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(71) Applicant *(for all designated States except US)*: **UNIVERSITE LIBRE DE BRUXELLES** [BE/BE]; Avenue F.D. Roosevelt 50, CP 161, B-1050 Brussels (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HAMDI, Haïfa** [TN/FR]; rue Marc Lefrancq 141, Appart 1, Résidence Les Harpignies, F-59300 Valenciennes (FR). **STORDEUR, Patrick** [BE/BE]; rue du Croissant 158, B-1190 Brussels (BE). **OCMANT, Annick** [BE/BE]; Chaussée de Soignies 34 J, B-7070 Le Roeulx (BE).

(74) Agents: **VANHALST, Koen Victor Rachel** et al.; E. Gevaertdreef 10a, B-9830 Sint-Martens-Latem (BE).

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(54) **Title**: NEW BIOMARKERS FOR DETERMINING ALLERGY STATUS

(57) **Abstract**: The present invention provides new methods for diagnosing, predicting and/or prognosticating the allergic response of a subject to a certain allergen, based on the expression levels of 4 biomarkers identified in Basophils, after stimulation with a real allergen, and kits for carrying out said methods.

NEW BIOMARKERS FOR DETERMINING ALLERGY STATUS

FIELD OF THE INVENTION

The present invention lies in the field of medical diagnostics, more particularly in the diagnosis, prediction and prognosis of the allergic state of a subject. The present invention particularly provides new tools and methods for assessing or predicting the allergic state and/or response of subjects to allergens or to immunotherapy.

BACKGROUND OF THE INVENTION

The prevalence of atopic disease has been increasing for the last decades in the Western World, becoming a major problem of public health. However, the diagnosis of allergic status remains a challenging area.

Indeed, the procedures routinely used in clinical practice (i.e. skin testing and detection of serum IgE) do not allow a clear distinction between non-symptomatic IgE sensitization and symptomatic IgE-mediated allergy although cut-off values for specific IgE levels have been determined for a few allergens (Sampson H.A., J Allergy Clin Immunol 2001;107:891e6). About 50% of subjects that are “diagnosed” as being allergic to a certain allergen based on elevated IgE levels are actually misdiagnosed due to a non-allergen-specific (non-symptomatic) raise in IgE levels. Therefore new easy to use and more accurate diagnostic tests allowing the clear identification of the offending allergen are urgently required.

Recently, several functional tests focusing on basophils, which play a key role in IgE-mediated reactions, have been developed. The detection of mediator release (histamine, leukotrienes) and cell activation markers (expression of CD63, up-regulation of CD203c) upon stimulation with allergens has been proved to be useful in selected cases (Hamilton R.G. et al., 2004, J Allergy Clin Immunol 2004;114:213e25). As Th2 cytokines are potent actors of the atopic process, the quantification of their synthesis could be another valuable approach to investigate the response to an allergen. However, most experiments focusing on Th2 cytokine production have been performed on purified cells rather than on whole blood samples or fractions thereof.

In order to overcome the above mentioned problems and drawbacks, the inventors identified new allergy-specific diagnostic and/or prognostic markers of peripheral blood basophils using a quantitative real-time PCR test performed and validated on PBMC's and on whole blood samples and evaluated the diagnostic power of said new markers.

SUMMARY OF THE INVENTION

In order to overcome the above stated problems, the inventors investigated the differential expression of genes of Basophil cells, involved in allergy, before and after stimulation with real allergens. Several new biomarkers were identified and validated that can predict the allergic response of a subject to a specific allergen.

The present invention provides a method for determining whether a subject is allergic to or is prone to becoming allergic to a certain allergen comprising the steps of:

a) contacting a part of a whole blood sample of the subject with said allergen,

b) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in the part of the sample contacted with said allergen and in the part not contacted with said allergen,

c) comparing the expression level(s) in both parts of the sample, wherein an increase of the expression level of said one or more biomarkers in the part contacted with the allergen as compared to the non-contacted part indicates the tested subject is allergic to, or is prone to become allergic to said tested allergen.

Preferably, said expression level is assessed using PCR analysis, preferably quantitative PCR analysis.

In a preferred embodiment, the sample is selected from the group consisting of: blood, whole blood, peripheral blood mononuclear cells (PBMC) depleted from basophils or not, purified basophils, plasma or serum, preferably, a whole blood sample.

In a further embodiment, the allergen was added to the subject in vivo, i.e. by administering the allergen to the subject under investigation or under desensitisation treatment. A sample taken before said administration than acts as the reference sample. Alternatively, the allergen is added to a sample of the subject in vitro, i.e. by adding the allergen to the sample after it was obtained from the subject.

The invention further provides a method for diagnosing or predicting the allergic response of a subject to a certain allergen comprising the steps of:

a) contacting part of a whole blood sample of the subject with said allergen,

b) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in the part of the sample contacted with the allergen and in the part of the sample not contacted with the allergen,

c) comparing the expression level(s) in both parts, wherein an increase of the expression level of said one or more biomarkers in the part contacted with the allergen as compared to the non-contacted part indicates the tested subject is allergic or is prone to become allergic to said tested allergen.

The invention further provides a method of defining immunotherapy in a subject allergic to a certain allergen comprising the steps of:

a) contacting part of a whole blood sample of the subject with said allergen,

b) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in the part contacted with the allergen and in the part not contacted with the allergen,

c) comparing the expression level(s) in both parts of the sample, wherein an increase of the expression level of said one or more biomarkers in the part contacted with the sample as compared to the non-contacted part indicates the tested subject is allergic or is prone to become allergic to said tested allergen and could benefit from immuno-therapy specific for said allergen.

In addition, the invention provides a method for monitoring the response of a subject to an immunotherapy treatment comprising the steps of:

a) contacting a whole blood sample of the subject with said allergen at a first time point, before or at an early stage of the immunotherapy treatment,

b) contacting a whole blood sample of the subject with said allergen at a second time point, at a later stage of the immunotherapy treatment,

c) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein), in said samples at both time points.

d) comparing the expression level(s) at the two time points, wherein a decrease of the expression level of said one or more biomarkers measured at the second time point indicates the immunotherapy is beneficial for the subject.

In one preferred embodiment of the methods according to the invention, the addition of the allergen or allergen extract for immunotherapy treatment is, or has been done in vivo, i.e. by administering said allergen to the subject under investigation e.g. by sublingual, oral, or nasal administration, or by intraperitoneal, subcutaneous or intravenous injection.

The invention further provides a method for predicting the response to a vaccine or medicament comprising the steps of:

a) contacting part of a whole blood sample of the subject with the vaccine,

b) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in the part contacted with the vaccine and in the part not contacted with the vaccine,

c) comparing the expression level(s) in both parts of the sample, wherein an increase of the expression level of said one or more biomarkers in the part contacted with the vaccine or medicine as compared to the non-contacted part of the sample indicates the tested subject is allergic or is prone to become allergic to said tested vaccine.

In a preferred embodiment, the subject is human. In a further preferred embodiment, the subject is an adult, an adolescent, a child, a toddler, a baby or a neonate.

In a further preferred embodiment, the allergen is selected from the group consisting of foodstuffs such as cowmilk, gluten, wheat, peanuts, tree nuts, soy, egg, fish, shell fish, chocolate, strawberries etc.; insect venoms such as venom from bees, wasps, ants, mosquitos, flies etc.; venom from other animals such as snakes, spiders, etc; irritating secretions from plants, such as Bigsting (stinging nettle), Hogweed, thistle species etc.; tree, flower or grass-pollen; dust; small insects such as (house)mites etc.; other animal allergens such as hairs form e.g. cats and dogs; medicinal substances such as antibiotics such as penicillin etc.; topical (medicinal) creams; latex or other chemicals, polymers and plastics; perfumes and deodorants and the like.

The invention thus provides methods for determining the response of a subject to any known substance or allergen (e.g. such as those exemplified herein) using the method according to the invention.

In an alternative embodiment, the stimulation with an allergen is done in vitro, by adding said allergen to the sample, after it was obtained from, or is taken from, the subject.

In a preferred embodiment of the method of the invention, the mRNA or protein level of the biomarkers identified by the present invention is determined.

In a preferred embodiment of the method of the invention, the sample is selected from the group consisting of: blood, whole blood, plasma or serum, purified Basophiles, or PBMCs. Preferably, said sample is a whole blood sample.

Alternatively, in all methods as defined herein, a single sample can be used, wherein the expression level of said one or more biomarkers is tested before and after addition of the allergen. An increase of the expression level of said one or more biomarkers after addition of the allergen indicates the subject is allergic to or prone to become allergic to said allergen.

In preferred embodiments of the methods according to the invention, the expression level of all 4 biomarkers listed therein is measured and optionally also that of the following biomarkers: ENPP3, CISH and/or Dcun1D3.

The invention further provides a kit for diagnosing allergy in a subject comprising or consisting of:
(i) means for measuring the expression level of one or more of the biomarkers selected from the group consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in a sample of the subject;
(ii) optionally one or more allergens or allergen extracts to be tested;
(iii) means and/or instructions for performing the diagnosis according to the method of the present invention.

