Title: METHOD FOR EVALUATING THE RESPONSE OF RHEUMATOID ARTHRITIS PATIENTS TO THERAPY AND FOR DIAGNOSING DISEASE SEVERITY

Abstract: The present invention identified sIL-7R as a serum marker of fibroblast and CD4 T cell activation in RA. Elevated sIL-7R serum levels reflect RA synovial cell exposure to combinations of pro-inflammatory cytokines, including TNF-alpha, IL-1 beta, IL-17 and synergistic combinations of these cytokines. Elevated baseline serum levels predict response to methotrexate therapy in early RA patients. Elevated baseline sIL-7R serum levels strongly predict poor-response to TNF blockade in DMARD-resistant patients and, potentially, poor-response to IL-1 and IL-17 blocking agents. The invention thus provides for methods and tools for predicting disease severity and the responsiveness of a subject to treatment.
METHOD FOR EVALUATING THE RESPONSE OF RHEUMATOID ARTHRITIS PATIENTS TO THERAPY AND FOR DIAGNOSING DISEASE SEVERITY

Field of the invention

The present invention is directed to methods for determining the response to a treatment of individuals suffering from rheumatoid arthritis (RA). In particular, the invention relates to methods to predict the responsiveness of a patient with rheumatoid arthritis to methotrexate, and biologics including, but not limited to, a TNF blocking drug, IL-1 blocking agents and/or IL-17 blocking agents.

Background of the invention

Rheumatoid arthritis (RA) has a worldwide distribution and involves all ethnic groups. Although the disease can occur at any age, the prevalence increases with age and the peak incidence is between the fourth and sixth decade. The prevalence estimates for the North American population vary from 0.3% to 1.5%. Today, over 2,500,000 individuals are diagnosed with rheumatoid arthritis in the United States alone, with some statistics indicating from 6.5 to 8 million potentially afflicted with the disease. Women are affected 2-3 times more often than men.

The early symptoms of rheumatoid arthritis are mostly joint specific such as painful joints with joint swelling or tenderness, but may also include rather non-specific manifestations like stiffness, fever, subcutaneous nodules, and fatigue. Very characteristic is the symmetric involvement of joints. The joints of the hands, feet, knees and wrists are most commonly affected, with eventual involvement of the hips, elbows and shoulders. As the disease progresses, any type of motion becomes very painful and difficult leading eventually to a loss of function of the involved joints. The more severe cases of rheumatoid arthritis can lead to intense pain and joint destruction. Some 300,000 bone and joint replacement surgical procedures are performed annually in an effort to alleviate the pain and mobility loss resultant from arthritis related joint destruction.

RA is a chronic inflammatory disease of the synovium that can lead to severe joint damage if insufficiently treated. Central to the pathogenesis of RA is the proliferation of synovial fibroblasts (synovial “pannus”) in response to the production of autocrine, but also paracrine molecules produced by infiltrating mononuclear cells. Among these molecules, pro-inflammatory cytokines such as TNF-alpha, IL-1-beta, IL-6 or IL-17 play an important role, and this observation led to the development of targeted therapies. In this context, TNF blocking agents are used routinely in RA patients who have failed first-line Disease Modifying Anti-Rheumatic Drugs (DMARD) therapy.
The use of biologics has considerably improved clinical and functional outcomes in RA. At the present time, clinicians in charge of RA patients have access to several TNF blocking agents, but also other biologics that are either already available (tocilizumab, rituximab, abatacept) or are still in development or in clinical studies (anti-IL-17 antibodies, anti-IL-1β antibodies, new TNF blocking agents). The availability of these new drugs opens new perspectives for patients, but also new challenges for clinicians, in particular in terms of treatment selection based on objective criteria. As a general rule, a good-response is obtained in about 20-30% of patients treated with biologics, while a moderate-response is obtained in an additional 40-45%. At the present time, no rational treatment algorithms are available that help in prescribing the right biologic to the right patient based on an a priori estimation of response to therapy. Given the rates of non-response (25 to 30%), as well as the significant medical risks associated with disease progression in non-responders, and last but not least, the high cost of these drugs, new biomarkers of response to therapy are urgently needed in the field of RA.

Therefore, there is a need for a means to determine whether a subject having RA is likely to respond to TNF blockade therapy and to diagnose disease severity.

Summary of the invention

The present invention provides several methods to predict or estimate the likelihood or probability that a patient with rheumatoid arthritis will respond with positive or favorable clinical results to treatment with methotrexate or with biological agents including, but not limited to, TNF blocking agent, IL-1 blocking agents and/or IL-17 blocking agents and to diagnose the RA disease severity or progression in a subject.

The invention is based on the unexpected finding that in RA patients, especially in patients with severe or advanced RA, the FLS cells of the synovial tissue show an increased expression of a specific transcript of the IL7 receptor, lacking exon 6. The protein derived from said transcript is a soluble truncated form of the IL7 receptor, which is secreted by the FLS cells and is capable of binding IL7, thereby functioning as an IL7 sequester molecule. The inventors found that said soluble IL-7 receptor protein is induced in FLS by pro-inflammatory cytokines, such as TNF-alpha, IL-1beta or IL-17 and synergistic combinations of these cytokines, which play an important pathogenic role in RA. In addition, the inventors showed that surprisingly said secreted soluble IL7 receptor protein fragment can be detected in synovial fluid and, more surprisingly, in serum samples of both healthy subjects and RA patients, wherein the amount or concentration of the protein increases with the disease severity and with resistance to treatment with methotrexate (in early RA patients) or biologics including, but not limited to, TNF-blocking agents (especially in DMARD-resistant patients), IL-1 blocking and/or IL-17 blocking agents (in DMARD-resistant patients).
These findings indicate that the soluble form of the IL7 receptor is an interesting diagnostic marker, both for assessing disease progression and for predicting response to treatment with methotrexate, TNF-blocking agents, IL-1 blocking and/or IL-17 blocking agents.

One aspect of the invention thus provides a method for predicting the response to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agents and/or IL-17 blocking agents, in a patient comprising or consisting of evaluating the amount of soluble IL7R protein in a sample of the patient, preferably a bodily fluid sample of the patient, more preferably a synovial fluid sample or a blood, or serum sample.

In a further embodiment, the invention provides a method for evaluating the disease severity of a RA patient comprising or consisting of evaluating the amount of soluble IL7R protein in a sample of the patient, preferably a bodily fluid sample of the patient, more preferably a synovial fluid sample or a blood, or serum sample.

Another aspect of the invention relates to a method to determine the probability of a positive clinical response in a patient, with early rheumatoid arthritis, to treatment with a methotrexate based on the amount of soluble IL7 receptor protein in a serum or synovial sample of said patient.

Another aspect of the invention relates to a method to determine the probability of a positive clinical response in a DMARD-resistant patient, to treatment with IL-1 blocking agents and/or IL-17 blocking agents based on the amount of soluble IL7 receptor protein in a serum or synovial sample of said patient.

The present inventors have designed methods based on the identification of the level of the sIL7R biomarker in serum as being predictive of the response to TNF blocking agents in severe RA, and that are useful for determining whether an individual with rheumatoid arthritis will be a poor, moderate or good responder to TNF blocking therapy. In addition, the level of soluble IL7R protein secreted by fibroblast-like synovial cells (FLS) in synovial fluid has the potential to be useful for identifying the probability of a positive clinical response in a patient, with rheumatoid arthritis, to treatment with a TNF blocking drug.

The methods according to the present invention is based on the comparison of the level, amount or concentration of the sIL7R protein as compared to standard values that can be determined beforehand or simultaneously based on samples from pre-diagnosed or predetermined samples using an identical assay.

The invention further provides for a method for predicting the response to a treatment with methotrexate in a subject with early RA comprising the steps of:

(a) measuring in a sample from said patient the amount of soluble IL-7R, and
(b) predicting the response to the treatment with methotrexate in said patient by evaluating the results of step (a).

The invention thus provides for a method for predicting the response to a treatment with a TNF blocking agent in a DMARD-resistant RA patient comprising the steps of:

(a) measuring in a sample from said patient the amount of soluble IL-7R, and

(b) predicting the response to the treatment with the TNF blocking agent in said patient by evaluating the results of step (a).

The invention further provides for a method for predicting the response to a treatment with IL-1 blocking agents and/or IL-17 blocking agents in a subject with DMARD-resistant RA comprising the steps of:

(a) measuring in a sample from said patient the amount of soluble IL-7R, and

(b) predicting the response to the treatment with IL-1 blocking agents and/or IL-17 blocking agents in said patient by evaluating the results of step (a).

The invention further provides for a method for diagnosing the RA disease severity in a subject comprising the steps of:

(a) measuring in a sample from said patient the amount of soluble IL-7R, and

(b) predicting the disease severity in said patient by evaluating the results of step (a).

In any one of the methods defined herein, measuring the amount of sIL7R is preferably performed by measuring the concentration of soluble IL-7R protein in a blood, serum, or synovial fluid sample of said subject. In a preferred embodiment, said method comprises the steps of:

i) providing a sample from a subject,

ii) measuring the concentration of soluble IL-7R in the sample,

iii) predicting the response to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent or the RA disease severity in a subject when detectable soluble IL-7R is present in the sample or when the amount of soluble IL-7R in the sample is greater than or equal to a threshold value.

In a preferred embodiment of any one of the methods as defined herein, the amount of the sIL7R protein is detected using a reagent which specifically binds with said protein, preferably selected from the group consisting of: an aptamer, a photoaptamer, a protein, a peptide, a peptidomimetic, an antibody or a fragment or a derivative thereof, a polyclonal antibody, a monoclonal antibody, a humanised or a chimeric antibody, an engineered antibody, or a biologically functional antibody fragment sufficient for binding to the soluble form of the IL7R
alpha chain protein, or wherein the amount of soluble IL7R is measured using any of biochemical assay, immunoassay, surface plasmon resonance, fluorescence resonance energy transfer, bioluminescence resonance energy transfer or quenching is detected.

In addition, the step of determining the threshold value of responders vs. non-responders in any one of the above methods can optionally be determined before step (i) by:

(i1) assessing the amount of soluble IL-7R in a plurality of samples from patients before treatment with said methotrexate, TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent

(i2) assessing the amount of soluble IL-7R in a plurality of samples from patients after treatment with said methotrexate, TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent and

(i3) correlating the response of the patients treated with said methotrexate, TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent to the amount of soluble IL7R, thereby determining the threshold value of responders.

In addition, the step of determining the threshold value of low vs. high RA disease severity is optionally determined before step (i) by:

(i1) assessing the amount of soluble IL-7R in a plurality of samples from patients with low RA disease severity or from healthy subjects,

(i2) assessing the amount of soluble IL-7R in a plurality of samples from patients with high RA disease severity, and

(i3) correlating the response of the two patient/subject groups to the amount of soluble IL7R, thereby determining the threshold value corresponding to disease severity.

