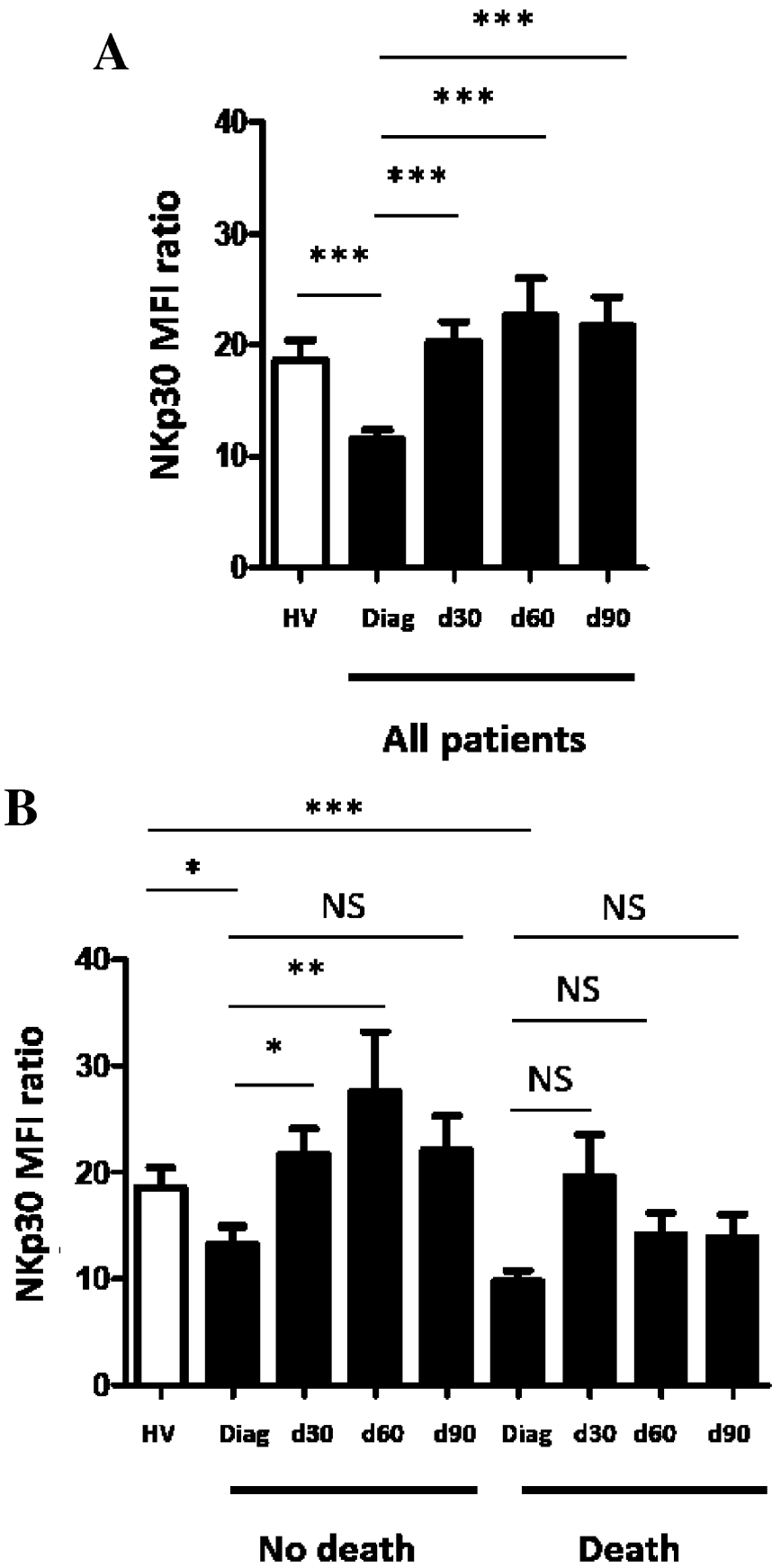
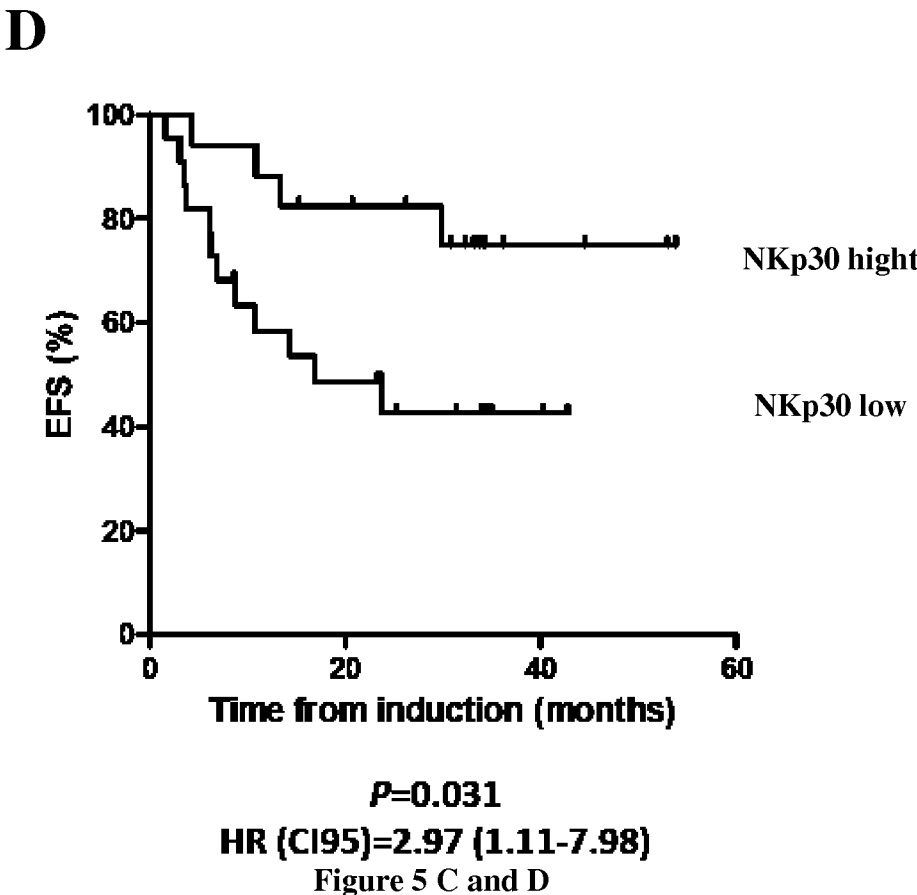