In a further embodiment, the kit of the invention comprises:
a) one or more vessel(s) suitable for accepting a blood sample,
b) one or more primer pair(s), each pair being specific to the mRNA of one member of the

group of biomarkers consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein),

c) optionally one or more allergen(s) or allergen extracts,

d) optionally an RNA stabilizing agent,

wherein said vessel comprises: i) a vessel capable of accepting a blood sample, and optionally: ii) a container in which a stabilizing agent is present, iii) a connection between the inside of said vessel (i) and the inside of said container (ii), (iv) a physical barrier that temporarily blocks said connection; and

optionally: (v) a container in which the allergen is present, vi) a connection between the inside of said vessel (i) and the inside of said container (v), and (vii) a physical barrier that temporarily blocks said connection between (i) and (v).

In a one embodiment, the allergen is already present in said vessel (i).

The invention further provides for the use of a kit according to the invention, for diagnosing, predicting or prognosticating the allergic active state in a subject, preferably by performing the method according to the invention.

In a preferred embodiment of the kits and/or methods of the invention, the means for determining either biomarker level is a means for determining the mRNA or protein level, such as end-point-PCR, real-time-PCR, quantitative-PCR, digital-PCR, or northern blot, capable of determining the mRNA level of the biomarkers, or ELISA and other EIA, ELISPOT, Luminex's xMAP technology, flow cytometry, nephelometry, turbidimetry, immunoprecipitation, capable of determining the concentration of the specific biomarker protein(s).

In another embodiment of the kit of the invention, means for determining the expression level of said one or more biomarker(s) is a specific binding assay, immunodetection assay, Mass-spectrometry assay, capable of determining the protein level of the biomarkers.

Preferably, the kits comprise primer pairs suitable for detecting the expression level of all 4 biomarkers listed therein and optionally also for the following biomarkers: ENPP3, CISH and/or Dcun1D3.

Preferably, the kits comprise primer pairs suitable for detecting the expression level of all 4 biomarkers listed therein and optionally also for the IL-6 biomarker.

Finally, the invention provides for the use of the methods and kits of the invention for determining the treatment needed for an allergic patient comprising performing the method steps or using the kits as defined herein at different time points during the treatment, wherein increased expression levels of said one or more biomarkers of the invention point to an active allergic phase in the subject under observation, indicating the need for anti allergic treatment.

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.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Histogram with means +/- SEMs per group for marker HAS1. Using the 1 way ANOVA (Kruskal-Wallis) test , we calculated the following p-value: p = 0.0018**

Figure 2: Histogram with means +/- SEMs per group for marker CD44. Using the 1 way ANOVA (Kruskal-Wallis) test , we calculated the following p-value: p = 0.0018**

Figure 3: Histogram with means +/- SEMs per group for marker RYBP. Using the 1 way ANOVA (Kruskal-Wallis) test , we calculated the following p-value: p = 0.0869

Figure 4: Histogram with means +/- SEMs per group for marker EGR1. Using the 1 way ANOVA (Kruskal-Wallis) test , we calculated the following p-value: p = 0.288

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In a survey for new biomarkers that can effectively predict allergy response in a subject, the inventors used basophil cells or PBMCs or basophils-depleted PBMCs, stimulated or not with an allergen in order to identify genes that are specifically upregulated upon stimulation with allergens. Using a real cat allergen such as Fel d1 (formerly called “cat allergen 1”) as a test case, the inventors analysed changes in the expression level genes in the transcriptome of PBMC’s, basophils and basophils-depleted PBMCs, isolated from peripheral blood of subjects allergic to the cat. This study was approved by the local ethics committee and patients and controls gave informed consent.

The results show that a panel of 4 genes was obtained which are significantly up-regulated upon stimulation of PBMC's or basophils with the cat allergen Fel d1 which is known to be the most important allergen of the cat, found in hair, dander and saliva of the animal. The genes are listed in Table 1 below. Expression levels of these genes remained unchanged when PBMC's, basophils and basophils-depleted PBMCs, isolated from peripheral blood of subjects allergic to the cat, were stimulated with the major allergen of birch tree pollen, Bet v1, validating the specificity of the response.

The validation of the markers listed above was done using qPCR on LDA microfluidic cards comprising whole blood samples.

Table 1: List of up-regulated genes after allergen stimulation

Gene names	Affymetrix ID	Full name	Database Acc. No.
EGR1	227404_s_at 201694_s_at	Early growth response 1	NP_001955 (SEQ ID NO. 1)
CD44	1565868_at 217523_at	CD44 molecule (Indian blood group)	NP_000601 (isoform 1 precursor) (SEQ ID NO. 2)
HAS1	207316_at	Hyaluronan synthase 1	NP_001514 (SEQ ID NO. 3)
Rybp	237456_at	RING1 and YY1 binding protein	NP_036366 (SEQ ID NO. 4)

Two different allergens (Fel d1 and Bet v1) were tested in the current experiments. These experiments will be repeated using other specific allergens in order to confirm the general utility of the methods and kits as provided in the present invention.

Methods of the invention

The present method thus comprises obtaining or providing two blood samples from a subject, one sample before the subject is treated with the allergen and one sample after the subject is treated with the allergen. The blood samples are not further treated and are analyzed as such. In-between the taking of both blood samples, the subject is submitted to a treatment with the allergen during a suitable period of time which may vary from 1 to 24 hours, depending on the allergen that is administrated to the patient and which for instance may be about 2, 3, 4, 5, 6, 7 or 8 hours. Other allergens may be administrated for months, like for desensitization therapy.

Alternatively, the allergen is not administered to the subject under treatment, but is added in vitro to the sample or a portion thereof obtained from the subject. In this case, a single sample of the subject can be divided in two portions: one portion to which the allergen is added in vitro and one portion to which no allergen is added. The increased expression level of the one or more biomarkers of the invention can then be calculated based on the differential mRNA levels of said biomarkers between both samples, i.e. with and without allergen added.

In a preferred embodiment, before mRNA determination, all blood samples (i.e. the blood samples obtained from a subject before and after treatment with the allergen or the blood samples incubated or not with an allergen in vitro) are all treated with a stabilizing agent. In an embodiment, the stabilizing agent is an inhibitor of cellular RNA degradation and/or gene induction. For example, said inhibitor of cellular RNA degradation and/or gene induction is that as found in a PAXgene™ Blood RNA Tube or alternatively in a Tempus™ Blood RNA Tube. For example, a quaternary amine surfactant may be used as a stabilizing agent. Suitable quaternary amine surfactants, able to stabilize RNA from biological samples, are described in US Pat. No. 5,985,572, WO94/18156 and WO02/00599. One example of a quaternary amine which can be used in the method of the present invention is tetradecyltrimethyl-ammonium oxalate. (US Pat. No 5,985,572). Alternatively, said cationic detergent may be Catrimox-14TM (US Pat. No 5,010,183).

The method of the invention can comprise the steps of:

a) providing a first and a second blood sample (or a first and second portion of a blood sample) of a subject, wherein the first sample is taken prior to the treatment with an allergen and the second sample is taken after the treatment with the allergen,
b) adding to said first and second (portion of the) blood sample a stabilizing agent,
c) determining the mRNA levels of the one or more biomarkers selected from the group consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in said first and second stabilized blood samples;
d) comparing the mRNA levels of said one or more biomarkers determined in said first and second (part of the) blood sample, and evaluating the allergic status in said individual, wherein increased expression levels of said one or more biomarkers upon allergen addition indicates the patient is allergic to said allergen.

Preferably, said increase in expression level is 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5 fold.

In a preferred embodiment, all 4 biomarkers are used in parallel, i.e. the expression levels of all 4 genes is determined in the sample of the subject, in order to obtain an expression profile encompassing data of all 4 biomarkers.

In a preferred embodiment, the method of the present invention may use a blood collecting vessel and a container in which a stabilizing agent is present. Preferably, the inside of said blood collecting vessel and the inside of said container are connected, and a physical barrier temporarily blocks said connection.

The method of the invention can comprise the steps of:

a) providing a first and a second blood sample (or a first and second portion of a blood sample) of a subject, wherein the first sample is taken prior to the treatment with an allergen and the second sample is taken after the treatment with the allergen,
b) adding said first and second (portion of a) blood sample in a separate device comprising: (i) a collection vessel, (ii) a container in which a stabilizing agent is present, (iii) a connection between the inside of said vessel and the inside of said container, and (iv) a physical barrier that temporarily blocks said connection.
c) adding to said first and second (portion of a) blood sample the stabilizing agent by removing said physical barrier,
d) determining the mRNA levels of the one or more biomarkers selected form the group consisting of: in said first and second stabilized blood samples;
e) comparing the mRNA levels of said biomarkers determined in step c) and d), and evaluating the allergy status of the individual, wherein an increase in the expression levels of the one or more biomarkers after addition of the allergen indicates the patient is allergic to the allergen.

Alternatively, the method of the present invention may use a blood collecting vessel and a container in which a stabilizing agent is present as described above, wherein either an amount of allergen is present in the vessel (i) used for collecting the second blood sample, or wherein said blood collecting vessel additionally comprises a container (v) comprising an amount of allergen, connected with vessel (i) by a connection (vi), said connection being temporarily blocked by a physical barrier (vii).