In an embodiment, said sample, is a serum sample. In another embodiment, said sample is a synovial fluid sample.

Another aspect of the invention relates to a kit for predicting the response of a RA patient to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent comprising:

a) optionally a means for providing a sample from the subject, e.g. a blood, or serum sample

b) a means for assessing the protein level of the soluble IL7R protein in said sample, and

c) one or more reference values reflecting the sIL7R protein level in RA patients that either do or do not respond to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agent.
In a further aspect, the invention provides for the use of the kit as described herein, in a method for predicting the response of a subject to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agent and/or IL-17 blocking agents as described herein.

Another aspect of the invention relates to a kit for predicting the severity of the RA condition of a RA patient comprising:

5 a) optionally a means for drawing blood,

b) a means for assessing the protein level of the soluble IL7R protein in said serum sample, and

c) one or more reference values reflecting the sIL7R protein level in RA patients with previously established RA severities.

In addition, the invention provides for the use of the kit as described herein, in a method for predicting the severity of the RA condition of a subject as described herein.

In a further embodiment, the method of the invention comprises:

- providing in vivo or in said biological sample, preferably in said sample, the amount or concentration of the soluble IL7R protein as defined herein,

- providing reference levels by establishing the amount or concentration of the soluble IL7R protein as defined herein for reference samples, preferably reference samples, from reference subjects which are poor, moderate and good responder to TNF inhibition,

- comparing the subject level with the reference level, and

- determining whether said biological sample, preferably said sample, is from a poor, moderate or good responder to TNF inhibition.

Those skilled in the art will immediate recognize the many other effects and advantages of the present method and the numerous possibilities for end uses of the present invention from the detailed description and examples provided below.

25 **Brief description of the figures**

**Figure 1: FLS and activated CD4 T cells produce two IL-7R alpha-chain isoforms.**

A) IL-7R Western Blots on protein extracts from FLS and PBMC, and IL-7R PCR on cDNA from TNF-alpha and IL-1-beta-activated FLS (1), IL-2 and PHA activated CD4 T cells (2), antigen-activated CD8 T cell clones (3) and B-EBV cells (4). Arrows indicate the expected sizes of the membrane-bound IL-7R.
B) Sequencing of purified IL-7R PCR fragments indicate that FLS produce a full-length IL-7R-alpha-chain and a truncated form of the IL-7R-alpha-chain lacking exon 6 encoding the transmembrane domain, thereby resulting in a secreted form of the molecule.

**Figure 2: Pro-inflammatory cytokines stimulate sIL-7R production by FLS.**

A) IL-7R (open bars) and sIL-7R (closed bars) real-time qPCR studies carried out on FLS stimulated with the indicated cytokines. Results are expressed as mean fold changes in IL-7R and sIL-7R gene expression (+/- SEM) over unstimulated FLS, obtained from 2 to 5 different experiments each.

B) Flow cytometric evaluation of IL-7R expression by PBMC and FLS. Cells were incubated with a PE-conjugated IL-7R antibody (red) or a PE-conjugated isotype control (blue). Autofluorescence of the cells is depicted in green. Graphs are representative of 3 different experiments. Similar results were obtained using FLS stimulated with pro-inflammatory cytokines, alone or in combination.

C) sIL-7R measurements were performed by sandwich-ELISA in supernatants of FLS cultures stimulated with the indicated cytokines. Results are expressed as mean optical density (O.D.) units x 1,000 (after subtraction of the baseline O.D.) +/- SEM obtained from 3 different experiments. D. Effect of IL-7 and sIL-7R-Fc fusion protein on proliferation of synovial CD4 T cells cultured in the presence of autologous FLS and autologous serum. Results are expressed as mean cpm (+/- SEM) obtained from two different experiments. * p < 0.05; ** p < 0.005; *** p < 0.0005 versus unstimulated cells.

**Figure 3: Serum sIL-7R titers are significantly higher in early and DMARD-resistant RA patients.**

sIL-7R titers measured by sandwich ELISA in duplicate serum samples from healthy individuals (n=75), early RA (n=52) and DMARD-resistant RA (n=76) patients.

**Figure 4: sIL-7R serum concentrations predict response to TNF blockade in DMARD-resistant RA patients.**

A) sIL-7R titers measured by sandwich ELISA in duplicate baseline serum samples obtained in DMARD-resistant RA patients treated with 3 mg/kg infliximab. Patients were categorized in responders versus non-responders according to EULAR response criteria.

B) Linear correlation between baseline sIL-7R serum levels and DAS-Score differences (follow-up minus baseline DAS28-CRP).

C) Receiving Operating Characteristic curve evaluating the value of baseline sIL-7R in predicting response to therapy. The curve was plotted by calculating sensitivity and specificity of the test at several cut-off values.
Figure 5: sIL-7R serum concentrations predict response to methotrexate therapy in early RA

A) sIL-7R titers measured by sandwich ELISA in duplicate baseline serum samples obtained in early RA patients treated with methotrexate. Patients were categorized in responders versus non-responders according to EULAR response criteria.

B) Linear correlation between baseline sIL-7R serum levels and DAS-Score differences (follow-up minus baseline DAS28-CRP).

Figure 6: represents in (A) SEQ ID NO:1 (mRNA sequence corresponding to the membrane bound form of IL-7R) in (B) SEQ ID NO:2 (amino acid sequence of the membrane bound form of IL-7R).

Figure 7: represents in (A) SEQ ID NO:3 (mRNA sequence corresponding to the soluble form of IL-7R), and in (B) SEQ ID NO:4 (amino acid sequence corresponding to the soluble form of IL-7R).

Detailed description

The present inventors show that serum and synovial fluid levels of a soluble form of the interleukin-7 receptor alpha-chain (sIL-7R) are significantly associated with response to therapy in early RA patients treated with methotrexate and in DMARD-resistant RA patients treated with TNF blocking agents. Soluble IL-7R is produced by RA synovial fibroblasts after exposure to pro-inflammatory cytokines and, to a lesser extent, by activated CD4 T cells. By reflecting synovial cell exposure to TNF-alpha, IL-1-beta, IL-17 and synergistic combinations of these cytokines, sIL-7R is identified as a marker of disease severity and response to therapy in RA, detectable in the serum and synovial fluid of the subject.

The inventors first studied the expression of the IL-7R alpha-chain in synovial cells from RA patients, and found that cultured fibroblast-like synovial cells (FLS) produce the secreted form of the molecule. Although PCR and Western Blot experiments indicate that FLS also display a positive signal for the full length IL-7R alpha-chain, it could not be detected by flow cytometry at the cell surface of resting or stimulated cells, and neither was any proliferative effect or induction of cytokines by FLS found in response to IL-7 (data not shown).

The soluble IL-7R has been described in fibroblast cell lines as the result of alternative splicing of the gene, leading to a deletion of exon 6 that contains the transmembrane domain (Goodwin et al., Cell 1990; 60: 941-51; Pleiman et al., Mol Cell Biol 1991; 11: 3052-9). The present invention shows that addition of pro-inflammatory cytokines, such as TNF-alpha and IL-1-beta, or various combinations of TNF-alpha, IL-1-beta and IL-17, stimulate the expression
and secretion of sIL-7R by fibroblast-like synovial cells (FLS). Other than FLS, expression of the sIL-7R gene in activated CD4 T-cells was also detected, although to a lesser extent.

It has been demonstrated in the past that soluble IL-7R is able to bind and inhibit IL-7. The present invention confirms that such a mechanism is also present in the context of RA and demonstrated that, while IL-7 stimulates synovial CD4 T-cell proliferation in response to FLS, IL-7 inhibition by a siL-7R-Fc fusion protein induces the opposite effect. Taken together, these results indicate that sIL-7R is induced by pro-inflammatory cytokines in FLS and plays a role in a negative feed-back loop through the inhibition of T-cell proliferation and activation.

Next, the inventors studied whether sIL-7R was detectable in synovial fluid and/or serum of RA patients and controls. Since the molecule is secreted by FLS in response to pro-inflammatory cytokines, the correlation of the synovial fluid and/or serum titers with RA disease severity and progression were evaluated and sIL-7R could be readily detectable in the sera and synovial fluid samples of healthy individuals, an observation that confirms recent findings (Faucher et al., PLoS One 2009; 4: e6690; Rose et al., J Immunol 2009; 182: 7389-97). In particular, Rose et al. studied soluble IL-7R serum levels in HIV patients and controls. They produced convincing evidence that soluble IL-7R is produced by alternative splicing of the IL-7 gene in T-cells, and not by proteolytic cleavage from the surface of T-cells. The present invention established that synovial fluid and serum levels of the soluble IL7R molecule are higher in patients with early RA as compared to healthy subjects and are even higher in DMARD-resistant RA patients, an observation consistent with the data indicating that soluble IL-7R reflects synovial fibroblast activation and, to a lesser extent, CD4 T-cell activation.

Strikingly, high baseline soluble IL-7R serum levels in early RA patients are associated with poor-response to methotrexate therapy. Similarly, high baseline soluble IL-7R serum levels in DMARD-resistant RA patients are significantly associated with poor-response to TNF blockade. Conversely, lower serum levels of the molecule are strongly associated with adequate response to therapy. With a sensitivity of 88% and a specificity of 63% for the prediction of good- and moderate-response to TNF blockade, serum soluble IL-7R is a promising clinical biomarker that can aid the individual therapeutic decision-making process in RA in order to evaluate the usefulness of therapy with not only TNF-blocking agents, but also methotrexate and potentially also other biologicals such as IL-1 and IL-17 blocking agents.

Without wanting to be bound by any theory, it appears that the sIL7R is a marker of severity in RA because it reflects synovial cell exposure to cytokines with a strong pro-inflammatory action, such as TNF, IL-1, IL-17 and potentially other cytokines. The sIL7R level therefore also predicts absence of response to therapy in that the higher the amount of these cytokines in the synovium, the harder it will be for (any) therapy to counteract them. It is therefore expected that high baseline sIL7R serum levels could actually very well predict absence of response to
IL-1 blockade or IL-17 blockade and other biologics such as rituximab (Mabthera) therapy which down-regulates IL-17 production in the synovium (data not shown), CTLA4-Ig (Orencia), or tocilizumab (Ro-Actemra).

Using soluble IL7R as a biomarker in clinical practice would have an important impact in terms of patients’ outcomes and drug expenses. In the absence of selection, 25% of the patients described in this work did not respond to TNF blockade, a proportion in accordance with published data. If the patients had been selected based on baseline soluble IL-7R serum levels, the proportion of non-responders would have dropped to 11%, while only 5% would have been switched to an alternative therapy although they would have responded to TNF blockade. Such differences are highly significant from a clinical point of view and make the serum soluble IL-7R an important new clinical biomarker in RA.