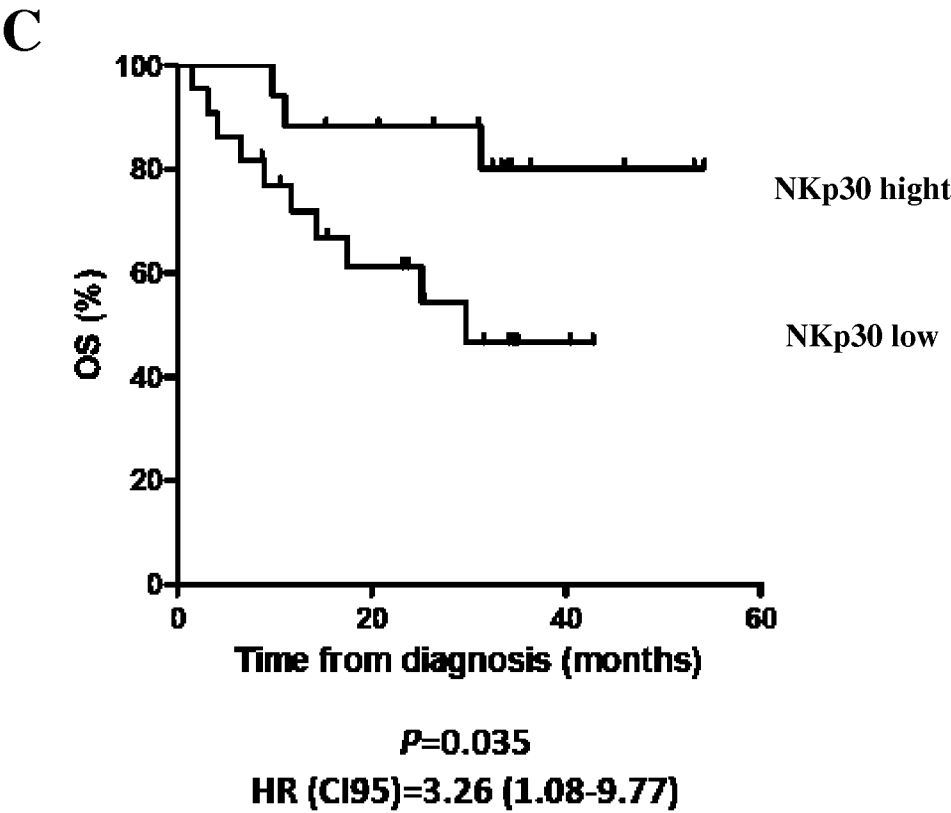


