



Computational modelling

Development of systems biology-oriented biomarkers by permuted stepwise regression for the monitoring of seasonal allergic rhinitis treatment effects

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ARTICLE INFO

Article history:

Received 11 August 2011

Received in revised form 23 January 2012

Accepted 6 February 2012

Available online 12 February 2012

Keywords:

Biomarkers

Cytokines

Systems biology

Permuted stepwise regression

Seasonal allergic rhinitis

Citrus/Cydonia

ABSTRACT

Background: The immune system, a complex set of integrated responses, often cannot be explained, predicted, or monitored by examining its separate components as biomarkers. Combining different components may therefore be a suitable approach to develop relevant biomarkers reflecting immune system functioning in an appropriate way.

Methods: Here we compute and test pattern variables that should reflect immune system functioning on the systems level. Computation was based on a dataset (from a randomized controlled trial comparing two routes of administration) of allergen-specifically induced expression levels of cytokines (IL-1 β , IL-5, IL-10, IL-12, IL-13, IL-17, IFN- γ and TNF- α) and symptom severity scores from 22 seasonal allergic rhinitis (SAR) patients measured before and after six weeks of treatment with medicinal products containing Citrus and Cydonia. By means of stepwise regression analyses we explored and tested pattern variables of the immunological data using permuted stepwise regression (PStR) to distinguish optimally between (immunological) baseline and post-baseline data for the whole treatment group (22 patients) and the two separate treatment groups (11 patients in each group). The validity of the stepwise selection method for the computed pattern variables was tested by means of random permutation tests and evaluated with the cross-validated correct rate of classification (CV correct).

Results: For the total group a pattern variable was computed with three variables: IL-10 (day 7), TNF- α (day 1) and IL-10 (day 1) (CV correct: 0.91; $p < 0.001$; $R^2 = 0.66$), demonstrating a small improvement from the model with IL-10 (day 7) only (CV correct: 0.84; $p < 0.001$; $R^2 = 0.47$). For the subcutaneous injection group a pattern variable was computed with four variables: IL-10 (day 7), IL-10 (day 1), IL-17 (day 7) and IFN- γ (day 7) (CV correct: 0.90; $p < 0.01$; $R^2 = 0.78$), demonstrating a very small improvement from the model with IL-10 (day 7) only (CV correct: 0.86; $p < 0.01$; $R^2 = 0.58$). For the nasal spray group a pattern variable was computed with three variables: IL-10 (day 7), TNF- α (day 1) and IL-10 (day 1) (CV correct: 0.95; $p < 0.01$; $R^2 = 0.79$), demonstrating a moderate improvement from the model with IL-10 (day 7) only (CV correct: 0.79; $p < 0.05$; $R^2 = 0.37$).

Conclusion/discussion: In this study three robust systems biology-oriented biomarkers for the monitoring of SAR were computed that demonstrated small to moderate improvement compared to monitoring of a single cytokine (IL-10 (day 7)) (CV correct improvement: 0.07 (total group), 0.04 (subcutaneous injection group), 0.16 (nasal spray group)). Further computation and biomarker validation with larger datasets, including data from healthy persons and SAR patients, are indicated.

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1. Introduction

The immune system is increasingly found to participate in the development of several chronic illnesses including allergy, thus motivating the targeting of the immune system to sustain health at all ages and to modulate the risk of certain chronic illnesses. Biomarker examination is a means of monitoring and validating such immunomodulatory approaches (Lassere et al., 2007; Ptolemy and Rifai, 2010). These biomarkers are generally associated with communicatory molecules including cytokines, chemokines, acute-phase proteins, heat-shock proteins and antibodies. Nevertheless, consensus on the appropriate markers to monitor the maintenance of a healthy immune system or the improvement of an imbalanced immune system remains elusive, primarily because biological heterogeneity precludes readily identifiable thresholds for the prevention of chronic disease (Albers et al., 2005; Kant, 2010). However, the use of a constellation of markers, of which cytokines and cytokine receptors would be a major part, represent potential novel avenues of exploration (Jacobs et al., 2005).