All documents cited in the present specification are hereby incorporated by reference in their entirety.

When describing the invention, the terms used are to be construed in accordance with the following definitions, unless a context dictates otherwise:

The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps.

The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

The term "about" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of and from the specified value, in particular variations of +/-10% or less, preferably +/-5% or less, more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" refers is itself also specifically, and preferably, disclosed.

As used herein the term "biological sample" or "sample" refers to a sample that comprises a biomolecule that permits the expression level of a gene or an amount of protein to be determined. Representative biomolecules include, but are not limited to total RNA, mRNA, and polypeptides, and derivatives of these molecules such as cDNAs and ESTs. As such, a biological sample can comprise a cell or a group of cells. Samples may include, without limitation, whole blood, plasma, serum, red blood cells, white blood cells (e.g., peripheral blood mononuclear cells), saliva, urine, stool (i.e., faeces), tears, sweat, sebum, nipple aspirate, ductal lavage, tumour exudates, joint or synovial fluid, cerebrospinal fluid, lymph, fine
needle aspirate, amniotic fluid, any other bodily fluid, cell lysates, cellular secretion products, inflammation fluid, semen and vaginal secretions. Preferred samples may include ones comprising soluble IL7R protein or mRNA coding therefore in detectable quantities. In preferred embodiments, the sample for protein detection is whole blood or preferably plasma (i.e. whole blood minus the cells), more preferably serum (i.e., whole blood minus both the cells and the clotting factors) or joint or synovial fluid. Serum comprises all proteins not used in blood clotting and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances. Alternatively, a preferred sample for mRNA detection is a sample comprising cells expressing the mRNA encoding for the soluble form of the IL7R, i.e. the splice variant as defined herein. Preferably, said sample is joint fluid, synovial fluid, more preferably a sample of FLS-cells (fibroblast-like synovial cells).

The sample can, of course, be subjected to a variety of well-known post-collection preparative and storage techniques (e.g. fixation, storage, freezing, lysis, homogenization, DNA or RNA extraction, ultrafiltration, concentration, evaporation, centrifugation, etc.) prior to determining the level of expression in the sample. The RNA may be stabilized using known RNA stabilizers upon or shortly after obtaining the sample.

The methods of the present invention are particularly useful for subjects with rheumatic arthritis, preferably severe rheumatic arthritis (e.g. for anti-TNF-blocking therapy evaluation) or early RA (e.g. for methotrexate therapy evaluation), defined by appropriate RA severity scales known in the art and defined herein.

As used herein the terms "subject", "patient" or "individual" refer to any vertebrate species. Preferably, the term subject encompasses warm-blooded vertebrates, more preferably mammals. More particularly contemplated are mammals such as humans, as well as animals such as carnivores other than humans (such as cats and dogs), swine (pigs, hogs, and wild boars), poultry, ruminants (such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses.

As used in the specification and the appended claims, the singular forms "a", "an," and "the" include plural referents unless the context clearly dictates otherwise. By way of example, "a method" means one method or more than one method.

The term "and/or" as used in the present specification and in the claims implies that the phrases before and after this term are to be considered either as alternatives or in combination.

As used herein, the term "level" or "expression level" refers to the expression level data that can be used to compare the expression levels of different genes among various samples and/or subjects.
The term "amount" or "concentration" of certain protein refers respectively to the effective (i.e. total protein amount measured) or relative amount (i.e. total protein amount measured in relation to the sample size used) of the protein in a certain sample.

The term "sIL7R protein" or "soluble IL7R protein" refers to the soluble form of the IL7 receptor alpha chain protein, which is the result of an mRNA isoform or splice variant of the gene encoding for the IL7 receptor alpha chain protein, as described by Goodwin et al., 1990 and Pleiman et al., 1991 (Goodwin et al., Cell 1990; 60: 941-51; Pleiman et al., Mol Cell Biol 1991; 11: 3052-9). In essence, the soluble form is the product of a splice variant originating from deletion of exon 6, encoding the transmembrane region.

The term "gene encoding for the sIL7R protein" or "isoform encoding the soluble IL7R protein" refers to the mRNA isoform or splice variant of the gene encoding for the IL7 receptor alpha chain protein, as described by Goodwin et al., 1990 and Pleiman et al., 1991 (Goodwin et al., Cell 1990; 60: 941-51; Pleiman et al., Mol Cell Biol 1991; 11: 3052-9). In essence, said splice variant originates from deletion of exon 6, encoding the transmembrane region.

The mRNA sequence corresponding to the membrane bound form is represented by SEQ ID NO:1, the resulting amino acid sequence is represented by SEQ ID NO:2. The mRNA sequence corresponding to the soluble form is represented by SEQ ID NO:3, the resulting amino acid sequence is represented by SEQ ID NO:4.

As used herein the term "gene" or gene-transcript" encompasses sequences including, but not limited to a coding sequence, a promoter region, a transcriptional regulatory sequence, a non-expressed DNA segment that is a specific recognition sequence for regulatory proteins, a non-expressed DNA segment that contributes to gene expression, a DNA segment designed to have desired parameters, sense and anti-sense strands of genomic DNA (i.e. including any introns occurring therein), EST, RNA generated by transcription of genomic DNA (i.e. prior to splicing), RNA generated by splicing of RNA transcribed from genomic DNA, and proteins generated by translation of spliced RNA (e.g. including proteins both before and after cleavage of normally cleaved regions such as transmembrane signal sequences), cDNA made by reverse transcription of an RNA generated by transcription of genomic DNA (including spliced RNA) and fragments thereof, or combinations thereof. In particular, the present invention includes methods and kits for detecting the presence and/or amount of a certain splice variant of the gene encoding the IL7R alpha chain protein, especially the isoform missing exon 6 encoding the soluble IL7R protein fragment.

As used herein the term "fragment" shall be understood to mean a nucleic acid that is the same as part of, but not all of a nucleic acid that forms a gene. The term "fragment" also encompasses a part, but not all of an intergenic region.
The term “increased expression” and “decreased expression” refers to expression of the gene in a sample, at a greater or lesser level, respectively, than the level of expression of said gene (e. g. at least two-fold greater or lesser level) in a control (reference sample). The gene is said to be up-regulated or over-expressed or down-regulated or under-expressed if either the gene is present at a greater or lesser level, respectively, than the level in a control. Expression of a gene in a sample is "significantly" higher or lower than the level of expression of a gene in a control if the level of expression of the gene is greater or less, respectively, than the level by an amount greater than the standard error of the assay employed to assess expression, and preferably at least twice, and more preferably three, four, five or ten times that amount. Alternately, expression of the gene in the sample can be considered "significantly" higher or lower than the level of expression in a control if the level of expression is at least about two, and preferably at least about three, four, or five times, higher or lower, respectively, than the level of expression of the gene in said control.

The terms “increased amount or concentration” or “decreased amount or concentration” of a certain protein respectively refer to a higher or lower presence of the amount of protein in a certain sample as compared to the level in a control sample. The amount or concentration of the protein is "significantly" higher or lower than the amount or concentration of a protein in a control sample if said change exceeds the standard error of the assay employed to assess the amount or concentration of the protein, and is preferably at least 1.5, 2, 3, 4, 5, or 10 times the control amount or concentration.

As used herein, the term "profile" refers to a repository of the expression level data that can be used to compare the expression levels of different genes among various subjects.

The terms "predicting" or "prediction", "diagnosing" or "diagnosis" and "prognosticating" or "prognosis" are commonplace and well-understood in medical and clinical practice. By means of further explanation and without limitation, "predicting" or "prediction" generally refer to an advance declaration, indication or foretelling of a disease or condition in a subject not (yet) having said disease or condition. For example, a prediction of a disease or condition in a subject may indicate a probability, chance or risk that the subject will develop said disease or condition, for example within a certain time period or by a certain age. Said probability, chance or risk may be indicated inter alia as an absolute value, range or statistics, or may be indicated relative to a suitable control subject or subject population (such as, e.g., relative to a general, normal or healthy subject or subject population). Hence, the probability, chance or risk that a subject will develop a disease or condition may be advantageously indicated as increased or decreased, or as fold-increased or fold-decreased relative to a suitable control subject or subject population.
As used herein, the term "prediction of response" in a subject may also particularly mean that the subject has a 'positive' prediction of responding to treatment with methotrexate, TNF-blocking agents, IL-1 blocking agents and/or IL-17 blocking agents. The term "prediction of no response" in a subject may particularly mean that the subject has a 'negative' prediction of responding to treatment with methotrexate, TNF-blocking agents, IL-1 blocking agents and/or IL-17 blocking agents vis-à-vis a control subject or subject population.

The term "severity" or progression" used in relation to Rheumatoid Arthritis can be presented by several scale systems such as: the rheumatoid arthritis medical records based index of severity (RARBIS: Ting et al., 2005, *J Rheumatol*, 32:1679-1687 and Cabral et al., 2005, *Arthritis Rheum*, 53:61-66.), the 28-joint Disease Activity Score (DAS28: van der Heijde et al., 1993, *J Rheumatol* 20:579-581 and Prevoo et al., 1995, *Arthritis Rheum*, 38:44-48), or the RA Severity Scale (RASS: Bardwell et al., 2002, *Rheumatology* (Oxford), Jan;41(1):38-45). Any one of these scales can be used to distinguish between healthy subjects, early RA subjects, DMARD-resistant progressive RA patients. All scales are generally designed around three visual analogue scales: Disease Activity, Functional Impairment and Physical Damage, based e.g. on number of swollen joints, number of tender joints, erythrocyte sedimentation rate (ESR), and general health. Preferably, the severity is established based on persistant disease activity (i.e. elevated DAS28-CRP scores) despite therapy, leading to an increased probability of radiological damage, joint destruction and, finally, disability.

In an embodiment, the level of expression of said gene or fragment thereof in (vivo or in) said sample, preferably serum or synovial fluid sample, is assessed by detecting the level of expression of the soluble form of the IL-7 alpha chain protein or a fragment thereof. In a preferred embodiment, the level of expression of said protein or fragment thereof is detected using a binding agent which specifically binds with said protein or fragment thereof.

The term "binding agent" is defined as specifically binding to the soluble IL7 receptor protein alpha chain or a fragment thereof and may include *inter alia* an antibody, aptamer, photoaptamer, protein, peptide, peptidomimetic or a small molecule.