Figure 5 A and B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/053248

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 C12Q1/68 C12Q1/6886
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)
G01N C12Q

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)

EPO-Internal, WPI Data, BIOSIS, EMBASE, COMPENDEX, FSTA, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. FAURIAT ET AL: "Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction", BLOOD, vol. 109, no. 1, 1 January 2007 (2007-01-01), pages 323-330, XP055128290, ISSN: 0006-4971, DOI: 10.1182/blood-2005-08-027979 the whole document abstract table 1 page 328, left-hand column, paragraph 3 - right-hand column, paragraph 1; figure 6A page 329, right-hand column, paragraph 1 ----- -/--	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search

27 February 2018

Date of mailing of the international search report

13/03/2018

Name and mailing address of the ISA/

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Authorized officer

Gall-Truchot, A

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/053248

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANNA MARTNER ET AL: "Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia", ONCOIMMUNOLOGY, vol. 5, no. 1, 5 May 2015 (2015-05-05), page e1041701, XP055394636, DOI: 10.1080/2162402X.2015.1041701 the whole document abstract page 3, left-hand column, paragraph 2 figures 5A, 5D -----	1-6
A	"ACUTE LEUKEMIAS XVI ED - Hehlmann Rüdiger", ANNALS OF HEMATOLOGY, BERLIN, DE, vol. 96, no. 1, 20 January 2017 (2017-01-20), pages S1-S89, XP036138939, ISSN: 0939-5555, DOI: 10.1007/S00277-017-2921-1 [retrieved on 2017-01-20] page S87 Role of Cytotoxic Lymphocyte Subsets in the Outcome of Immunotherapy with HDC (Ceplene) and Low Dose IL-2 in AML -----	1-6
A	ANNA MARTNER ET AL: "NK cell expression of natural cytotoxicity receptors may determine relapse risk in older AML patients undergoing immunotherapy for remission maintenance", ONCOTARGET, vol. 6, no. 40, 30 October 2015 (2015-10-30), pages 42569-42574, XP055394637, DOI: 10.18632/oncotarget.5559 the whole document abstract page 42570, left-hand column, paragraph 1 - right-hand column, paragraph 1 figures 2A, 2C ----- -/--	1-6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/053248

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANNA MARTNER ET AL: "Natural Killer Cell Expression of Natural Cytotoxicity Receptors Determines Relapse Risk in Elderly Patients with Acute Myeloid Leukemia Receiving Immunotherapy with Histamine Dihydrochloride and Interleukin-2", 56TH ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY; SAN FRANCISCO, CA, USA; DECEMBER 06 -09, 2014 (BLOOD VOL 124, NR 21), 4 December 2014 (2014-12-04), XP055394654, the whole document	1-6
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