The method of the invention comprises the steps of:

a) providing a first and a second blood sample (or a first and second portion of a blood sample) of a subject, preferably before the subject was treated with an allergen,

b) adding said first and second blood sample in a separate device comprising: (i) a collection vessel, (ii) a container in which a stabilizing agent is present, (iii) a connection between the inside of said vessel and the inside of said container, and (iv) a physical barrier that temporarily blocks said connection, additionally comprising a container (v) comprising an amount of allergen, connected with vessel (i) by a connection (vi), said connection being temporarily blocked by a physical barrier (vii).

c) contacting only one of the samples or portion of the samples with an allergen e.g. by removing a temporary physical barrier (vii) separating the sample vessel (i) and the container (v) with the allergen,

d) incubate the samples or portion of the samples in the separate device for a suitable time period

e) adding to said first and second (portion of the) blood sample the stabilizing agent e.g. by removing a temporary physical barrier (iv) separating the sample vessel (i) and the container (ii) with the stabilizing agent,

f) determining the mRNA levels of one or more genes selected from the group consisting of: in said first and second stabilized (portions of) blood samples;

g) comparing the mRNA levels of said biomarkers determined in step d) and e), and evaluating the allergy status of the individual, wherein an increase in the expression levels of the one or more biomarkers after addition of the allergen indicates the patient is allergic to the allergen.

The first and second (portions of) blood samples are preferably stabilized with stabilizing agent, as fast as possible after blood collection or after the ex vivo incubation.

In any one of the embodiments listed or defined herein, the allergen can also be added in vitro, i.e. after the sample of e.g. blood has been taken from the subject under investigation. One part of the blood sample is then contacted with the allergen and the resulting RNA expression pattern resulting there from is determined subsequently by fixing the RNA with an RNA stabilizing agent, followed by RNA analysis as indicated for the other embodiments. The remaining part of the blood sample is also analysed for its RNA expression pattern in the same manner, safe from the addition of the allergen. The two expression patterns can then be compared to give an indication of the allergic state of the subject.

In another preferred embodiment, the method involves incubating a blood sample in the presence of an allergen in an incubator, preferably at a temperature of about 37°C. Preferably, the method is performed in the absence of controlling the air composition during incubation. In accordance with the invention, as incubator an oven can be used that is working under ambient atmospheric conditions, i.e. without any regulation of the amounts of CO_2 and H_2O present in the oven. In said oven, the sample is preferably maintained at 37°C. However, a cell culture incubator, wherein atmospheric conditions and the concentration of e.g. CO_2 are controlled, is not required for carrying out the incubation step of the present method.

The method of the invention can comprise the steps of:

a) providing a first and a second blood sample (or a first and second portion of a blood sample) of an individual,

b) adding an allergen only to said second blood sample (or said second blood sample portion), present in a vessel comprising: (i) a suitable amount of allergen present inside said vessel, (ii) a container in which a stabilizing agent is present, (iii) a connection between the inside of said vessel and the inside of said container, and (iv) a physical barrier that temporarily blocks said connection.

c) incubating said second blood sample (or second portion) *in vitro* for a suitable period of time;

d) adding to said second blood sample (or second portion) the stabilizing agent by removing said physical barrier,

e) determining mRNA levels of said one or more biomarkers according to the invention in said incubated and stabilized second blood sample (or second blood portion);

f) adding to said first blood sample (or first blood portion) a stabilizing agent and determining mRNA levels of said one or more biomarkers according to the invention in said first blood sample (or first blood portion) from said patient,

g) comparing the expression levels in e) and f) and evaluating the allergic state of the subject, wherein an increased expression of said one or more biomarkers in step e) as compared to step f) indicates the subject is allergic to the tested allergen.

In an embodiment, said first blood sample (or first blood portion) is also incubated, similarly to said second sample (or blood portion), in a vessel as described above but free of allergen. This first blood sample is also stabilized with stabilizing agent, as fast as possible after blood collection, or alternatively, after incubation as done for the tube

containing the allergen. This can again be done by perforating or removing the temporary physical barrier (iv) between the sample vessel (i) and the container (ii) comprising the stabilizing agent. In a preferred embodiment, the allergen is present in the sample vessel prior to the addition of the second (portion of the) sample.

In an alternative embodiment, said allergen is present in an additional container (v), in connection with said sample vessel, wherein the connection (vi) is temporary blocked by a physical barrier (vii). In this embodiment, two identical vessels can be used for the first and second (portion of) the blood sample, wherein for the first (portion of the) sample, the physical barrier between the allergen container and the sample vessel is left intact, and wherein for the second (portion of the) sample, said barrier is perforated, thereby only bringing the second (portion of the) sample in contact with the allergen.

The method comprises the determination of mRNA levels of the one or more biomarkers of the invention in a first blood sample of an individual and in a second blood sample that has been incubated with the allergen. The mRNA level of the one or more biomarkers is compared in both samples and based on the results thereof, the allergy status of the individual is evaluated.

In another embodiment, the present invention therefore also provides a method for monitoring the *in vivo* response of an individual to a treatment with an anti-allergic agent, comprising incubating a first blood sample of said individual *in vitro* with a suitable amount of the allergen and a second blood sample of said individual *in vitro* with a suitable amount of the allergen and the anti-allergic agent for a suitable period of time, and comparing mRNA levels of the one or more biomarkers of the invention in both blood sample. If the mRNA levels are reduced or modified in any way, upon addition of the anti allergic agent, it indicates that the symptoms of the patient can likely be reduced or alleviated when the anti-allergic agent is administered to the patient. The method can be applied using the steps, conditions, amounts, examples of incubation times and conditions as described above.

In yet another embodiment the invention also provides a method for identifying an individual as a responder or non responder to a treatment with an anti allergic agent, comprising incubating a blood sample of said individual *in vitro* with a suitable amount of said allergen for a suitable period of time, and determining mRNA levels of the one or

more biomarkers in said blood sample. The method can be applied using the steps, conditions, amounts, examples and incubation times and conditions as described above.

The invention further provides a method for adjusting an anti-allergy therapy in a patient, comprising the step of identifying an individual as a responder or non responder to a treatment using the method of the present invention, and adjusting said therapy when the patient is a non-responder. In an embodiment, said adjusting step comprises discontinuing the therapy. In another embodiment, said adjusting step comprises using a different anti-allergic agent. Preferably, the identification step is performed at least twice before adjusting the therapy. Preferably, the at least two successive identification steps are separated by 3 to 6 months. The method can be applied using the steps, conditions, amounts, examples and conditions or as described above.

The term “blood sample” applied in the present method generally refers to a “whole blood sample”. The term “whole blood” as used herein refers to blood as it is collected by venous sampling, i.e. containing white and red cells, platelets, and plasma. Alternatively, the sample can be purified basophils or PBMCs, or depleted-PBMCs, all obtained from blood, e.g. peripheral blood.

The mRNA levels of the biomarkers of the invention can be determined using any known method in the art. Examples are: Polymerase Chain Reaction (PCR), Real-Time quantitative PCR (RT-qPCR), End-Point PCR, digital PCR (dPCR), RNA or cDNA hybridization techniques, microarrays, RNA-in-situ hybridization (RISH), Northern-Blotting, digital analysis of gene expression (DAGE), sequence-analysis based expression analysis, Supported oligonucleotide detection, Pyrosequencing, Polony Cyclic Sequencing by Synthesis, Simultaneous Bi-directional Sequencing, Single-molecule sequencing, Single molecule real time sequencing, True Single Molecule Sequencing, Hybridization-Assisted Nanopore Sequencing or Sequencing by synthesis.

In a preferred embodiment, the biomarker mRNA levels are determined by real-time quantitative polymerase chain reaction (qPCR, qc-PCR, RT-PCR). As used herein, "Real-time quantitative rt-PCR" relates to a method that monitors the degradation of a dual-labeled fluorescent probe in real time concomitant with PCR amplification. Input target RNA levels are correlated with the time (measured in PCR cycles) at which the reporter fluorescent emission increases beyond a threshold level. In example 1, a real-time quantitative polymerase chain reaction (qc-PCR) as can be applied in accordance with the present invention is illustrated.

Kits

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.

A kit for diagnosing allergic response to an allergen in a subject according to the invention typically comprises:

a) one or more vessel(s) suitable for accepting a blood sample,

b) one or more primer pairs specific to the mRNA of the one or more biomarkers according to the invention which is suitable for the transcription of mRNA of said biomarker genes into cDNA and the amplification of the latter, and a probe designed to anneal to an internal region of the produced cDNA,

wherein said vessel comprises: i) a vessel capable of accepting a blood sample, and optionally ii) a container in which a stabilizing agent is present, iii) a connection between the inside of said vessel (i) and the inside of said container (ii), and iv) a physical barrier that temporarily blocks said connection.

The kit according to the invention optionally additionally comprises:

c) a control primer pair specific to the mRNA of a control gene which is suitable for the transcription of mRNA of said control gene into cDNA and the amplification of the latter, and a control probe designed to anneal to an internal region of the produced control cDNA.