The term "specifically bind" as used throughout this specification means that an agent (denoted herein also as "specific-binding agent") binds to one or more desired molecules or analytes, such as to one or more proteins, polypeptides or peptides of interest or fragments thereof substantially to the exclusion of other molecules which are random or unrelated, and optionally substantially to the exclusion of other molecules that are structurally related. The term "specifically bind" does not necessarily require that an agent binds exclusively to its intended target(s). For example, an agent may be said to specifically bind to protein(s) polypeptide(s), peptide(s) and/or fragment(s) thereof of interest if its affinity for such intended target(s) under the conditions of binding is at least about 2-fold greater, preferably at least
about 5-fold greater, more preferably at least about 10-fold greater, yet more preferably at least about 25-fold greater, still more preferably at least about 50-fold greater, and even more preferably at least about 100-fold or more greater, than its affinity for a non-target molecule. Preferably, the agent may bind to its intended target(s) with affinity constant \( K_A \) of such binding \( K_A \geq 1 \times 10^6 \text{ M}^{-1} \), more preferably \( K_A \geq 1 \times 10^7 \text{ M}^{-1} \), yet more preferably \( K_A \geq 1 \times 10^8 \text{ M}^{-1} \), even more preferably \( K_A \geq 1 \times 10^9 \text{ M}^{-1} \), and still more preferably \( K_A \geq 1 \times 10^{10} \text{ M}^{-1} \) or \( K_A \geq 1 \times 10^{11} \text{ M}^{-1} \), wherein \( K_A = [\text{SBA}_T]/[\text{SBA}][T] \), SBA denotes the specific-binding agent, T denotes the intended target. Determination of \( K_A \) can be carried out by methods known in the art, such as for example, using equilibrium dialysis and Scatchard plot analysis.

As used herein, the term "antibody" is used in its broadest sense and generally refers to any immunologic binding agent. The term specifically encompasses intact monoclonal antibodies, polyclonal antibodies, multivalent (e.g., 2-, 3- or more-valent) and/or multi-specific antibodies (e.g., bi- or more-specific antibodies) formed from at least two intact antibodies, and antibody fragments insofar they exhibit the desired biological activity (particularly, ability to specifically bind an antigen of interest), as well as multivalent and/or multi-specific composites of such fragments. The term "antibody" is not only inclusive of antibodies generated by methods comprising immunisation, but also includes any polypeptide, e.g., a recombinantly expressed polypeptide, which is made to encompass at least one complementarity-determining region (CDR) capable of specifically binding to an epitope on an antigen of interest. Hence, the term applies to such molecules regardless whether they are produced in vitro or in vivo. In an embodiment, an antibody may be any of IgA, IgD, IgE, IgG and IgM classes, and preferably IgG class antibody. In an embodiment, the antibody may be a polyclonal antibody, e.g., an antiserum or immunoglobulins purified therefrom (e.g., affinity-purified). In another preferred embodiment, the antibody may be a monoclonal antibody or a mixture of monoclonal antibodies. Monoclonal antibodies can target a particular antigen or a particular epitope within an antigen with greater selectivity and reproducibility. By means of example and not limitation, monoclonal antibodies may be made by the hybridoma method first described by Kohler et al. 1975 (Nature 256: 495), or may be made by recombinant DNA methods (e.g., as in US 4,816,567). Monoclonal antibodies may also be isolated from phage antibody libraries using techniques as described by Clackson et al. 1991 (Nature 352: 624-628) and Marks et al. 1991 (J Mol Biol 222: 581-597), for example. In further embodiments, the antibody binding agents may be antibody fragments. "Antibody fragments" comprise a portion of an intact antibody, comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(\text{ab}')\text{2}, Fv and scFv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multivalent and/or multispecific antibodies formed from antibody fragment(s), e.g., dibodies, tribodies, and multibodies. The above designations Fab, Fab', F(\text{ab}')\text{2}, Fv, scFv etc. are intended to have their art-established meaning. The term antibody
includes antibodies originating from or comprising one or more portions derived from any animal species, preferably vertebrate species, including, e.g., birds and mammals. Without limitation, the antibodies may be chicken, turkey, goose, duck, guinea fowl, quail or pheasant. Also without limitation, the antibodies may be human, murine (e.g., mouse, rat, etc.), donkey, rabbit, goat, sheep, guinea pig, camel (e.g., Camelus bactrianus and Camelus dromaderius), llama (e.g., Lama pacos, Lama glama or Lama vicugna) or horse. A skilled person will understand that an antibody can include one or more amino acid deletions, additions and/or substitutions (e.g., conservative substitutions), insofar such alterations preserve its binding of the respective antigen. An antibody may also include one or more native or artificial modifications of its constituent amino acid residues (e.g., glycosylation, etc.).


The term "aptamer" refers to single-stranded or double-stranded oligo-DNA, oligo-RNA or oligo-DNA/RNA or any analogue thereof, that can specifically bind to a target molecule such as a the siL7 protein alpha chain or a fragment thereof. Advantageously, aptamers can display fairly high specificity and affinity (e.g., \( K_A \) in the order \( 1 \times 10^9 \) M\(^{-1} \)) for their targets. Aptamer production is described \textit{inter alia} in US 5,270,163; Ellington & Szostak 1990 (Nature 346: 818-822); Tuerk & Gold 1990 (Science 249: 505-510); or "The Aptamer Handbook: Functional Oligonucleotides and Their Applications", by Klussmann, ed., Wiley-VCH 2006, ISBN 3527310592, incorporated by reference herein. The term "photoaptamer" refers to an aptamer that contains one or more photoreactive functional groups that can covalently bind to or crosslink with a target molecule. The term "peptidomimetic" refers to a non-peptide agent that is a topological analogue of a corresponding peptide. Methods of rationally designing peptidomimetics of peptides are known in the art. For example, the rational design of three peptidomimetics based on the sulphated 8-mer peptide CCK26-33, and of two peptidomimetics based on the 11-mer peptide Substance P, and related peptidomimetic design principles, are described in Howell 1995 (Trends Biotechnol 13: 132-134).

The term "small molecule" refers to compounds, preferably organic compounds, with a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes
biological macromolecules (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, e.g., up to about 4000, preferably up to 3000 Da, more preferably up to 2000 Da, even more preferably up to about 1000 Da, e.g., up to about 900, 800, 700, 600 or up to about 500 Da.

Any existing, available or conventional separation, detection and quantification methods can be used herein to measure the presence or absence (e.g., readout being present vs. absent; or detectable amount vs. undetectable amount) and/or quantity (e.g., readout being an absolute or relative quantity, such as, for example, absolute or relative concentration) of sIL7R and/or fragments.

For example, such methods may include immunoassay methods, mass spectrometry analysis methods, or chromatography methods, or combinations thereof.

The term "immunoassay" generally refers to methods known as such for detecting one or more molecules or analytes of interest in a sample, wherein specificity of an immunoassay for the molecule(s) or analyte(s) of interest is conferred by specific binding between a specific-binding agent, commonly an antibody, and the molecule(s) or analyte(s) of interest.

Immunoassay technologies include without limitation direct ELISA (enzyme-linked immunosorbent assay), indirect ELISA, sandwich ELISA, competitive ELISA, multiplex ELISA, radioimmunoassay (RIA), ELISPOT technologies, and other similar techniques known in the art. Principles of these immunoassay methods are known in the art, for example John R. Crowther, "The ELISA Guidebook", 1st ed., Humana Press 2000, ISBN 0896037282.

By means of further explanation and not limitation, direct ELISA employs a labelled primary antibody to bind to and thereby quantify target antigen in a sample immobilised on a solid support such as a microwell plate. Indirect ELISA uses a non-labelled primary antibody which binds to the target antigen and a secondary labelled antibody that recognises and allows quantifying the antigen-bound primary antibody. In sandwich ELISA the target antigen is captured from a sample using an immobilised ‘capture’ antibody which binds to one antigenic site within the antigen, and subsequent to removal of non-bound analytes the so-captured antigen is detected using a ‘detection’ antibody which binds to another antigenic site within said antigen, where the detection antibody may be directly labelled or indirectly detectable as above. Competitive ELISA uses a labelled ‘competitor’ that may either be the primary antibody or the target antigen. In an example, non-labelled immobilised primary antibody is incubated with a sample, this reaction is allowed to reach equilibrium, and then labelled target antigen is added. The latter will bind to the primary antibody wherever its binding sites are not yet occupied by non-labelled target antigen from the sample. Thus, the detected amount of bound labelled antigen inversely correlates with the amount of non-labelled antigen in the sample. Multiplex ELISA allows simultaneous detection of two or more analytes within a single
compartment (e.g., microplate well) usually at a plurality of array addresses (see, for example, Nielsen & Geierstanger 2004. J Immunol Methods 290: 107-20 and Ling et al. 2007. Expert Rev Mol Diagn 7: 87-98 for further guidance). As appreciated, labelling in ELISA technologies is usually by enzyme (such as, e.g., horse-radish peroxidase) conjugation and the end-point is typically colorimetric, chemiluminescent or fluorescent.

Radioimmunoassay (RIA) is a competition-based technique and involves mixing known quantities of radioactively-labelled (e.g., $^{125}$I- or $^{131}$I-labelled) target antigen with antibody to said antigen, then adding non-labelled or ‘cold’ antigen from a sample and measuring the amount of labelled antigen displaced (see, e.g., "An Introduction to Radioimmunoassay and Related Techniques", by Chard T, ed., Elsevier Science 1995, ISBN 0444821198 for guidance).

Further, mass spectrometry methods are suitable for measuring biomarkers. Generally, any mass spectrometric (MS) techniques that can obtain precise information on the mass of peptides, and preferably also on fragmentation and/or (partial) amino acid sequence of selected peptides (e.g., in tandem mass spectrometry, MS/MS; or in post source decay, TOF MS), are useful herein. Suitable peptide MS and MS/MS techniques and systems are well-known per se (see, e.g., Methods in Molecular Biology, vol. 146: "Mass Spectrometry of Proteins and Peptides", by Chapman, ed., Humana Press 2000, ISBN 089603609x; Biemann 1990. Methods Enzymol 193: 455-79; or Methods in Enzymology, vol. 402: "Biological Mass Spectrometry", by Burlingame, ed., Academic Press 2005, ISBN 9780121828073) and may be used herein. MS arrangements, instruments and systems suitable for biomarker peptide analysis may include, without limitation, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS; MALDI-TOF post-source-decay (PSD); MALDI-TOF/TOF; surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF) MS; electrospray ionization mass spectrometry (ESI-MS); ESI-MS/MS; ESI-MS/(MS)$^n$ (n is an integer greater than zero); ESI 3D or linear (2D) ion trap MS; ESI triple quadrupole MS; ESI quadrupole orthogonal TOF (Q-TOF); ESI Fourier transform MS systems; desorption/ionization on silicon (DIOS); secondary ion mass spectrometry (SIMS); atmospheric pressure chemical ionization mass spectrometry (APCI-MS); APCI-MS/MS; APCI- (MS)$^n$; atmospheric pressure photoionization mass spectrometry (APPI-MS); APPI-MS/MS; and APPI- (MS)$^n$. Peptide ion fragmentation in tandem MS (MS/MS) arrangements may be achieved using manners established in the art, such as, e.g., collision induced dissociation (CID). In an embodiment, detection and quantification of biomarkers by mass spectrometry may involve multiple reaction monitoring (MRM), such as described among others by Kuhn et al. 2004 (Proteomics 4: 1175-86). In an embodiment, MS peptide analysis methods may be advantageously combined with upstream peptide or protein separation or
fractionation methods, such as for example with the chromatographic and other methods described herein below.