Since we expect no single marker to fully reflect immune status, since the analyses of multiple separate biomarkers can be difficult to interpret, and since most physiological systems are characterized by homeostatic control with large interconnectedness between the elements of the system (Gardy et al., 2009), combining analyses of various physiologically related biomarkers may prove suitable and efficient (Kohl et al., 2010; Noble, 2011; Van der Greef et al., 2007; Van Ommen et al., 2009). To achieve this goal, analysis methods are used that are generally based on prevailing immunological hypotheses about the role of environmental factors (including allergens) in maintaining health and preventing disease. Alternatively, one can focus on generated applications that provide selected clusters of biomarkers indicative of immune activity and permit objective and quantitative estimates of the effect of immune interventions and can predict associations with particular health outcomes (Gardy et al., 2009). These (systems biology-orientated) methods usually combine feature selection with repeated sampling to investigate the main relations in the data, like for instance with permuted stepwise regression (PStR) (Andersson et al., 2009, 2011).

In this study we focus on biomarkers for seasonal allergic rhinitis (SAR) or “hay fever”, a type I immediate hypersensitivity reaction mediated by specific IgE antibodies to a seasonal allergen, leading to chronic mucosal inflammation characterized by sneezing, itching, rhinorrhoea, and nasal blockage (Bousquet et al., 2008). An overactive population of allergen-specific Th2-type cells is generally detected not only in the affected organs – the nose, eyes, and upper respiratory system in SAR, but also in the peripheral blood mononuclear cell fraction (PBMC) (Kirmaz et al., 2011). The diverse CD4+ helper T cell populations consist of Th0, Th1, Th2, Th3, Th17, and Th22 cells, as well as various subsets of regulatory T cells (Akdis et al., 2004; Akdis and Akdis, 2009; Bonilla and Oettgen, 2010). These T cell subsets are identified based on the production of signature cytokines after exposure to the specific allergen, such as interferon-gamma (IFN- γ) from Th1 cells, interleukin (IL)-5 and IL-13 from Th2 cells, and IL-10 from regulatory T cells (Treg). The differentiation of Th1 cells is induced after activation of monocytes as antigen-presenting cells, subsequently producing IL-12p70

as the active cytokine. The generation of Th2 cells is dependent upon other environmental factors and various cytokines, including IL-10 itself (Fujimura and Okamoto, 2010; Chaplin, 2010).

Cytokine genes are only expressed after activation and full differentiation of the T cells, and this production is highly variable among humans, partly due to an extensive degree of polymorphism in the cytokine genes (Smith and Humphries, 2009). Therefore, in order to establish the effects of interventions, it is our hypothesis that it is critical to compare sets or patterns of cytokines rather than absolute levels of cytokine production in stimulated, cultured PBMCs with full differentiation of T cell subsets.

Here we demonstrate the development and testing of a set of pattern variables for monitoring intervention effects in SAR patients. At first we explored and tested pattern variables of the immunological data using permuted stepwise regression (PStR) to distinguish optimally between (immunological) baseline and post-baseline data. PStR is a technique for building statistical models automatically, by selecting variables from a pre-defined set of candidate variables and testing the significance of the complete selection procedure with random permutation (Andersson et al., 2009, 2011). Computation was based on a dataset (from a randomized controlled trial comparing two routes of administration) of allergen-stimulated PBMC culture supernatant levels of cytokines (IL-1 β , IL-5, IL-10, IL-12, IL-13, IL-17, IFN- γ , and TNF- α) from 22 seasonal allergic rhinitis (SAR) patients measured before and after six weeks of treatment with either Citrus/Cydonia comp.® (compositum) 1% solution for injection or Gencydo® nasal spray (Baars et al., 2011).