In any one of the embodiments listed or defined herein, the allergen can either be added in vitro, i.e. after the sample of e.g. blood has been taken from the subject under investigation. One part of the blood sample is then contacted with the allergen and the resulting RNA expression pattern resulting from the stimulation is determined subsequently by fixing the RNA with an RNA stabilizing agent, followed by RNA analysis as indicated for the other embodiments. The remaining part of the blood sample is also analysed for its RNA expression pattern in the same manner, safe from the addition of the allergen. The two expression patterns can then be compared to give an indication of the active state of the MS in the subject.

A typical kit according to the invention can thus comprise:

a) one or more vessel suitable for accepting a blood sample,

b) one or more primer pairs specific to the one or more biomarkers of the invention, and

c) a probe designed to anneal to an internal region of the produced cDNAs of the one or more biomarkers,

wherein said vessel (i) comprises: a) an allergen present inside said vessel, optionally present in a container (v) separated from said vessel by a physical barrier (vii) temporarily blocking the connection (vi), b) a container (ii) in which an RNA-stabilizing agent is present, c) a connection between the inside of said vessel (i) and the inside of said container (ii), d) a physical barrier (iv) that temporarily blocks said connection (iii).

In use, any one of the physical barrier(s) in the kits or methods of the invention may be opened by the application of physical force to said vessel. Said force may transmit an opening means to said physical barrier. Examples of such physical barriers include rotary valve, aperture valve, slit valve, diaphragm valve, ball valve, flap valve. Alternatively, said force may irreversibly open said physical barrier. Other examples of such physical barriers include a plug which is forced out of position, a barrier which shatters upon the application of force. In an embodiment, the inside of said container and the inside of said vessel are connected, and the flow of stabilizing agent from the container to the vessel is prevented by the surface tension of the stabilizing agent in combination with the aperture size of the connection. According to this aspect, at an appropriate time an application of force which transmits to the stabilizing agent, forces the stabilizing agent from the container into the vessel. The force may be applied, for example, by squeezing, continually inverting, and agitating.

The allergen can be provided in said vessel in a liquid or lyophilized form, not immobilized. The allergen can also be immobilized on part or all of the inside surface of said vessel. The inside wall of the vessel may be lined with a suitable coating enabling the allergen to be attached. In another embodiment, said allergen is immobilized on a solid support. The solid support may be attached to the inside of the vessel. Alternatively, the solid support may be free of the inside of the vessel. Examples of solid supports include, but are not limited to, chromatography matrix, magnetic beads.

The vessel may be sealed with resealing means such as a screw-cap, push-on cap, and a flip-cap. Said vessel may comprise one or more openings. In a particular embodiment, the vessel as described above comprises one or more areas suitable for puncture by a

syringe needle, such as a re-sealable septum. The vessel may comprise a fitting suitable for receiving a syringe or a syringe needle and transmitting the contents therein to the interior of said vessel. Suitable vessel may further comprise cannular suitable for withdrawing bodily fluids.

Suitable vessel may further comprise a valve which is capable of minimizing the flow of gas/liquid from vessel, and allowing the flow of biological sample into the vessel. Suitable vessel may further comprise a means through which displaced gas may be expelled. Said means are known the art and include valves, non-drip holes, vents, clothed-vents, expandable vessel walls, use of negative pressure within said vessel. Said vessel may further be held under negative pressure. The negative pressure may be utilized to relieve the pressure build-up upon introduction of whole blood into said sealed vessel. Alternatively, or in addition, the negative pressure may be at a predetermined level and may be utilized so as to allow the introduction of a fixed volume of whole blood. Suitable vessel may comprise an indication for dispensing a known volume of stabilizing agent therein.

In an embodiment, the qPCR control gene used in some of the kits or methods according to the invention is selected from the group comprising mRNAs for certain ribosomal proteins such as RPLP0 (ribosomal protein, large, P0), glyceraldehyde-3-phosphate dehydrogenase mRNA, beta actin mRNA, MHC I (major histocompatibility complex I) mRNA, cyclophilin mRNA, 28S or 18S rRNAs (ribosomal RNAs).

The kit can further comprise additional components for carrying out the method of the invention, such as RNA extraction solutions, purification column 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. Any convenient means may be present in the kits.

The allergens

The allergy of the subject can be to any product, e.g. foodstuffs such as cowmilk, gluten, wheat, peanuts tree nuts, hazelnuts, soy, egg, fish, shell fish, chocolate, strawberries, apple etc.; insect venoms such as from bees, wasps, ants, etc.; venom from other animals such as snakes, spiders, etc; irritating secretions and other allergens from plants, such as Bigsting (stinging nettle), timothy grass etc.; pollen from trees such as birch; dust or small insects such as (house)mites etc.; animal allergens such as hairs form e.g. cats and dogs; medicinal substances such as antibiotics such as penicillin etc.; topical (medicinal) creams; latex or other chemicals, polymers and plastics; perfumes and deodorants, etc.; heavy metals such as chromium, nickel, titanium, etc. Exposure to such an allergen can sometimes lead to an anaphylactic shock in an allergic subject and eventually to death. Knowing that a subject is allergic to a certain allergen beforehand is therefore of paramount importance in order to avoid serious health problems. A selection of animal and plant allergens for use in the invention include, but are not limited to the list available on the World-Wide Web at http://www.allergen.org. The examples given herein and used in the following examples are in no way meant to be limiting and serve the purpose of providing a proof of concept of the methods of the present invention only.

Treatment of allergy

The general treatment of an allergic reaction is nowadays mainly focussed on alleviating the symptoms of the allergic reaction, which is of course highly desired for the subject, but does in no way prevent the recurrence of the allergic reaction upon new exposure to the allergen in question. Immunosuppressants such as e.g. antihistamines or corticosteroids can therefore help the patient to feel better, but can never cure him. One way of actually curing the subject is using allergy immunotherapy. A three-to-five-year individually tailored regimen of injections may result in long-term benefits.

Allergy immunotherapy (also called hyposensitization therapy, immunologic desensitization or allergen-specific immunotherapy) involves a series of injections with an allergy extract given regularly for several years (usually 3 to 5 years). The first injections

only contain very small amounts of the allergen or antigen to which the subject is allergic and this dose is progressively increased over time. The immune system of the subject is thus gradually exposed to higher dosages of the allergen and becomes less sensitive to it. This process is called desensitization. A good example of such an immunotherapy is the sublingual (under the tongue) tablet Grazax, containing a grass pollen extract which has been shown effective with few side effects and can even be self-administered at home. Allergen specific immunotherapy is the only treatment strategy which treats the underlying cause of the allergic disorder and has been shown to produce long term remission of allergic symptoms, reduce severity of associated asthma as well as reduce the chances of new sensitizations to allergens developing. The allergy extract is usually administered sublingually (under the tongue), by nasal or oral administration or by injections under the skin (subcutaneous). Subcutaneous injection immunotherapy has been shown to be highly efficient treatment for allergic disease, but can lead to anaphylaxis and is therefore restricted to specialized clinical settings. Sublingual therapy which can be safely administered at home is currently preferred. Another possibility is the use of DNA vaccines that incorporate a gene encoding the entire or partial allergen sequence and containing at least one T-cell epitope sequence. Possible immunotherapy strategies can be found in the art and the examples given herein are not to be seen as limiting. The methods and kits of the present invention are helpful in determining the exact allergens to which a subject is allergic. After identification of the exact allergen according to the methods of the present invention, a specific immunotherapy procedure can be developed for said subject.

For easy of comparison, the expression levels of the biomarkers are often depicted as “ratios” or “stimulation index” or “stimulation ratio” herein. Said ratios are calculated by dividing the expression level of a certain biomarker of allergen-treated blood samples by the expression level of said biomarker in the non-allergen-treated blood sample. If the ratio is close to 1, there is no or little response of the biomarker expression level to the allergen. If on the other hand the ratio is significantly higher than 1, there is an increase in biomarker expression upon stimulation with the allergen. The ratio represents the “fold induction” of the expression level of the biomarker. As indicated above, the goal of the diagnostic methods according to the invention is to provide a more accurate indication whether or not a patient can be classified as allergic to a certain allergen. A significant increase in expression level for classifying or diagnosing a subject of being allergic to said tested allergen or allergen extract is e.g. 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, or more than 20, wherein a ratio of 1.5 indicates that the expression level of said biomarker

after stimulation with the allergen is 1.5 times higher than the expression level without stimulation with the allergen.

In order to increase the diagnostic power of the current methods of the invention, it is possible to combine the above defined ratios of all 4 biomarkers as identified herein, and optionally also that of the IL-6, ENPP3, CISH and/or Dcun1D3 biomarker. Since some biomarkers are more or less responsive to a certain allergen in a certain subject as compared to another subject, combining the ratios of all 4 biomarkers and IL6, ENPP3, CISH and/or Dcun1D3 can enable the reduction of such asymptomatic fluctuations.

The following examples are intended to illustrate and to substantiate the present invention are not to be seen as limiting the invention. It is understood that the skilled person can think of other non-mentioned embodiments of the methods and kits of the present invention without departing from the spirit and scope thereof.