Chromatography can also be used for measuring biomarkers. As used herein, the term "chromatography" encompasses methods for separating chemical substances, referred to as such and vastly available in the art. In a preferred approach, chromatography refers to a process in which a mixture of chemical substances (analytes) carried by a moving stream of liquid or gas ("mobile phase") is separated into components as a result of differential distribution of the analytes, as they flow around or over a stationary liquid or solid phase ("stationary phase"), between said mobile phase and said stationary phase. The stationary phase may be usually a finely divided solid, a sheet of filter material, or a thin film of a liquid on the surface of a solid, or the like. Chromatography is also widely applicable for the separation of chemical compounds of biological origin, such as, e.g., amino acids, proteins, fragments of proteins or peptides, etc. Chromatography as used herein may be preferably columnar (i.e., wherein the stationary phase is deposited or packed in a column), preferably liquid chromatography, and yet more preferably HPLC. While particulars of chromatography are well known in the art, for further guidance see, e.g., Meyer M., 1998, ISBN: 047198373X, and "Practical HPLC Methodology and Applications", Bidlingmeyer, B. A., John Wiley & Sons Inc., 1993. Exemplary types of chromatography include, without limitation, high-performance liquid chromatography (HPLC), normal phase HPLC (NP-HPLC), reversed phase HPLC (RP-HPLC), ion exchange chromatography (IEC), such as cation or anion exchange chromatography, hydrophilic interaction chromatography (HILIC), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC) including gel filtration chromatography or gel permeation chromatography, chromatofocusing, affinity chromatography such as immuno-affinity, immobilised metal affinity chromatography, and the like. In an embodiment, chromatography, including single-, two- or more-dimensional chromatography, may be used as a peptide fractionation method in conjunction with a further peptide analysis method, such as for example, with a downstream mass spectrometry analysis as described elsewhere in this specification.

Further peptide or polypeptide separation, identification or quantification methods may be used, optionally in conjunction with any of the above described analysis methods, for measuring biomarkers in the present disclosure. Such methods include, without limitation, chemical extraction partitioning, isoelectric focusing (IEF) including capillary isoelectric focusing (CIEF), capillary isoelectrophoresis (CIEP), capillary electrophoresis (CE), capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), free flow electrophoresis (FFE), etc.
In some embodiments, the degree of gene expression of said gene or fragment thereof can be measured in e.g. a synovial fluid sample comprising FLS cells that show increased expression of the gene-transcript encoding the soluble form of the IL7 receptor in case of RA. The level of gene expression of the gene is able to distinguish between those patients who will respond well and those patients who will not respond well to a TNF blocking agent. In practice, the level of expression of the gene or fragments thereof in a patient whose response status is unknown is compared to the level of expression of the same gene or fragment thereof in patients whose response status is known. The mathematical similarity between the two expression patterns determines the probability that the unknown patient response will be similar to response of the known patient.

In various embodiments the gene expression pattern can be determined in a wide variety of ways including, but not limited to, measuring mRNA levels in a biological sample or measuring protein expression products in a biological sample. These can be performed either ex vivo for example in a blood or serum sample using low-density microarrays, or in vivo after injection of isotopic tracers allowing to identify and quantify the presence of specific markers in affected patients.

In an embodiment, the TNF blocking agents (also referred as TNF inhibitors) can be adalimumab (HUMIRA®, Abbott), infliximab (REMICADE®, Schering-Plough), etanercept (ENBREL®, Wyeth), certolizumab pegol (CIMZIA®, UCB) or Golimumab (Schering-Plough).

In a further embodiment, the IL-1 blocking agents can be: blocking antibodies such as canakinumab (Ilaris) currently tested in juvenile arthritis; Anakinra (Kineret) a recombinant protein (IL-1 receptor antagonist, which competitively binds to the IL-1 receptor and, thereby, blocks IL-1 binding and signal transduction.

In a further embodiment, the IL-17 blocking agents can be: IL-17 blocking antibodies; other biologics such as rituximab (Mabthera) therapy (down-regulating IL-17 production in the synovium), and CTLA4-Ig (Orencia) or tocilizumab (Ro-Actemra).

In an embodiment, the level of expression is determined using a method selected from the group consisting of DNA microarray, reverse transcriptase polymerase chain reaction (RT PCR), immunohistochemistry, immunoblotting, and protein microarray. Preferably, the level of expression is determined using DNA-microarray, preferably low-density DNA-spotted microarray.

In an embodiment, the level of expression of said gene or fragment thereof in said biological sample, preferably in a serum or synovial fluid sample, is assessed by detecting the level of expression of at least one transcribed polynucleotide or fragment thereof encoded by said gene or fragment thereof. Preferably, said at least one transcribed polynucleotide or fragment
thereof is a cDNA, or mRNA. In an embodiment, the step of detecting further comprises amplifying the transcribed polynucleotide. The step of detecting can be done using the method of quantitative RT PCR.

In an embodiment, the level of expression of the gene or fragments thereof in said biological sample, preferably a serum or synovial fluid sample, is assessed by detecting the level of expression of the transcribed polynucleotide or fragments thereof encoded by said gene or fragments thereof. Preferably, said transcribed polynucleotide or fragments thereof is a cDNA, or mRNA.

In an embodiment, the level of expression of said gene or fragment thereof is assessed by detecting the presence of at least one transcribed polynucleotide or fragment thereof in a sample, preferably in a serum or synovial fluid sample, with a probe which anneals with the transcribed polynucleotide or fragment thereof under stringent hybridization conditions, known in the art (cf. Sambrook et al., Molecular cloning, a laboratory manual).

The present invention therefore also provides arrays comprising probes for detection of polynucleotides (transcriptional state) or for detection of proteins (translational state) in order to detect differentially-expressed genes of the invention. By “array” is intended a solid support or substrate with peptide or nucleic acid probes attached to said support or substrate. Arrays typically comprise a plurality of different nucleic acid or peptide capture probes that are coupled to a surface of a substrate in different, known locations. These arrays, also described as “microarrays” or colloquially “chips” have been generally described in the art. These arrays may generally be produced using mechanical synthesis methods or light directed synthesis methods which incorporate a combination of photolithographic methods and solid phase synthesis methods.

The skilled person is capable of designing oligonucleotide probes that can be used in methods of the present invention. Preferably, such probes are immobilized on a solid surface as to form an oligonucleotide microarray of the invention. The oligonucleotide probes useful in methods of the present invention are capable of hybridizing under stringent conditions to the at least one, at least two, at least three, at least five, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 100, at least 120, at least 150, at least 180, at least 200, at least 220, at least 240, at least 250, at least 260, at least 264, at least 270, or at least 300 nucleic acids as described herein.

In a preferred embodiment, the probes in the array are designed so as to detect specifically the gene-transcript or mRNA molecule encoding for the soluble form of the IL7 receptor protein as described herein. In particular, the probe may be so designed so as to distinguish between mRNA molecules lacking exon 6 and mRNA molecules comprising exon 6.
Although a planar array surface is preferred, the array may be fabricated on a surface of virtually any shape or even a multiplicity of surfaces. Arrays may be peptides or nucleic acids on beads, gels, polymeric surfaces, and fibers such as fiber optics, glass or any other appropriate substrate. Arrays may be packaged in such a manner as to allow for diagnostics or other manipulation of an all-inclusive device.

In a preferred embodiment, the step of determination of the level of expression is performed using microarray, preferably DNA-microarray, more preferably low-density DNA-spotted microarray. Suitable probes for said microarray are identified hereunder.

In particular, a mixture of transcribed polynucleotides obtained from the sample, preferably from a serum or synovial fluid sample, is contacted with a substrate having fixed thereto a polynucleotide complementary to or homologous with at least a portion (e.g. at least 7, 10, 15, 20, 25, 30, 40, 50, 100, 250, 300, or more nucleotide residue) of a RNA transcript encoded by a gene for use in the invention. If polynucleotides complementary to or homologous with a RNA transcript encoded by the gene for use in the invention are differentially detectable on the substrate (e.g. detectable using radioactivity, different chromophores or fluorophores), are fixed to different selected positions, then the levels of expression of a plurality of genes can be assessed simultaneously using a single substrate.

When the assay has an internal control, which can be, for example, a known quantity of a nucleic acid derived from a gene for which the expression level is either known or can be accurately determined, unknown expression levels of other genes can be compared to the known internal control. More specifically, when the assay involves hybridizing labelled total RNA to a solid support comprising a known amount of nucleic acid derived from reference genes, an appropriate internal control could be a housekeeping gene (e.g. glucose-6-phosphate dehydrogenase or elongation factor-1), a housekeeping gene being defined as a gene for which the expression level in all cell types and under all conditions is substantially the same. Use of such an internal control allows a discrete expression level for a gene to be determined (e.g. relative to the expression of the housekeeping gene) both for the nucleic acids present on the solid support and also between different experiments using the same solid support. This discrete expression level can then be normalized to a value relative to the expression level of the control gene (for example, a housekeeping gene). As used herein, the term "normalized", and grammatical derivatives thereof, refers to a manipulation of discrete expression level data wherein the expression level of a reference gene is expressed relative to the expression level of a control gene. For example, the expression level of the control gene can be set at 1, and the expression levels of all reference genes can be expressed in units relative to the expression of the control gene.
In one embodiment, nucleic acids isolated from a biological sample, preferably from a serum or synovial fluid sample, are hybridized to a microarray, wherein the microarray comprises nucleic acids corresponding to those genes to be tested as well as internal control genes. The genes are immobilized on a solid support, such that each position on the support identifies a particular gene. Solid supports include, but are not limited to nitrocellulose and nylon membranes. Solid supports can also be glass or silicon-based (i.e. gene "chips"). Any solid support can be used in the methods of the presently claimed subject matter, so long as the support provides a substrate for the localization of a known amount of a nucleic acid in a specific position that can be identified subsequent to the hybridization and detection steps.

A microarray can be assembled using any suitable method known to one of skill in the art, and any one microarray configuration or method of construction is not considered to be a limitation of the disclosure.

The present invention also encompasses a method for predicting the response to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agents and/or IL-17 blocking agents in a patient, said method comprising:

- first obtaining a polynucleotide sample from a biological sample, preferably from a serum or synovial fluid sample, and

- then reacting the sample polynucleotide obtained in the first step with a probe immobilized on a solid support having polynucleotide sequences corresponding to all or part of the sIL7R-gene-transcript or fragment thereof, and

- detecting the reaction product and comparing with a reference reaction product.