The use of systems biology-orientated pattern variables should improve the separate biomarker approach in monitoring health related changes as a result of the effect of immune interventions. Therefore the following hypotheses were tested in this study:

1. Even in a homogeneous group of SAR patients the individual production of cytokines is highly variable among humans, partly due to an extensive degree of polymorphism in the cytokine genes (Smith and Humphries, 2009). This high variability will result in a broad range of cytokine production measurements and a non-normally distributed dataset.
2. The computed (data-driven) pattern variables demonstrate larger cross-validated correct rate of classification (CV correct) values (Hellgren et al., 2008) than the separate cytokines with regard to the classification of cytokine samples in baseline and post-baseline.
3. The computed pattern variables demonstrate statistically significant baseline to post-baseline changes.

2. Materials and methods

2.1. Dataset

Our dataset derived from a randomized, controlled trial with 22 SAR patients (Baars et al., 2011) that studied the efficacy and safety of Citrus/Cydonia comp.® 1% solution of injection or Gencydo® nasal spray for the treatment of SAR. The two medicinal products are identical in their composition; the objective of the trial was identification of the more

favorable route of administration (subcutaneous injections versus nasal spray). The trial was a national (The Netherlands), stratified (age: 18–40 years or 41–60 years; and radioallergosorbent testing (RAST) scores (Quillen and Feller, 2006) for birch pollen: >2 or <3 ; with a balanced randomization for age and RAST scores), comparative, single-blind laboratory clinical trial with two parallel groups with 11 patients in each group. All eligible patients were recruited from a single center, the Louis Bolk Institute in Driebergen, NL. The first patient was enrolled on May 19, 2009 and the last patient completed the study on August 11, 2009.

2.2. Pollen counts

Pollen counts were acquired on a daily basis for grass pollen and birch pollen from the Leiden University Medical Centre (<http://www.lumc.nl/con/1070/85683/105795/105824/>) during both the wash-out period and the treatment period.

2.3. Patients

Eligible participants were all adults, aged 18 to 60 years, suffering from SAR for at least two years, with a RAST for grass pollen ≥ 2 , and suffering from sneezing, itching nose, and watery nasal discharge, with a severity score of at least two of the three symptoms ≥ 2 (ranging from 0 = not present to 3 = severe) and requiring the use of antihistamines and/or corticosteroids for treatment of SAR symptoms for at least the preceding two years. Exclusion criteria were chronic inflammatory autoimmune diseases, allergy (hypersensitive) to one of the constituents of Citrus/Cydonia comp.® or Gencydo® nasal spray, pharmacological treatment of allergic rhinitis or use of other preparations containing Citrus and/or Cydonia extracts within the two weeks prior to study enrollment, use of cromoglycates in the last month before study onset, pregnancy or lactation, and severe internal or systemic disease. We also excluded patients with concomitant pharmacological treatment for SAR such as antihistamines, corticosteroids, or other preparations and patients who participated in another clinical trial simultaneously or within the four weeks prior to enrollment in this study.

Baseline homogeneity of the treatment groups was accomplished with regard to the following SAR related aspects: RAST scores (grass pollen and birch pollen), worst SAR symptom severity during the previous pollen season (anamnestically), SAR symptom severity scores in the morning and the evening during the wash-out period and onset of interventions (data not shown). Homogeneity of the treatment groups was accomplished with regard to the following SAR non-related aspects: sex, age, height, weight, smoking status, ethnic origin, remaining medical history, prior medication, vital signs and physical examination (Table 1).

2.4. Treatment

After a one-week (for patients that had not been treated for SAR in the week before enrollment) or a two-week (for patients that had been treated for SAR in the week before enrollment) wash-out period, patients were assigned to four strata and then randomized to a six-week treatment period. A randomization list was generated with the Random

Allocation Software Program version 1.0 (Saghaei, Isfahan University of Medical Sciences, Iran) using a random block size of two in order to guarantee a balanced allocation.