EXAMPLES

The invention is illustrated by the following non-limiting examples

Example 1: Identification of new allergy specific genes in activated basophils with DNA Microarrays

We first studied the gene expression profile in activated Basophils. We used purified basophils from healthy donors. DNA microarray analysis shows that Basophil activation (2 hours) induced up regulation of 180 genes (n=3 experiments). Because of lack of specificity with anti FcεRI stimulus, that directly targets IgE receptor and triggers the corresponding signaling cascade, we studied Basophilic activation among PBMCs from cat allergic patients. Moreover Basophils could be activated during purification and that could boost the allergic stimuli and induce non specific genes. The PBMC gene expression profile was studied in parallel to Basophil depleted PBMCs. We used the cat allergen (Fel d1) for cell activation. DNA microarray analysis shows that short term PBMC activation induced up regulation of 77 genes.

The identified genes in Basophils (n=3, activated with anti-FceRI) were compared and combined with the gene list identified in Fel d-1 activated allergic PBMCs (n=2). The up-regulated genes (the ratio of expression levels when stimulated versus non-stimulated is above 2) simultaneously in Basophils and PBMCs have been identified. When the up-

regulation was reverted (with more than 50 % of inhibition) in Basophil depleted PBMCs we considered that the corresponding gene is specific to Basophils in this setting. Thus a list of five genes was obtained (Table 1).

Table 1: List of up-regulated genes upon stimulation of basophils or PBMCs with an allergen.

Gene names	Affymetrix ID	Full name	Database Acc. No.
EGR1	227404_s_at 201694_s_at	Early growth response 1	NP_001955 (SEQ ID NO. 1)
CD44	1565868_at 217523_at	CD44 molecule (Indian blood group)	NP_000601 (isoform 1 precursor) (SEQ ID NO. 2)
HAS1	207316_at	Hyaluronan synthase 1	NP_001514 (SEQ ID NO. 3)
Rybp	237456_at	RING1 and YY1 binding protein	NP_036366 (SEQ ID NO. 4)

Example 2: Expression of Basophil LDA cards qPCRs

To be closer to physiological conditions we worked on whole blood from cat allergic patients stimulated with the Fel d1 allergen in same conditions indicated previously (Tab2). In parallel to Fel d1 stimulation, we used Betv1 (Birch allergen), as a control stimulation which should normally not lead to allergic reaction in previously diagnosed cat allergic patients who were also diagnosed non-allergic to birch tree pollen (Table 3).

Table 2: qPCRs in cat allergic patients (Fel d1 stimulation)

	Patient's stimulation ratios							
	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
Genes	1	2	3	4	5	6	7	8
HAS1	27	8.3	14.8	18.1	0.4	28.3	18.7	16.7
CD44	2.4	1.4	1.8	3.5	1.8	1.7	1.57	1.9
RYBP	1.3	1.3	0.7	1.5	1.1	1.2	1.1	2.0
EGR1	0.99	1.6	2.3	2.2	0.9	0.8	2.4	1.3

Table 3: qPCRs in cat allergic patients (Bet v1 stimulation)

Patient's stimulation ratios

Genes	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
HAS1	0.5	1.8	1.7	1.3	0.1
CD44	0.7	0.99	0.9	1.2	0.8
RYBP	0.96	0.99	0.90	1.34	0.93
EGR1	0.7	0.87	1.1	1.4	0.67

Patient 5 was left out of the test, since a further analysis revealed unresponsiveness to anti-Fce receptor antibody, which is used as a positive control. Unresponsiveness to the positive control is considered as unresponsiveness of the basophils, and is one of the criteria of exclusion. This also explains the absence of response to the 4 studied genes.

Table 4: qPCRs in 8 healthy controls (Fel d11 stimulation)

Genes	Control's stimulation ratio.							
	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Control 7	Control 8
HAS1	0.2	1.3	2.2	1.7	0.2	0.7	2.1	1.9
CD44	0.3	0.4	1.4	1.5	0.8	0.9	1.4	0.9
RYBP	0.2	0.4	1.2	1.4	0.6	1.0	0.7	0.8
EGR1	1.2	1.3	0.9	1.1	1.0	0.7	1.4	1.9

As can be seen from the comparison of Tables 2 and 3, the cat allergen Fel d1 leads to an increased stimulation ratio in previously diagnosed cat allergic patients, while the unrelated Betv1 allergen does not. This implies that the response of said 5 identified biomarkers is specific to the concerned allergen and thus related to the clinics, i.e. in cat allergic patients, a cat allergen induces their expression, while a non-related allergen does not.

The ratios are calculated by dividing the expression level of a certain biomarker of allergen-treated blood samples by the expression level of said biomarker in the non-allergen-treated blood sample. If the ratio is close to 1, there is no or little response of the biomarker expression level to the allergen. If on the other hand the ratio is significantly higher than 1, there is an increase in biomarker expression upon stimulation with the allergen.

Example 3: Analysis of markers in pediatric patients tested for food allergy

Blood samples from 13 children allergic to peanut (positive IgE and /or skin test to peanut, and clinical manifestations), 8 children sensitized to peanut (positive IgE and /or skin test to peanut, but no clinical manifestation), and 18 healthy controls were stimulated or not with peanut extract and anti-IgE antibody (positive control).

The expression level of the 4 genes of the present invention will be tested on mRNA isolated form said samples.

Material and methods

Cell and Blood stimulation

Peripheral blood cells were purified from Buffy coats using Hetasep™ solution. Basophils were purified using the Basophil purification kit from Stemcell Technologies, Grenoble, France, and subsequently activated for 2 hours with an anti-FceRI antibody, (0.2 ug/ml, obtained from Bühlmann Laboratories AG, Switzerland).

Peripheral blood mononuclear cells (PBMCs) from blood of cat allergic patients were isolated through a ficoll gradient. A fraction of blood was depleted in Basophils using the Basophil depletion kit from Stemcell Technologies, Grenoble, France.

PBMC and Basophil depleted PBMCs were stimulated in parallel with the allergens for 2 hours. Whole blood was stimulated for 2 hours with allergens (12,5 ug/ml Fel d1 or Betv1, obtained from Indoor Biotechnologies Wiltshire, UK) and Paxgene (1.5 ml for 500 µl of whole blood) was added (Becton Dickinson, Erembodegem, Belgium) at this end of the incubation in order to stabilize total RNA.

Microarrays

Basophils were isolated from Buffy coats using Hetasep™ (Stemcell Technologies, Grenoble, France) density gradient centrifugation followed by a negative selection kit: EasySep™ Human Basophil Enrichment Kit (Stemcell Technologies, Grenoble, France) as described previously (B. F. Gibbs et al., 2008, Clin Exp Allergy, 2008 Mar; 38(3):480-5). Basophils were then activated for 2 hours with an anti-FceRI antibody (0.2 ug/ml, obtained from Bühlmann Laboratories AG, Switzerland). PBMCs were separated from the

heparinized peripheral blood of two cat allergic patients by Lymphoprep™ (NYCOMED PHARMA, Switzerland) density gradient centrifugation. A fraction of blood was depleted in Basophils using the Basophil RosseteSep™ depletion kit (Stemcell Technologies, Grenoble, France) and Basophil depleted PBMCs were separated by Lymphoprep™ (NYCOMED PHARMA, Switzerland) density gradient centrifugation. PBMCs and the corresponding Basophil depleted PBMCs were stimulated in parallel with Fel d1 (an uteroglobin-like protein that is the most important cat allergen) for 2 hours. RNA from Basophils or PBMCs and the corresponding Basophil depleted PBMCs were extracted with the micro-RNAeasy™ kit (Qiagen). RNA quality was controlled using the Agilent 2100 Bioanalyser. Using the MessageAmp™ Premier RNA Amplification Kit (Ambion). Double strand cDNA was synthesized from 30 ng total RNA to which Affymetrix Genechip™ Poly-A Controls were added. The cDNA was transcribed in vitro in order to produce biotin labeled aRNA. 10 µg of the cleaned up aRNA was then fragmented using the Genechip™ Sample Cleanup module (Affymetrix) in order to produce fragments of 35 to 200 bases. 300 µl of hybridization mix was produced including the 10 µg of fragmented aRNA, the oligonucleotide B2 control, the Affymetrix hybridization controls, herring sperm DNA, acetylated BSA, hybridization buffer and DMSO. This mix was engaged on GeneChip™ Human Genome U133 Plus 2.0 Array at 45°C during 16 to 18 hours. After hybridization the GeneChips were washed and stained using the EukGE-WS2v5 fluidics protocol and finally scanned using the Affymetrix GeneChip™ Scanner 3000.

The quality of the GeneChips results was assessed using the BioConductor™ (Gentleman R. C. et al., 2004, Genome Biol; 5(10):R80) yaqcaffy library. Then, the raw data have been normalized together using the RMA algorithm (Irizarry. R. A. et al., 2003, Nucleic Acids Res.; 31(4):e15; Irizarry. R. A. et al., 2003, Biostatistics; 4(2):249-264).