The present invention also encompasses the use of a probe that hybridizes under stringent conditions to the sIL7R-gene-transcript or fragment thereof, preferably the use of probes that hybridizes under stringent conditions to said gene-transcript or fragments thereof, from a biological sample, preferably from a synovial sample; or an antibody that binds to the sIL7R protein or fragment thereof, from a biological sample, preferably from a serum sample, for predicting the response to a treatment with a TNF blocking agent in a patient or for diagnosing the severity or disease progression in an RA patient.

Also provided are kits for use in practicing the subject methods. The term "kit" as used herein refers to any combination of reagents or apparatus that can be used to perform a method of the invention.

The kit may comprise a plurality of reagents, each of which is capable of binding specifically with a nucleic acid or polypeptide corresponding to the gene-transcript encoding the soluble IL7 receptor protein for use in the invention. Suitable probe for binding with a nucleic acid (e. g. a genomic DNA, an mRNA, a spliced mRNA, a cDNA, or the like) include complementary
nucleic acids. For example, the nucleic acid reagents may include oligonucleotides (labelled or non-labelled) fixed to a substrate, labeled oligonucleotides not bound with a substrate, pairs of PCR primers, molecular beacon probes, and the like.

In an embodiment, the kit comprises a nucleic acid probe that binds specifically with a gene nucleic acid or a fragment of the nucleic acid.

Alternatively, the kit according to the invention can comprise a protein array enabling the specific and quantitative detection of the soluble form of the IL7 receptor protein, or enabled to detect both the membrane bound and soluble form of the IL7 receptor protein at different spots of the array. In addition to the IL7 receptor proteins, further RA-related protein detection means can be present on the array. The kit may comprise a plurality of binding agents, each of which is capable of binding specifically with the soluble IL7R protein, such as antibodies, aptamers, small molecules, peptides etc. as defined above, that are linked to a solid phase using known techniques.

The kit may further comprise means for performing a protein detection assay on the array as known in the art.

The present invention thus also encompasses a method for predicting the response to a treatment with methotrexate, a TNF blocking agent, IL-1 blocking agents and/or IL-17 blocking agents in a patient, said method comprising:

- first obtaining a protein sample from a biological sample, preferably from a serum sample, and

- then reacting the sample protein obtained in the first step with a binding agent immobilized on a solid support having high specific affinity for the siIL7R protein or fragment thereof, and

- detecting the reaction product and comparing with a reference reaction product.

The kit may further comprise means for performing PCR reactions. The kit may further comprise media and solution suitable for taking a sample, preferably a synovial and/or serum sample, and for extracting RNA, respectively protein, from said sample.

The kit can further comprise additional components for carrying out the method of the invention, such as RNA extraction solutions, purification column(s) and buffers and the like. The kit of the invention can further include any additional reagents, reporter molecules, buffers, excipients, containers and/or devices as required described herein or known in the art, to practice a method of the invention.

The various components of the kit may be present in separate containers or certain compatible components may be pre-combined into a single container, as desired. In addition to the above components, the kits may further include instructions for practicing the present
invention. These instructions may be present in the kits in a variety of forms, one or more of which may be present in the kit.

One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g., diskette, CD, etc., on which the information has been recorded. In an embodiment, said kit further comprises a computer-readable medium comprising one or more digitally encoded expression profiles, where each profile has one or more values representing the expression of said gene-transcript encoding the soluble IL7 receptor protein or fragment thereof. Said digitally encoded expression profiles are preferably profiles of poor, moderate and good responder to methotrexate or to biologicals including TNF blocking, IL-1 blocking or IL-17 blocking agents.

The invention also provides a computer-readable medium comprising one or more digitally encoded expression profiles, where each profile has one or more values representing the expression of the gene-transcript encoding the soluble IL7 receptor protein or fragment thereof that is differentially-expressed in a poor, moderate or good responders to methotrexate or TNF blockade, IL-1 blockade or IL-17 blockade.

In some embodiments, the digitally-encoded expression profiles are comprised in a database.

The kits according to the invention may comprise a microarray as defined above and a computer readable medium as described above. The array comprises a substrate having addresses, where each address has a probe that can specifically bind a nucleic acid molecule (by using an oligonucleotide array) or a peptide (by using a peptide array) that is differentially-expressed in at least one poor, moderate or good responder. The results can be converted into a computer-readable medium that has digitally-encoded expression profiles containing values representing the expression level of the nucleic acid molecule or protein detected by the array. Any other convenient means may be present in the kits. Said microarray may be able to specifically detect the membrane-bound form of the IL7 receptor or the gene-transcript encoding therefore at one position and the soluble form of the IL7 receptor protein or the gene-transcript encoding therefore at a different position. This enables the simultaneous detection and quantification of both isoforms in the sample. The microarray may of course encompass further addresses whereon additional relevant markers can be detected and quantified.

The invention also provides for the storage and retrieval of a collection of data relating to poor, moderate or good responder to TNF blockade therapy specific gene expression data of the present invention, including expression levels and/or protein amounts or concentrations in a computer data storage apparatus. The same holds true for the storage and retrieval of a
collection of data relating to healthy subjects, early RA patients, DMARD-resistant RA patients, or progressed RA patients. These reference values or data sets can be used in the methods and kits according to the invention.

In an embodiment, the level of expression of said at least one gene or fragment thereof in said patient is assessed by detecting the level of expression of a protein or a fragment thereof encoded by said at least one gene or fragment thereof. Preferably, the level of expression of said protein or fragment thereof is detected using a reagent which specifically binds with said protein or fragment thereof. Said reagent can be any binding molecule as defined herein e. g. a peptide, an antibody, or a fragment thereof.

Preferably, the level of expression of the mRNA, gene or isoform coding for the sIL7R or fragment thereof is detected by measuring or detecting joint uptake of the reagent. Preferably, said reagent is labeled with a radioactive isotope, which can be detected by radio-imaging. Suitable radioactive isotope can be selected from the group comprising Technetium$^{99m}$, Carbon$^{11}$, Oxygen$^{15}$, Nitrogen$^{13}$, Rubidium$^{82}$, Gallium$^{67}$, Gallium$^{68}$, Yttrium$^{90}$, Molybdenum$^{99}$, Iodine$^{123,124,131}$, Fluorine$^{18}$, Phosphorus$^{32}$, Copper$^{62}$, Thallium$^{201}$, Copper$^{64}$, Copper$^{62}$, Indium$^{111}$, and Xenon$^{133}$. Suitable radio-imaging method can be selected from the group consisting of single photon emission computed tomography (SPECT), positron emission tomography (PET) and gamma cameras.

The method of the invention can also be performed in vivo on a patient after injection of isotopic tracers allowing to identify and quantify the presence of the genes or of the encoded protein thereof in affected patients.

The present invention discloses that the soluble IL7R protein is differentially-expressed in poor, moderate or good responders to methotrexate or TNF blockade or, potentially, IL-1 blockade or IL-17 blockade. Accordingly, said protein and the gene encoding it are potential therapeutic targets that are useful in methods of screening test compounds to identify therapeutic compounds for the treatment of rheumatic arthritis.

The differentially-expressed sIL7R gene or isoform of the invention may be used in cell-based screening assays involving recombinant host cells expressing the differentially-expressed gene product. The recombinant host cells are then screened to identify compounds that can activate the product of the differentially-expressed gene (i.e. agonists) or inactivate the product of the differentially-expressed gene (i.e. antagonists), or increase or decrease its expression.

The following examples are intended to illustrate and to substantiate the present invention.
Examples

Example 1: Serum soluble interleukin-7 Receptor concentrations predict response to methotrexate in early rheumatoid arthritis and to TNF-blockade in DMARD-resistant rheumatoid arthritis.

MATERIAL AND METHODS

Patients

Sera were collected from healthy individuals (n=75), patients with early (n=52) or DMARD-resistant (n=76) RA, patients with SLE (n=30) and stored at -80°C. All RA patients met the American College of Rheumatology (ACR) criteria for the diagnosis of RA (Arnett et al., Arthritis Rheum 1988; 31: 315-24.). Patients with early RA had disease duration of less than one year. They were (mean ± standard deviation) 45 ± 14 years old. All had active disease at the time of serum sampling (mean DAS28-CRP score: 5.87 ± 1.28) and none of them was treated, except for non-steroidal anti-inflammatory drugs.

DMARD-resistant RA patients were 55 ± 12 year old. Disease duration was 10.8 ± 6.7 years. They were treated at baseline with a median 15 mg methotrexate/week schedule (range 7.5 – 25 mg/week). 9% were treated with other DMARD’s after having failed methotrexate therapy in the past. 13% were treated with another DMARD together with methotrexate. Patients had taken an average 3 DMARD’s including methotrexate (range 1 – 7) before starting infliximab therapy. All of them had erosive changes imaged on conventional x-rays of the hands and/or the feet. They all had active disease at the time of serum sampling (mean DAS28-CRP score: 5.69 ± 1.12). After baseline serum collection, DMARD-resistant RA patients were treated with a standard schedule of infliximab: 3 mg/kg at week 0, week 2, week 6 and then every other month in addition to their DMARD therapy. Follow-up DAS-28CRP evaluation were performed between 4 and 6 months after baseline and patients were categorized into non-responders versus responders according to EULAR response criteria (van Gestel et al., Arthritis Rheum 1996; 39:34-40).

Isolation and culture of cells

FLS were purified from synovial biopsies from 8 additional RA patients as previously described (De Bari et al., Arthritis Rheum 2001; 44: 1928-1942). Briefly, minced synovial fragments were digested in 1 mg/mL hyaluronidase solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at 37°C and 6 mg/mL collagenase type IV (Invitrogen, Paisley, UK) for 2 hours at 37°C. Next, cells were washed, resuspended in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 1% antibiotics-antimycotics (Invitrogen) and 1% minimum essential medium sodium pyruvate (Invitrogen), and seeded at 10,000 cells per
square centimetre in six-well plates. When the cells reached confluence, adherent cells were detached using sterile 0.5% trypsin-ethylendiaminetetraacetic acid (Invitrogen) and used between passages 3 and 9. For the cytokine stimulation experiments, FLS were seeded in 24-well plates at 25,000 per well. Unless stated otherwise, the following cytokine concentrations were used: TNF-alpha (R&D Systems, Minneapolis, MN, USA) 10 ng/mL, IL-1-beta (R&D Systems) 10 ng/mL, and IL-17 (R&D Systems) 50 ng/mL. After overnight incubation with the indicated cytokines, supernatants were collected for sIL-7R determination and cells were harvested for total RNA extraction. For co-culture experiments, CD4 T cells were purified from the hyaluronidase- and collagenase-digested synovial biopsies using magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer’s instructions. FLS (5,000/well) and CD4 T cells (50,000/well) were seeded in flat-bottomed 96-well plates in the presence of autologous serum and 100 ng/ml IL-7 (R&D Systems) or 5 μg/ml sIL-7R-Fc fusion protein (R&D Systems), as indicated. After 3 days, proliferative responses were measured in quadruplicates after an overnight pulse with 0.5 μCi ³H-thymidine (GE Healthcare, United Kingdom).