Patients received the treatment in accordance with the Summary of Product Characteristics; either Citrus/Cydonia comp. 1% subcutaneous injections (1 mL ampoules, available under the trade name Gencydo® 1%, manufacturer Weleda AG, Schwäbisch Gmünd, Germany) twice per week, or the Gencydo nasal spray (available under the name “Gencydo neusspray” in the Netherlands, manufactured by Weleda AG, Schwäbisch Gmünd, Germany) four times per day (1–2 sprays in each nostril). This strategy resulted in the nasal spray group receiving four times the active dose compared with the injection group. Citrus/Cydonia comp. 1% solution for injection and Gencydo nasal spray are medicinal products that contain identical amounts of lemon juice (*Citrus limon*) and aqueous extract from the fruit of a quince (*Cydonia oblonga*). One milliliter of these preparations contains 8–12 mg *C. limon* juice corresponding to 0.65 mg fruit acid, calculated as citric acid, and 30 mg *C. oblonga* aqueous extract (drug-extraction-rate 1/2.1).

For more than eighty years, Citrus/Cydonia comp. has been prescribed for SAR patients. Clinical experiences (De Bruin and Baars, 2001), three in vitro studies (Baars and Savelkoul, 2008; Gründemann et al., 2010), two cohort studies (Baars and De Bruin, 2005; Rother and Oexle, 2008), and a randomized controlled trial (Baars et al., 2011) support the efficacy and safety of Citrus/Cydonia comp. and Gencydo, respectively, in SAR treatment.

2.5. Efficacy variables

2.5.1. Primary efficacy variables: cytokine analyses

Primary endpoints were SAR-related changes in immunological parameters between the start of the treatment (baseline) and after six weeks of treatment (post-baseline). From each patient 8 mL of peripheral blood were collected; PBMCs were isolated. PBMCs were cultured in Yssel's medium at 37 °C in a humidified atmosphere with 5% CO₂ at a density of 1×10^6 viable cells/mL. Cells were plated out in 48 well plates at a concentration of 1×10^6 cells/mL and cultured at 37 °C. After five hours, in which the cells adapted to the culture conditions, various stimuli or a matching volume of medium were added. Cultures were stimulated polyclonally with 150 ng/mL anti-CD3 plus 100 ng/mL anti-CD28 monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) or cultured in medium only (Jeurink et al., 2008). In addition, we performed allergen-specific stimulation of 10^5 cells/mL in 1 mL cultures with applied pollen extract (Phl p 1 from Timothy grass [*Phleum pratense*]; Biomay Vienna, Austria; 10 µg/mL in medium). The proliferation capacity, cell survival, toxicity, and total production capacity of several cytokines in the culture supernatants of the PBMCs were analyzed at day 1 (IL-1 β , IL-10, IL-12, IL-17, IFN- γ , and TNF- α : demonstrating the reaction of the innate immune system) and at day 7 (IL-5, IL-10, IL-12, IL-13, IL-17, and IFN- γ : demonstrating the reactions of specialized T cell subsets) (Akdis et al., 2004; Chaplin, 2010) essentially according to well-established procedures (Jeurink et al., 2008). Examples of the reasons to choose these specific cytokines to monitor immunological SAR treatment effect (Ozdemir et al.,

Table 1

Baseline characteristics for the two treatment groups.