LDA cards qPCRs

Heparinized whole Blood from cat allergic patients (1 ml) was activated with 10 µg/ml Feld1 or Betv1 for 2 hours. Paxgene™ (Qiagen) solution (1.250 ml/ 1ml whole blood) was then added for cell lysis and RNA stabilization. Total RNA was extracted using the PreAnalytix™ Paxgene™ RNA isolation kit (Qiagen) and cDNA was synthesized using the Quantitect™ kit (Qiagen). qPCR was performed on TaqMan™ Low Density Array (LDA) cards. The LDA cards were designed and purchased from ABI (Applied Biosystem Inc, Foster City, USA).

Quantitative real-time PCR (Taqman™) assays were performed using a 96-genes format Taqman™ Low Density Arrays (TLDA) on an ABI PRISM 7900 thermocycler. TaqMan™

Universal PCR Master Mix (ABI) was used for PCRs. The oligonucleotide primers and fluorogenic probes were prepared and spotted in the TLDA by ABI. The 96-gene format low-density array allowed simultaneous measurement of 92 selected target genes and 3 housekeeping genes (Rplp1, Rplp0, and β-actin). We used a relative quantification method (delta-delta-Ct method) to calculate the gene expression values. Genes where no amplified product could be detected were given an arbitrary Ct of 36 cycles, the maximum number of cycles carried out by the thermocycler.

CLAIMS

1. A method for determining whether a subject is allergic to a certain allergen comprising the steps of:

a) contacting a whole blood sample of the subject with said allergen,

b) measuring the expression level of one or more of the biomarkers selected from the group consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein),

c) comparing said expression level(s) with those of a sample of a non-allergic subject, wherein an increase of the expression level of said one or more biomarkers as compared to the non-allergic subject indicates the tested subject is allergic or is prone to become allergic to the tested allergen.

2. A method for determining whether a subject is allergic to a certain allergen comprising the steps of:

a) measuring the expression level of one or more of the biomarkers selected from the group consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein), before and after contacting the blood sample of the subject with said allergen,

b) comparing the expression level(s) measured before and after the addition of the allergen to the sample, wherein an increase of the expression level of said one or more biomarkers after addition with the allergen indicates the tested subject is allergic or is prone to become allergic to the tested allergen.

3. A method for determining whether a subject is allergic to a certain allergen comprising the steps of:

a) contacting a part of a whole blood sample of the subject with said allergen,

b) measuring the expression level of one or more of the following markers: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein) in the part of the sample contacted with said allergen and in the part not contacted with said allergen,

c) comparing the expression level(s) before in both parts of the sample, wherein an increase of the expression level of said one or more biomarkers in the part contacted with the allergen as compared to the non-contacted part indicates the tested subject is allergic or is prone to become allergic to said tested allergen

4. The method according to any one of claims 1-3, wherein the expression levels of all 4 biomarkers is assessed and wherein optionally also the expression level of ENPP3, CISH and Dcun1D3 are assessed.

5. The method according to any one of claims 1 to 4, wherein the sample is selected from the group consisting of: blood, whole blood, peripheral blood mononuclear cells (PBMC) depleted from basophils or not, plasma or serum, preferably, a whole blood sample.

6. The method according to any one of claims 1 to 5, wherein said stimulation with the allergen was done in vivo, i.e. by administering the allergen to the subject under investigation or wherein said stimulation with the allergen is done in vitro, i.e. by adding the allergen to the sample after it was obtained from the subject.

7. The method according to any one of claims 1 to 6, wherein the mRNA or protein level of the one or more biomarker(s) is determined, preferably by means of quantitative PCR.

8. The method according to any one of claims 1 to 7, wherein the subject is human and wherein preferably said subject is an adult, an adolescent, a child, a toddler, a baby or a neonate.

9. The method according to any one of claims 1 to 8, wherein the allergen is selected from the group consisting of foodstuffs such as cowmilk, gluten, wheat, peanuts tree nuts, hazelnuts, soy, egg, fish, shell fish, chocolate, strawberries, apple etc.; insect venoms such as venom from bees, wasps, ants, mosquitos, flies etc.; venom from other animals such as snakes, spiders, etc; irritating secretions from plants, such as Bigsting (stinging nettle), Hogweed, thistle species etc.; tree, flower or grass-pollen; dust; small insects such as (house)mites etc.; other animal allergens such as hairs form e.g. cats and dogs; medicinal substances such as antibiotics such as penicillin etc.; topical (medicinal) creams; latex or other chemicals, polymers and plastics; perfumes and deodorants and the like; heavy metals such as chromium, nickel, titanium, etc.

10. The method according to any one of claims 5-9, wherein the expression level of all 4 biomarkers listed therein and optionally also for that of the following biomarkers: ENPP3, CISH and/or Dcun1D3 is measured.

11. A kit for diagnosing allergy in a subject comprising or consisting of:
(i) means for measuring the expression level of one or more of the biomarkers selected from the group consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein), in a sample of the subject;
(ii) optionally an allergen;
(iii) means and/or instructions for performing the diagnosing according to the method of any one of claims 1-9.

12. The kit according to claim 11, wherein the means for determining the expression level of the one or more biomarker(s) is a means for determining the mRNA or protein level, such as end-point-PCR, real-time-PCR, quantitative-PCR, digital-PCR, or northern blot, capable of determining the mRNA level of said one or more biomarker(s).

13. The kit according to claims 11 or 12, wherein the kit comprises:
a) one or more vessel(s) suitable for accepting a blood sample,
b) one or more primer pair(s), each pair being specific to the mRNA of one member of the group of biomarkers consisting of: HAS1 (Hyaluronan synthase 1), EGR1 (Early Growth Factor 1), CD44, and/or Rybp (RING1 and YY1 binding protein), in a sample of the subject,
c) optionally an allergen,
d) optionally an RNA stabilizing agent,
wherein said vessel comprises: i) a vessel capable of accepting a blood sample, and optionally ii) a container in which a stabilizing agent is present, iii) a connection between the inside of said vessel (i) and the inside of said container (ii), (iv) a physical barrier that temporarily blocks said connection and optionally (v) a container in which the allergen is present, vi) a connection between the inside of said vessel (i) and the inside of said container (v), and (vii) a physical barrier that temporarily blocks said connection between (i) and (v).

14. The kit according to any one of claims 11 to 13, comprising primer pairs suitable for detecting the expression level of all 4 biomarkers listed therein and optionally also for that of the following biomarkers: ENPP3, CISH and/or Dcun1D3.

15. The use of a kit according to any one of claims 11-14, for diagnosing, predicting or prognosticating the allergic state in a subject, preferably by performing the method according to any one of claims 1-10.

16. A method for monitoring the treatment of an allergic patient comprising performing the method according to any one of claims 1-10 at different time points during the treatment, wherein reduced expression levels of the one or more biomarkers, points to a reduction in allergic state of the subject under treatment, indicating the treatment is indeed beneficial for reducing allergy in said subject.

17. A method for determining the treatment needed for an allergic subject comprising performing the method according to any one of claims 1-10 at different time points during the treatment, wherein increased expression levels of the one or more biomarkers points to an allergic state of the subject under observation, indicating the need for anti-allergic treatment.

18. The method of any one of claims 1-10, using a kit according to any one of claims 11-14.

19. The methods and/or kits according to any of the previous claims, wherein the means for determining either biomarker level is selected from the group consisting of: end-point-PCR, real-time-PCR, quantitative-PCR, digital-PCR, northern blot analysis, capable of determining the mRNA level of the biomarkers; or wherein the means for determining either biomarker level is selected from the group consisting of: ELISA, ELISPOT, Luminex's xMAP technology, flow cytometry, nephelometry, turbidimetry, immunoprecipitation, a specific binding assay, an immunodetection assay, or a Mass-spectrometry assay, capable of determining the protein level of the biomarker(s).