PBMC from healthy donors were purified by lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. In some experiments, PBMC were cultured for 24 hours in the presence of IL-2 and PHA, and CD4 T cells were purified using magnetic beads (Miltenyi Biotec) as per manufacturer’s instructions. CD8 T cells clones (obtained from Pierre van der Bruggen, Ludwig Institute for Cancer Research, Brussels Branch) were stimulated with their cognate antigen presented by irradiated autologous B-EBV cells in the presence of IL-2 (Chiron, Amsterdam, The Netherlands, 50 U/ml) and IL-7 (R&D Systems, 5 ng/ml). After 24 hours, CD8 T cells clones were purified using magnetic beads (Miltenyi Biotec) as per manufacturer’s protocol. cDNA from B-EBV cell derived from two healthy donors were obtained from Pierre Coulie (Unité de Génétique Cellulaire, Université Catholique de Louvain).

RT-PCR experiments

Total RNA was extracted using the Nucleospin® RNA II extraction kit (Macherey-Nagel, Düren, Germany), including DNase treatment of the samples. cDNA was synthesized using RevertAid Moloney murine leukemia virus RT (Fermentas, St. Leon-Rot, Germany) and Oligo(dT) primers. IL-7R PCR amplification was carried out using Taq DNA polymerase (Fermentas) and the following primers: Forward: tccctccctcttcctctctc (SEQ ID NO:5) and Reverse: tctggcagtcaggaactt – 5’ (SEQ ID NO:6). PCR products were analysed by agarose gel electrophoresis. PCR fragments were gel purified and sequenced using the same primers and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) before being analysed on a 3130xl Genetic Analyzer (Applied Biosystems). Quantitative RT-PCR was performed on a MyiQ single-color RT-PCR detection system (Bio-Rad Laboratories,
Nazareth Eke, Belgium) using SYBR Green detection mix. For each sample, 5 ng of cDNA was loaded in triplicate with 1x SYBR Green Mix (Applied Biosystems) and the following 10 mM primers: beta-Actin: Forward: ggcatacgtgatgactcgg (SEQ ID NO:7) and Reverse: ctggaagggtgcagcgcaga; (SEQ ID NO:8) IL-7R: Forward: ttctgaggattgacgctaa (SEQ ID NO:9) and Reverse: aagccaaccaacaaagagt (SEQ ID NO:10); sIL-7R: Forward: agccaagcattgtgggtgc (SEQ ID NO:11) and Reverse: tacgatatgttaatctgtcag (SEQ ID NO:12). The melting curves obtained after each QPCR amplification confirmed the specificity of the SYBR Green assays. Relative expression of the target genes in the studied samples was obtained using the difference in the comparative threshold ($\Delta \Delta Ct$) method. Briefly, for each sample, a value for the cycle threshold (Ct) was determined, which was defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold. The $\Delta Ct$ for each sample was then calculated according to the formula $Ct_{\text{target gene}} - Ct_{\text{actin}}$; $\Delta \Delta Ct$ values then were obtained by subtracting the $\Delta Ct$ of a reference sample from the $\Delta Ct$ of the studied samples. Finally, the levels of expression of the target genes in the studied samples as compared with the reference sample were calculated as $2^{-\Delta \Delta Ct}$.

Flow cytometry experiments

FLS were harvested and resuspended in a sodium phosphate (1 mM, pH = 7.4) buffer containing 137 mM NaCl, 5 mM KCl, 0.4 mM MgSO4, 0.3 mM MgCl2, 5 mM Glucose, 4 mM NaHCO3, 1 mM EDTA and 3% FCS in the presence of a PE-conjugated IL-7R antibody (Becton Dickinson, Mountain View, CA) or a control isotype (Becton Dickinson). Cells were washed and fixed in paraformaldehyde (0.6%) before being analysed by flow cytometry (Becton Dickinson).

Western Blot experiments

FLS and PBMC were washed with ice-cold PBS, and lysed in cold lysis buffer (20 mM Heps pH7.8, 75 mM KCl, 0.1 mM EDTA, 1 mM sodium-orthovanadate, 2 mM MgCl2, 1 mM DTT, 10% glycerol, 0.5% Triton-X 100 and 1 tablet of Complete Protease Inhibitor (Roche) per 20 ml). Lysates were subjected to sonication and spun at 13,000X g to remove debris. Supernatants were resolved on a polyacrylamide gel and immunoblotted with a monoclonal mouse anti-IL-7R antibody (Sigma-Aldrich) at 1:1,000. Immunoreactive proteins were visualized using a goat anti-mouse-HRP antibody (Santa Cruz Biotechnology, Heidelberg, Germany) at 1:1,000, with a femto-range-sensitive ECL detection system (Thermo Fisher Scientific, Rockford, Illinois, USA).

sIL-7R ELISA

sIL-7R serum titers were determined by sandwich-ELISA in serum samples and culture supernatants using a goat polyclonal hIL-7R antibody (Sigma-Aldrich) as coating antibody and
a mouse monoclonal hIL-7R antibody as detecting antibody (Sigma-Aldrich). Briefly, Microlon ELISA plates (Greiner Bio One, Wemmel, Belgium) were coated overnight at 4°C with a 100 microliters goat polyclonal hIL-7R antibody solution diluted at 0.5 μ/ml. Plates were blocked with milk and then incubated at 37°C with the control and patient’s sera diluted in PBS supplemented with 0.5% BSA. Sera were tested in duplicates. For the standard curve, serial dilutions of a commercial IL-7R-Fc fusion protein (R&D Systems) containing the extracellular portion of the receptor bound to a Fc fragment of human IgG was used. For this reason, the results were expressed in pM/ml instead of μg/ml. The detection antibody was used at 1 μ/ml in PBS 0.5% BSA. The third antibody was a rat HRPO-anti-mouse IgG monoclonal antibody (LO-MK1, Unit of Experimental Immunology, Université Catholique de Louvain, Brussels, Belgium), diluted at 0.5 μg/ml in PBS 0.5% BSA. That antibody does not cross-react with human IgG, and is therefore unable to detect IgG rheumatoid factors bound to the coated anti-IL-7R goat antibody. Each incubation step lasted 2 hours. Plates were washed 5 times with PBS Tween (Sigma-Aldrich) 1/1,000 between each step. The reactions were revealed with 1-Step Ultra-TMB (Thermo Fisher Scientific) and stopped by the addition of 2M H2SO4. The sensitivity of the ELISA is 20 pM/ml.

Statistical analyses

In vitro data were analyzed using unpaired Student t tests. Serum data were analyzed using Mann-Whitney U tests. Correlations between DAS-Scores, CRP values, Delta DAS-Scores and serum sIL-7R were analyzed using Spearman Rank tests.

RESULTS

Fibroblast-like synovial cells express two isoforms of the gene encoding the IL-7R alpha-chain.

The production and regulation of IL-7R by cultured fibroblast-like synovial cells (FLS) obtained from the joints of RA patients, was studied. Unexpectedly, Western Blot and real-time qPCR experiments indicated that FLS express two isoforms of the molecule (Figure 1A). Sequencing of the amplicons demonstrated that these isoforms correspond to the native IL-7R, and an alternatively spliced variant lacking exon 6 (transmembrane domain), which encodes a soluble form of the IL-7R (sIL-7R) (Figure 1B).

sIL-7R expression by FLS is induced by pro-inflammatory cytokines

Checking whether expression of both IL-7R isoforms is regulated in FLS was done by the addition of pro-inflammatory cytokines known to play a role in the pathogenesis of RA. mRNA levels of both the membrane-bound and soluble forms of IL-7R were found to be up-regulated by the addition of TNF-alpha, IL-1-beta and various combinations of TNF-alpha, IL-1-beta and IL-17 (Figure 2A). Flow cytometry experiments were, however, unable to detect any cell
surface expression of the membrane-bound IL-7R on FLS under any of these conditions (Figure 2B). By contrast, ELISA experiments performed on culture supernatants indicated that sIL-7R secretion is induced in FLS by TNF-alpha, IL-1-beta and the combination of both cytokines (Figure 2C). Interestingly, sIL-7R gene expression is negative in activated CD8 T- and B-cells but slightly positive in activated CD4 T-cells (Figure 1A). Taken together, these results indicate that sIL-7R is a marker of fibroblast and, to a lesser extent, CD4 T-cell activation.

**sIL-7R inhibits FLS-induced CD4 T-cell proliferation**

Since sIL-7R is known to display IL-7 binding and blocking properties, the present inventors investigated whether it could interfere with synovial CD4 T-cell proliferation. RA FLS are known to express MHC class II molecules and stimulate the proliferation of autologous synovial CD4 T-cells. The results showed that the addition of IL-7 stimulates the proliferation of synovial CD4 T-cells cultured in the presence of autologous FLS. By contrast, addition of a sIL-7R-Fc fusion protein blocks the proliferation of these cells (Figure 1D). These results indicate that sIL-7R production by activated synovial fibroblasts plays a role in a negative feedback loop resulting in a decreased proliferation of synovial CD4 T cells.

**sIL-7R serum levels are higher in RA patients compared to controls**

Unexpectedly, the present inventors have shown that sIL-7R could also be detected in the much easier accessable serum fluid of healthy individuals and in patients with inflammatory disorders. Soluble IL-7R is readily detectable in the sera of healthy individuals (n=75) (mean ± SEM: 688.1 ± 60.5 pM/ml), and more interestingly, serum levels are significantly higher in untreated early RA patients (n=52) and in treated RA patients with active disease despite DMARD therapy (n =76), sIL-7R (mean ± SEM: 1473.3 ± 235.5 pM/ml and 1877.9 ± 258.6 pM/ml, respectively) as compared to controls (Figure 3). In both early and DMARD-resistant (more progressive) RA patients, baseline sIL-7R serum levels did not correlate with serum CRP values or with DAS28-CRP scores. These results indicate that serum sIL7R values can be used as an independent diagnostic tool for establishing or predicting the RA disease severity in a subject.