Variable		Citrus/Cydonia comp. (n = 11)	Gencydo (n = 11)	p-value
Sex: number (percentage)	Male	6 (55%)	2 (18%)	ns
	Female	5 (45%)	9 (82%)	
Age (years) (sd)		37.0 (13.1)	37.6 (10.5)	ns
Height (cm) (sd)		176.3 (9.6)	170.6 (7.4)	ns
Weight (kg) (sd)		69.5 (10.6)	66.4 (8.9)	ns
Smokers: number (percentage)		2 (18%)	1 (9%)	ns
Alcohol consumption: number (percentage)	None	0 (0%)	3 (27.3%)	ns
	Occasionally	10 (91%)	7 (63.6%)	
	Regularly	1 (9%)	1 (9%)	
Ethnic origin: number (percentage)	Caucasian	11 (100%)	9 (82%)	ns
	Asian	0 (0%)	2 (18%)	
Blood pressure at screening (mm Hg) (sd)		119 (24)/73 (14)	104 (16)/72 (9)	ns/ns
Heart rate at screening (beats per minute)		72 (10)	70 (6)	ns
RAST grass pollen		3.8 (1.3)	3.9 (1.1)	ns
RAST birch pollen		1.9 (1.8)	2.3 (2.1)	ns
Usual SAR symptom severity during the pollen season (total score anamnestically) (sd)	Sneezing	1.9 (0.5)	2.3 (0.5)	ns
	Itching nose	1.8 (0.8)	2.1 (0.5)	
	Watery nasal discharge	1.9 (0.9)	2.1 (0.5)	
	Total score	5.6 (1.9)	6.5 (1.2)	
SAR symptom severity scores in the morning during the wash-out period (total score) (sd)		6.6 (4.5)	8.0 (4.6)	ns
SAR symptom severity scores in the evening during the wash-out period (total score) (sd)		6.1 (3.7)	9.7 (5.2)	ns

ns = not significant.

2009; Deraz, 2010) (for more detailed information, see: Baars et al., 2011) are:

1. (Grass pollen stimulated minus medium stimulated) IL-10 and TNF- α at day 1: representing the inflammatory state of the SAR-related immune system
2. (Grass pollen stimulated minus medium stimulated) IL-10 at day 7: representing (regulatory) T cells (Tregs)
3. (Grass pollen stimulated minus medium stimulated) IFN- γ at days 7: representing Th1 activity
4. (Grass pollen stimulated minus medium stimulated) IL-1 β at day 1; IL-5 and IL-13 at day 7: representing Th2 activity.

Cytokine production was detected with the Cytometric Bead Array (CBA, BD Biosciences, San Diego, CA, USA). All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen, San Diego, CA, USA) and the procedure was performed according to the manufacturer's protocol. The detection limits were 1.1 pg/mL for IL-1 β , 2.2 pg/mL for IL-12p70, 0.3 pg/mL for IFN- γ , 0.7 pg/mL for TNF- α , 1.1 pg/mL for IL-5, 0.13 pg/mL for IL-10, 0.6 pg/mL for IL-13, and 0.3 pg/mL for IL-17. Levels below the detection level could not be measured reliably, but still might have exerted a biological effect. Nevertheless for the statistical analyses of the data a real value needed to be entered. For that reason a value of "0" was selected for all production levels below the detection level. The samples were measured on the FACSCanto II, using Flow Cytometry Analysis (FCAP) software (BD Biosciences, San Diego, CA, USA).

2.5.2. Secondary efficacy variables: symptom severity

Secondary efficacy variables were the change in nasal and non-nasal allergic rhinitis symptom severity before treatment start and after each week of treatment. The severity of nasal symptoms (nasal obstruction, itching nose, sneezing, and watery nasal discharge) and non-nasal symptoms (itchy/burning

eyes, watery eyes, redness of eyes, and itching ears/throat) were recorded twice per day (in the mornings and evenings) by the patient. The disease-specific severity questionnaire was provided to the patient as an online questionnaire in Dutch: 0 = no symptom, 1 = mild, 2 = moderate, and 3 = severe. Completion of the online questionnaires by the participants was checked daily. Total symptom scores (TSS) were analyzed during wash-out, and in treatment weeks 1–5. Due to a very low pollen count during week 6, the data from week 6 were excluded from the analyses (for more detailed information see: Baars et al., 2011). Missing values were replaced in two ways, mean week scores and Last Observation Carried Forward (Waladkhani, 2008), and subsequently compared.