Figure 1

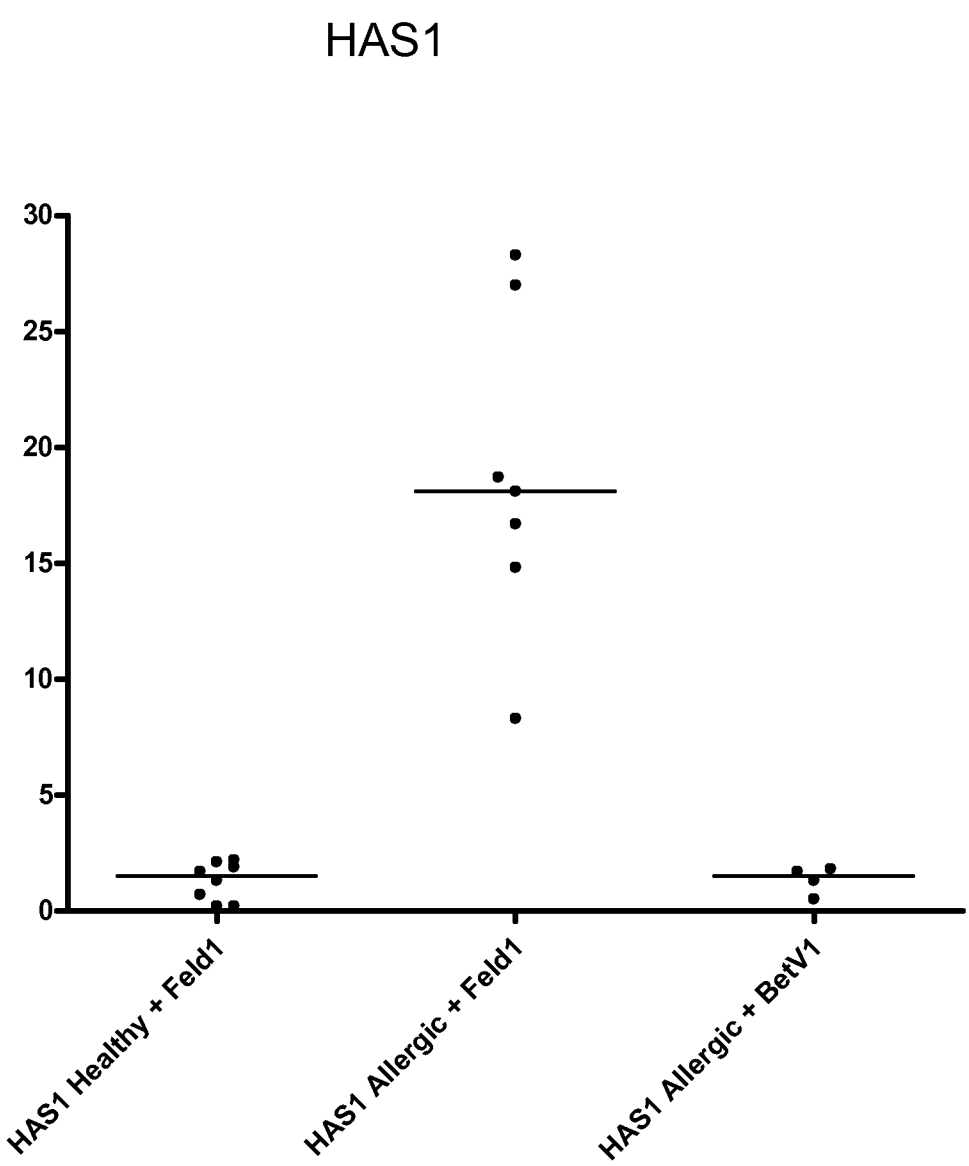

HAS1
30
25
20
15
10
5
0
HAS1 Healthy + Feld1
HAS1 Allergic + Feld1
HAS1 Allergic + BetV1

Figure 2

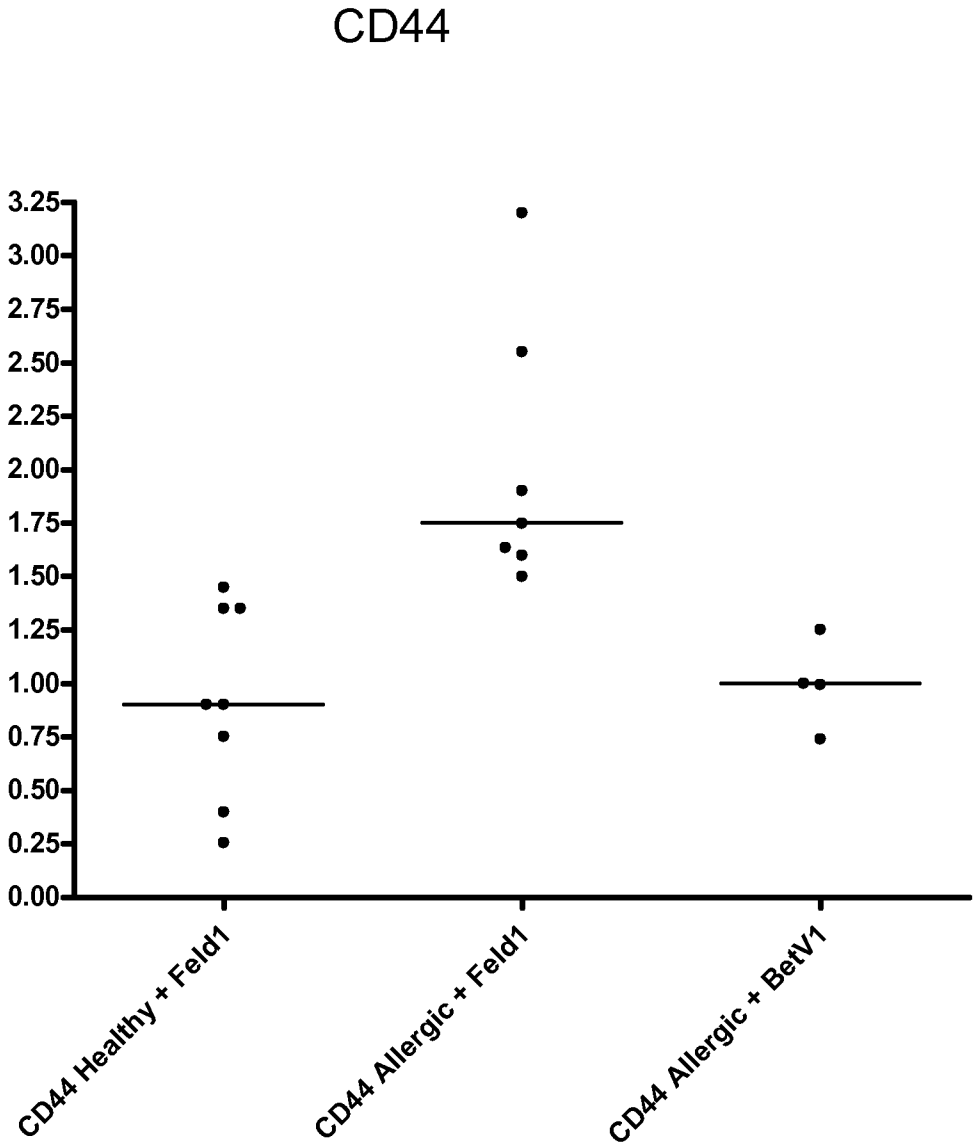

CD44
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
CD44 Healthy + Feld1
CD44 Allergic + Feld1
CD44 Allergic + BetV1