**Baseline sIL-7R serum levels predict response to therapy in DMARD-resistant RA patients**

As stated above, sIL-7R serum levels are higher in DMARD-resistant as compared to early RA patients, an observation suggesting that sIL-7R serum levels could be a marker of disease severity and treatment failure. The present inventors established whether baseline sIL-7R serum levels are able to predict disease progression and response to therapy in RA. Strikingly, in patients displaying active disease despite DMARD therapy and subsequently treated with
TNF blockade, elevated baseline sIL-7R serum levels strongly predicted poor-response to anti-TNF therapy (Figure 4A, 4B and 4C). Conversely, normal baseline sIL-7R serum levels were associated with adequate response to therapy, with a sensitivity of 93% and a specificity of 63%. In case of low sIL-7R serum titers, patients have an 88% probability of being responders (predictive positive value); by contrast, in case of elevated sIL-7R serum levels, patients have a 75% probability of being non-responders (predictive negative value).

Baseline sIL-7R serum levels predict response to methotrexate therapy in early RA patients

First-line therapy for early RA patients is based on the use of DMARD’s such as methotrexate, which induces an adequate response in about 60% of the cases. The present inventors have found that elevated baseline sIL-7R serum levels are associated with poor-response to methotrexate therapy in early RA. In particular, baseline sIL-7R serum concentrations are significantly higher in non-responders (786.2 ± 124.9 pmol/ml versus 2,132 ± 682 pmol/ml, p = 0.003) (Figure 5A and 5B).

Example 2: Soluble IL7R levels can help the rheumatologist to make decisions on therapy.

Below, three real-life cases were chosen to indicate how the serum soluble IL7R protein level is of help in the decision-making process of the rheumatologist.

Case 1: A 45-year-old patient has severe rheumatoid arthritis and is currently treated with 7.5 mg prednisolone/day, naproxen 1,000 mg/day and parenteral methotrexate 20 mg/day, started one year ago. He has 14 tender and 18 swollen joints, including most of the metacarpophalangeal joints. DAS28-CRP score is calculated at 7.3. Anti-citrullin antibodies and rheumatoid factors are positive; x-rays of the hands and feet show erosive and chondrolytic changes at the metacarlo- and metatarsophalangeal joints.

The appropriate therapy with biologics for this patient has to be chosen from: 1) a TNF-blocker and 2) tocilizumab (anti-IL-6R antibody), since both drugs are available in this indication. A serum sIL-7R determination is performed and the results of the test indicate that sIL-7R serum titers are in the range of normal. Based on this measurement, the rheumatologist can more easily decide in favour of a treatment with a TNF-blocker, since the probability of the patient to respond to TNF-blocking therapy is calculated as being around 90% in said patient, due to the normal serum sIL7R level value.

Case 2: A 62-year-old woman suffers from longstanding severe rheumatoid arthritis. She was initially treated with gold salts, next with salazopyrine, hydroxychloroquine and finally 25 mg/week oral methotrexate. She also takes prednisolone 10 mg/day and NSAID’s. Her disease is active (DAS28-CRP score 5.4) and conventional x-rays show disease progression
in the hands and feet. Her physician decides to initiate therapy with biological agents. Since her sIL-7R serum titers are very elevated, the probability that she does not respond to a TNF blocker is evaluated at 75%. For this reason, her rheumatologist prescribes tocilizumab therapy, in stead of TNF-blocking therapy.

5  **Case 3:** A 33-year-old man suffers from early rheumatoid arthritis, diagnosed based on his clinical presentation (pain and swelling in all metacarpophalangeal and proximal interphalangeal joints), high CRP serum levels and positive anti-CCP antibodies. His rheumatologist considers the prescription of first-line DMARD therapy with methotrexate. However, serum sIL-7R titers are very high, indicating that the probability of response to methotrexate therapy is low in this patient. For this reason, the rheumatologist urges the patient’s insurance company to consider the refunding of a combination therapy of methotrexate together with a biological agent, in order to avoid disease progression and long-term damage.
Claims

1. A method for predicting the response to a treatment with a TNF blocking agent in a DMARD-resistant RA patient comprising the steps of:
   (a) measuring in a sample from said patient the amount of soluble IL-7R, and
   (b) predicting the response to the treatment with the TNF blocking agent in said patient by evaluating the results of step (a).

2. A method for predicting the response to methotrexate therapy in early RA patients comprising the steps of:
   (a) measuring in a sample from said patient the amount of soluble IL-7R, and
   (b) predicting the response to methotrexate therapy in said patient by evaluating the results of step (a).

3. A method for predicting the response to therapy with other biologics such as IL-1 blocking agents and/or IL-17 blocking agents, in DMARD-resistant RA patients comprising the steps of:
   (a) measuring in a sample from said patient the amount of soluble IL-7R, and
   (b) predicting the response to therapy with other biologics such as IL-1 blocking agents and/or IL-17 blocking agents in said patient by evaluating the results of step (a).

4. A method for diagnosing the RA disease severity in a subject comprising the steps of:
   (a) measuring in a sample from said patient the amount of soluble IL-7R, and
   (b) predicting the RA disease severity in said patient by evaluating the results of step (a).

5. The method according to any one of claims 1-4, wherein measuring the amount is performed by measuring the concentration of soluble IL-7R protein in a serum or synovial sample of said subject.

6. The method according to any one of claims 1-5 comprising the steps of:
   i) providing a serum or synovial sample from a subject,
   ii) measuring the concentration of soluble IL-7R in the sample,
   iii) predicting the response to said treatment or the RA disease severity in a subject when detectable soluble IL-7R is present in the sample or when the amount of soluble IL-7R in the sample is greater than or equal to a threshold value.

7. The method according to any one of claims 1-6 wherein the amount of the sIL7R protein is detected using a reagent which specifically binds with said protein, preferably selected from the group consisting of: an aptamer, a photoaptamer, a protein, a peptide, a
peptidomimetic, an antibody or a fragment or a derivative thereof, a polyclonal antibody, a monoclonal antibody, a humanised or a chimeric antibody, an engineered antibody, or a biologically functional antibody fragment sufficient for binding to the soluble form of the IL7R alpha chain protein, or wherein the amount of soluble IL7R is measured using any of biochemical assay, immunoassay, surface plasmon resonance, fluorescence resonance energy transfer, bioluminescence resonance energy transfer or quenching is detected.

8. The method according to any one of claims 1-3, and 5-7, wherein the threshold value of responders vs. non-responders is determined before step (i) by

(i1) assessing the amount of soluble IL-7R in a plurality of serum or synovial samples from patients before treatment with said agent,

(i2) assessing the amount of soluble IL-7R in a plurality of serum or synovial samples from patients after treatment with said agent, and

(i3) correlating the response of the patients treated with said agent to the amount of soluble IL7R, thereby determining the threshold value of responders.

9. The method according to any one of claims 4 and 5-7, wherein the threshold value of low vs. high disease severity is determined before step (i) by

(i1) assessing the amount of soluble IL-7R in a plurality of serum or synovial samples from patients with low RA disease severity or from healthy subjects,

(i2) assessing the amount of soluble IL-7R in a plurality of serum or synovial samples from patients with high RA disease severity, and

(i3) correlating the response of the two patient/subject groups to the amount of soluble IL7R, thereby determining the threshold value corresponding to disease severity.

10. A kit for predicting the response of a RA patient to a treatment with methotrexate, a TNF blocking agent, IL1-blocking agents and/or IL17-blocking agents, comprising:

a) optionally a means for drawing a blood or synovial fluid sample,

b) a means for assessing the protein level of the soluble IL7R protein in said sample, and

c) one or more reference values reflecting the sIL7R protein level in RA patients that either do or do not respond to a treatment with methotrexate, a TNF blocking agent, IL1-blocking agents and/or IL17-blocking agents.

11. Another aspect of the invention relates to a kit for predicting the severity of the RA condition of a RA patient comprising:

a) optionally a means for drawing a blood or synovial fluid sample,
b) a means for assessing the protein level of the soluble IL7R protein in said sample, and

c) one or more reference values reflecting the sIL7R protein level in patients with different RA severity conditions.

12. The use of the kit according to claim 10, in the method according to any one of claims 1-3, and 5-8.

13. The use of the kit according to claim 11, in a method according to any one of claims 4-7 and 9.
**A**

FLS  FLS  PBMC

---

1  2  3  4

---

55 kD

**B**

Exon: 1 2 3 4 5 6 7 8

IL-7R

---

Extracellular domain  Intra-cellular domain

sIL-7R

---

1 2 3 4 5 7 8

**FIG. 1**
FIG. 2

A

TNF-α
IL-1β
IL-17

TNF-α + IL-1β
TNF-α + IL-17
IL-1β + IL-17

Normalized gene expression

B

Control isotype

IL-7R

PBMC

Counts

10^0
10^1
10^2

Control isotype

IL-7R

Fluorescence intensity

C

sIL-7R (optical density)

- TNF-α IL-1β TNF-α IL-1β

*** ***

D

H3-Thymidine incorporation

- IL-7 sIL7R-Fc

CD4 T cells CD4 T cells + FLS

*
FIG. 3

Serum sIL-7R (pg/ml)

DMARD-resistant RA
Early RA
Controls

$p < 0.0001$

$p < 0.005$
A: SEQ ID NO: 3
atgacaattctagttcacaacttttttgcttttcttatttcatcaagtcttttcttgagagagttggctatgtcacaattggagacactgggaacttgggagatgtactacacattctcatgtatt
agccagttggaatgtgactcgacactcactgacctgcttttgagacccagatgtcaacattcaccacacattggaatatgatgtgctggtgctggagga
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aaggttttaatgcacagtctaggcttaaccgcagaaaaaggtgaagaaaaagcaatgagacctggtgataatctcagcaaacagctgacacactctcatcgcaagaaagctcaacctggccagcaatgc
atgagatttaaagttgatccacatccctctgacactatattttttaaggttctttggaatggtgactgggattactactcagacaactccagagatcaataatagctcaggtttaaagctcatctg
atagccgccagtctcccccagctatgacagttcttgagacactcatttttggataagaaaaagaagaaaattttaa

B: SEQ ID NO: 4
MTILGTTFGMVFSLLQQVSGESGYAQNGLDLEADLDDYFSFCYSLQVLEVNGSQHSLTCAPEDPDVNTNLFEICGALVEVKTLMFRRKLQBIYFIETKFKLLIGKSNICVKGKSLTCKKIDLTI
VKEAPFDSLVYREGANDFVFVFTNSHLQKKYVKVLMDVAYRQERDENKTHVNLSTKLTLQRRKQLPAAMYIEKVRSPDHYFKGFSEWSPSYSSYFRTPFPINESSGLSLSYGFSPIIRLWNIFVRN
QKII

FIG. 7
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/564 G01N33/68
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

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

Electronic database consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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Further documents are listed in the continuation of Box C. See patent family annex.

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document 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)
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**"** 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.

A" document member of the same patent family

Date of the actual completion of the international search Date of mailing of the international search report
20 April 2010 04/05/2010

Name and mailing address of the ISA
European Patent Office, P.B. 5618 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer
Schindler-Bauer, P
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