2.6. Statistical analysis

2.6.1. Software

Statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, USA) and with Matlab version 7.11.0 (R2010b, The Mathworks, Natick, MA, USA).

2.6.2. Statistical analyses of the primary efficacy variables: cytokine analyses

To test the effectiveness of Citrus/Cydonia comp. 1% solution for injection as compared to Gencydo nasal spray with respect to the primary target variables, descriptive statistics, Student's *t*-tests, and non-parametric Wilcoxon Signed Ranks tests were performed to compare means and mean base-10 log transformed scores of the immunological parameters in both groups.

In order to demonstrate the high variability of the cytokine production levels in homogenous groups means, ranges and skewness (Kolmogorov–Smirnov Test) of all cytokines

were analyzed. An asymptotic significance <0.05 was regarded as a non-normal distribution.

2.6.3. Statistical analyses of the secondary efficacy variables: symptom severity

Mean scores and standard deviations were calculated for each week. Then, Student's *t*-tests, non-parametric Wilcoxon Signed Ranks tests, and multivariate analysis techniques were used to compare the symptom severity mean week scores in the subcutaneous and the nasal spray group for each of the five weeks.

2.6.4. Computation of pattern variables by means of permuted stepwise regression (PStR)

To identify potential pattern variable biomarkers of interest, immunological samples from all 22 SAR patients were analyzed at baseline and after 6 weeks of treatment. In addition, baseline to post-baseline changes were separately analyzed for the two treatment groups (Gencydo nasal spray and Citrus/Cydonia comp. 1% solution for injection).

The permuted stepwise regression procedure as described in Andersson et al. (2011) was applied on immunological data. Stepwise regressions with five different methods (forward stepwise selection (StepwiseForwardP) and stepwise selection (StepwiseP) using fine tuning with lower *p*-values to select variables for inclusion in the model (Draper and Smith, 1998), least absolute shrinkage and selection operator (Lasso) (Efron et al., 2004; Tibshirani, 1996), a rescaled version of Lasso (ConeLasso) and orthogonal matching pursuit (OMP) (Mallat and Zhang, 1993)) were computed to find subsets of cytokines that permitted the classification of cytokine samples in baseline and post-baseline. Sets of potential regression models were generated using a minimum of one and a maximum of nine cytokines. Final selection of reliable subsets of explanatory cytokines was based on random permutation tests with permuted subjects and repeated measures permuted within subjects. Random permutation tests were performed on the complete stepwise regression procedure including the selection of subsets of cytokines based on the highest R^2 . For each maximum number of cytokines, we tested for 9999 permutations whether the permuted R^2 was equal to or above 90% of the calculated true R^2 . In other words, we assessed if there was a significant gap (10%) between the calculated true R^2 and the distribution of all permuted R^2 s. Random permutation tests resulting in a *p*-value below 0.05 were considered significant. The cross-validated correct rate of classification, CV correct, was computed with 5-fold cross-validation averaged over 20 replications.

3. Theory

3.1. Computation of systems biology-orientated biomarkers

Systems biology aims at a more holistic understanding of biology (Chong and Ray, 2002), a systems-level understanding. This objective requires an examination of the structure and dynamics of function in cells and organisms, rather than the characterization of isolated parts of a cell or organism (Kitano, 2002).

In order to develop systems biology-oriented biomarkers capable of monitoring health and disease-specific changes at the systems level, one can compute pattern variables that

are either (more) data-driven or (more) hypothesis-driven. Computation of data-driven pattern variables investigates the relationships among all measured immunological components of the studied disease. Computation of hypothesis-driven pattern variables is based on investigating the relationships among a subset of previously, theoretically identified and relevant immunological components of the studied disease.