Figure 3

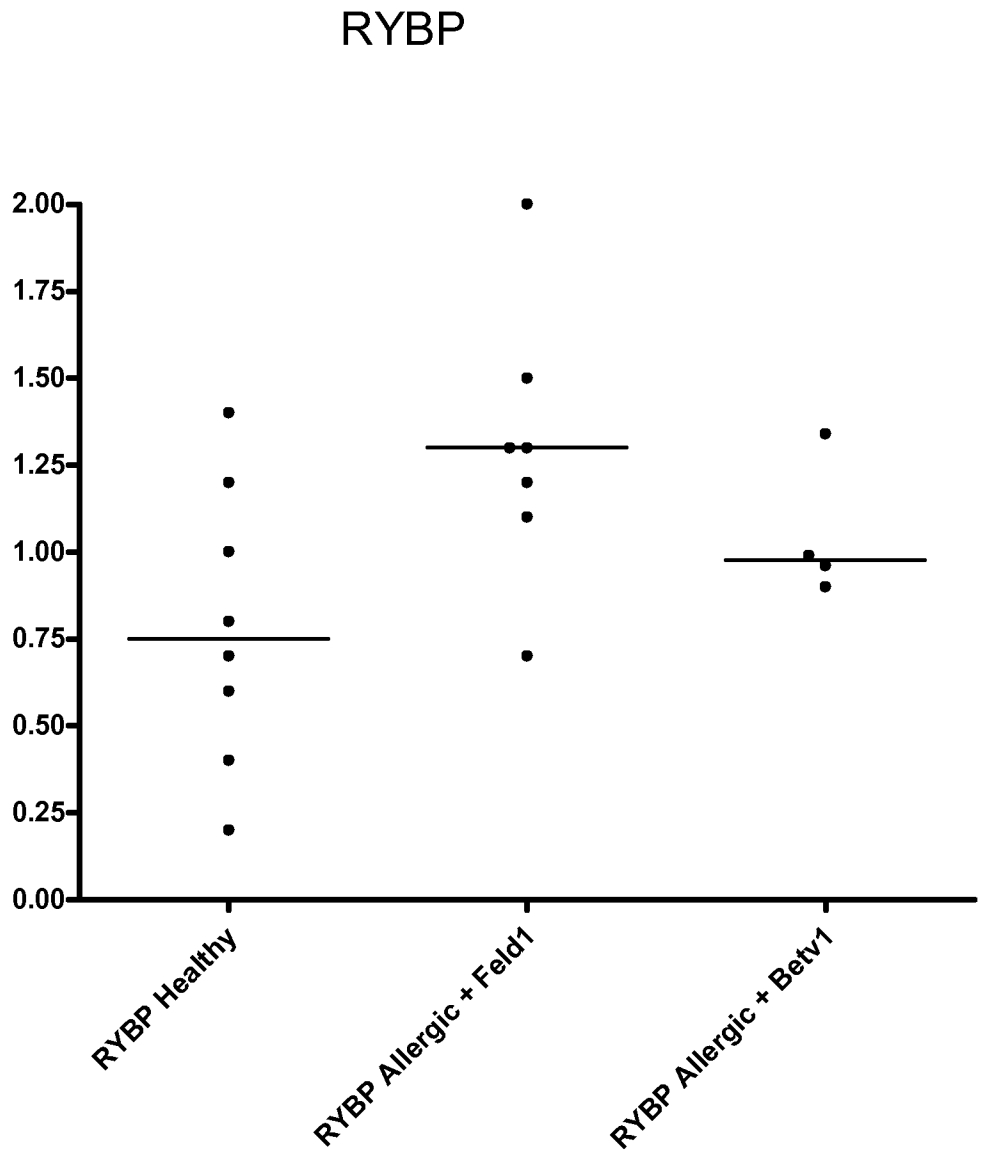
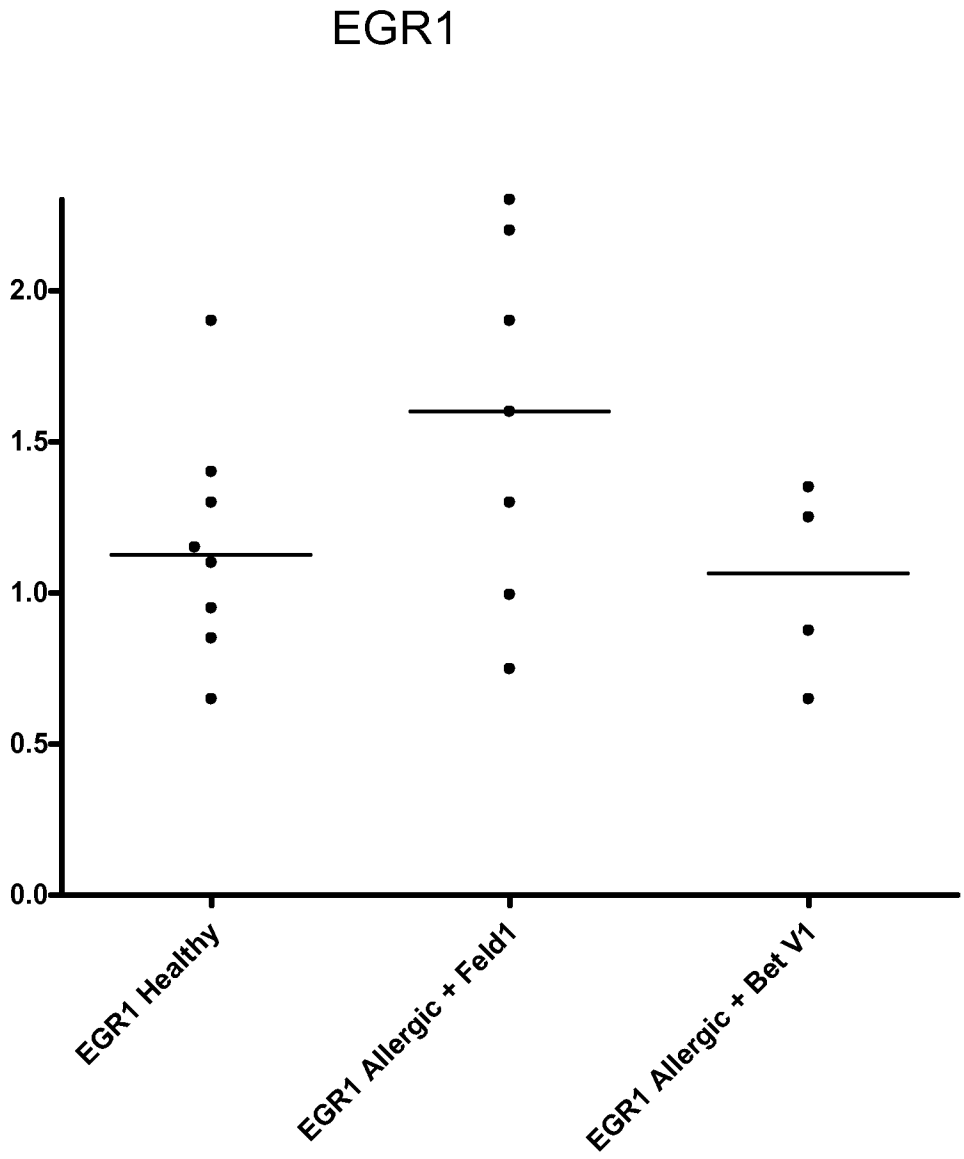

RYBP
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RYBP Healthy
RYBP Allergic + Feld1
RYBP Allergic + Betv1

Figure 4


EGR1
2.0
1.5
1.0
0.5
0.0
EGR1 Healthy
EGR1 Allergic + Feld1
EGR1 Allergic + Bet V1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/066398

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
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)
C12Q

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OCMANT ET AL: "IL-4 and IL-13 mRNA real-time PCR quantification on whole blood to assess allergic response", CYTOKINE, ACADEMIC PRESS LTD, PHILADELPHIA, PA, US, vol. 31, no. 5, 7 September 2005 (2005-09-07), pages 375-381, XP005012558, ISSN: 1043-4666 the whole document	1-19

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier 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)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 December 2010	12/01/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Botz, Jürgen

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OCMANT ANNICK ET AL: "Flow cytometry for basophil activation markers: the measurement of CD203c up-regulation is as reliable as CD63 expression in the diagnosis of cat allergy.", JOURNAL OF IMMUNOLOGICAL METHODS 30 MAR 2007 LNKD- PUBMED:17275019, vol. 320, no. 1-2, 30 March 2007 (2007-03-30), pages 40-48, XP002615390, ISSN: 0022-1759 the whole document	1-19
Y	WO 2007/101306 A1 (TELETHON INST FOR CHILD HEALTH [AU]; HOLT PATRICK [AU]; MCKENNA KATHER) 13 September 2007 (2007-09-13) the whole document	1-19
Y	SCHMIDT-WEBER CARSTEN B: "Gene expression profiling in allergy and asthma", CHEMICAL IMMUNOLOGY AND ALLERGY, KARGER, BASEL, CH, vol. 91, 1 January 2006 (2006-01-01), pages 188-194, XP008130929, ISSN: 1660-2242 the whole document	1-19
Y	EBO D G ET AL: "Basophil activation test by flow cytometry: Present and future applications in allergology", CYTOMETRY, vol. 74B, no. 4, July 2008 (2008-07), pages 201-210, XP002615391, the whole document	1-19
Y	SONNECK K ET AL: "Recombinant allergens promote expression of aminopeptidase-N (CD13) on basophils in allergic patients", INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY, vol. 21, no. 1, January 2008 (2008-01), pages 11-21, XP008130927, ISSN: 0394-6320 the whole document	1-13
Y	WO 2004/082610 A2 (BRIGHAM & WOMENS HOSPITAL [US]; SACKSTEIN ROBERT [US]) 30 September 2004 (2004-09-30) the whole document	1-19

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/066398

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KATOH SHIGEKI ET AL: "Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma", AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, vol. 176, no. 1, July 2007 (2007-07), pages 27-35, XP002615392, ISSN: 1073-449X the whole document	1-19
Y	KIM ET AL: "Hyaluronic acid targets CD44 and inhibits Fc@?RI signaling involving PKCdelta, Racl, ROS, and MAPK to exert anti-allergic effect", MOLECULAR IMMUNOLOGY, PERGAMON, GB, vol. 45, no. 9, 4 March 2008 (2008-03-04), pages 2537-2547, XP022540433, ISSN: 0161-5890, DOI: DOI:10.1016/J.MOLIMM.2008.01.008 the whole document	1-19
Y	SILVERMAN ERIC S ET AL: "The transcription factor early growth-response factor 1 modulates tumor necrosis factor-alpha, immunoglobulin E, and airway responsiveness in mice", AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, vol. 163, no. 3, March 2001 (2001-03), pages 778-785, XP002615393, ISSN: 1073-449X page 2001	1-19
Y	LI BO ET AL: "De novo synthesis of early growth response factor-1 is required for the full responsiveness of mast cells to produce TNF and IL-13 by IgE and antigen stimulation.", BLOOD 1 APR 2006 LNKD- PUBMED:16317093, vol. 107, no. 7, 1 April 2006 (2006-04-01) , pages 2814-2820, XP002615394, ISSN: 0006-4971 the whole document	1-19
Y	GUO J ET AL: "Yin-Yang 1 regulates effector cytokine gene expression and TH2 immune responses", JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, MOSBY, INC, US, vol. 122, no. 1, 1 July 2008 (2008-07-01), pages 195-201.E5, XP022831407, ISSN: 0091-6749, DOI: DOI:10.1016/J.JACI.2008.03.012 [retrieved on 2008-04-18] the whole document	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/066398

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/066398

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007101306 A1	13-09-2007	CA 2644162 A1	13-09-2007
		EP 1994172 A1	26-11-2008
		JP 2009528826 T	13-08-2009
		US 2009156540 A1	18-06-2009
WO 2004082610 A2	30-09-2004	NONE	