4. Results

4.1. Raw immunological data set of a homogeneous group of SAR patients demonstrating a broad range of cytokine production and a non-normal distribution

The hypothesis tested here was that even in a homogeneous group of SAR patients (see Section 2.3) the individual production of cytokines is highly variable among humans, partly due to an extensive degree of polymorphism in the cytokine genes as a potential, but likely, explanation. This high variability will result in a broad range of cytokine production measurements and a non-normally distributed dataset. Tables 2 and 3 show the baseline and post-baseline cytokine production levels of the patients included in the SAR study. The tested hypothesis was confirmed, since more than 56% (5/9) of the cytokine data were (IL-1, IL-10, IL-13, IL-17, IFN- γ) not normally distributed, and the broad range of all cytokine measurements in both treatment groups demonstrated the large variations from subject to subject. The non-normal distribution of the dataset implied the necessity of base-10 log transformation of all data.

4.2. The computation of pattern variables using PStR that would demonstrate larger CV correct values than the separate cytokines and statistically significant baseline to post-baseline changes

It was hypothesized that we were able to compute (data-driven) pattern variables that would demonstrate larger CV correct values than the separate cytokines with regard to the classification of cytokine samples in baseline and post-baseline. In addition it was hypothesized that these pattern variables would demonstrate statistically significant baseline to post-baseline changes. After base log 10 transformation of all cytokine data, we performed a permuted stepwise regression (PStR) on all cytokines for the total group, the Gencydo group and the Citrus/Cydonia group with all five different regression approaches. Only the statistically significant results are presented here.

For the total group a pattern variable was computed with three variables: IL-10 (day 7), TNF- α (day 1) and IL-10 (day 1) (CV correct: 0.91; $p < 0.001$; $R^2 = 0.66$) (Table 4, 4th row: N of variables = 3), demonstrating a small improvement compared to the model with IL-10 (day 7) only (CV correct: 0.84; $p < 0.001$; $R^2 = 0.47$) (Table 4, 1st row: N of variables = 1).

For the subcutaneous injection group a pattern variable was computed with four variables: IL-10 (day 7), IL-10 (day 1), IL-17 (day 7) and IFN- γ (day 7) (CV correct: 0.90; $p < 0.01$; $R^2 = 0.78$) (Table 5, 4th row: N of variables = 4), demonstrating a very small improvement compared to the model with IL-10 (day 7) only (CV correct: 0.86; $p < 0.01$;

Table 2

Baseline and post-baseline measurements of cytokine production levels of separate cytokines demonstrating broad ranges and non-normal distributions of data (Gencydo group).

Variable	Baseline			Post-baseline		
	Mean (SD)	Range	Skewness (asympt. sig.)	Mean (SD)	Range	Skewness (asympt. sig.)
Day 1: innate immunity						
IL-1 ^a	42 (106.5)	1–362	ns	70.7 (181.0)	2–615	0.018*
IL-10	79.4 (51.4)	11–187	ns	62.8 (41.5)	12–136	ns
IL-12	0.2 (0.5)	0–2	n.a.	0	0	0.011*
IL-17	0.4 (1.0)	0–3	0.004**	0.2 (0.8)	0–3	0.013*
IFN- γ	1.5 (2.4)	0–7	0.018*	2.8 (4.9)	0–15	ns
TNF- α	43.4 (46.9)	8.1–170.6	ns	48.1 (49.8)	3.8–156.3	ns
Day 7: specific T cell subsets						
IL-10	314.8 (250.7)	61.0–890.2	ns	172.3 (133.3)	54.4–500.9	ns
IL-12	0.6 (0.9)	0–2.5	ns	1.3 (1.7)	0–5.3	ns
IFN- γ	2010.4 (2361.8)	112.3–6578.3	ns	1089.1 (1231.8)	52.8–3345.6	ns
IL-5	301.7 (257.3)	12.7–881.2	ns	205.4 (126.8)	43.0–450.3	ns
IL-13	271.6 (281.9)	32.3–912.2	0.044*	176.3 (180.8)	30.7–656.7	ns
IL-17	168.4 (150.0)	4.4–483.7	ns	200.7 (265.6)	3.5–785.5	ns

SD = standard deviation.

SE = Standard error of skewness.

n.a. = Not applicable.

^a All cytokine measurements are in pg/mL.

* Kolmogorov–Smirnov asympt. sig. < 0.05.

** Kolmogorov–Smirnov asympt. sig. < 0.01.

$R^2 = 0.58$) (Table 5, 1st row: N of variables = 1). For the nasal spray group a pattern variable was computed with three variables: IL-10 (day 7), TNF- α (day 1) and IL-10 (day 1) (CV correct: 0.95; $p < 0.01$; $R^2 = 0.79$) (Table 6, 3rd row: N of variables = 3), demonstrating a moderate improvement compared to the model with IL-10 (day 7) only (CV correct: 0.79; $p < 0.05$; $R^2 = 0.37$) (Table 6, 1st row: N of variables = 1). The random permutation tests for the three pattern variables resulted in p-values of 0.0002 (total group), 0.0085 (Citrus/Cydonia comp.) and 0.0018 (Gencydo), demonstrating valid, robust prediction models with significant differences between baseline and post-baseline.

Fig. 1 demonstrates the importance of TNF- α (day 1) in addition to IL-10 (day 7) for the classification of cytokine samples in baseline and post-baseline, for the Gencydo group but not for the Citrus/Cydonia comp. group. For both groups there is a significant difference between baseline (0) and post-baseline (1) for IL-10 (day 7) (horizontal level of Fig. 1). For TNF- α (vertical level of Fig. 1) this was only the case for the Gencydo group.

The tested second and third hypotheses were confirmed, since we were able to compute three robust statistically significant pattern variables that demonstrated larger CV correct values than the separate cytokines with regard to the

Table 3

Baseline and post-baseline measurements of cytokine production levels of separate cytokines demonstrating broad ranges and non-normal distributions of data (Citrus/Cydonia comp. group).

Variable	Baseline			Post-baseline		
	Mean (SD)	Range	Skewness (asympt. sig.)	Mean (SD)	Range	Skewness (asympt. sig.)
Day 1: innate immunity						
IL-1 ^a	6.5 (3.0)	2–12	ns	146.9 (319.9)	2–1079	0.019*
IL-10	113.1 (80.9)	16–252	ns	122.2 (90.3)	34–330	ns
IL-12	0	0	0.01*	0.2 (0.4)	0–1	ns
IL-17	0.3 (1.0)	0–3	n.a.	0	0	0.004**
IFN- γ	1.0 (2.7)	0–9	0.025*	6.4 (19.8)	0–66	ns
TNF- α	51.9 (38.9)	15.7–136.2	ns	28.0 (33.4)	4.2–122.2	ns
Day 7: specific T cell subsets						
IL-10	252.3 (291.1)	34.5–1066.8	ns	297.1 (275.6)	82.6–1024.5	ns
IL-12	1.2 (1.2)	0–2.7	ns	1.1 (1.2)	0–3.7	ns
IFN- γ	2428.4 (2458.4)	124.2–7371.9	ns	1722.3 (1206.9)	635.7–5076.9	ns
IL-5	455.4 (518.4)	39.4–1747.4	ns	329.3 (219.0)	75.0–797.9	ns
IL-13	706.9 (1598.8)	16.2–5458.9	ns	412.6 (573.6)	34.7–1934.0	ns
IL-17	61.6 (65.7)	2.7–206.5	ns	152.6 (144.7)	12.4–433.3	ns

SD = standard deviation.

SE = Standard error of skewness.

^a All cytokine measurements are in pg/mL.

* Kolmogorov–Smirnov asympt. sig. < 0.05.

** Kolmogorov–Smirnov asympt. sig. < 0.01.

Table 4

The computation of an optimal pattern variable model by means of a permuted stepwise regression (PStR) on all cytokines for the total group.

N	R ²	P-value	Method	CV correct	N of var.	Variables in the model				Regression weights of variables			Intercept
44	0.47	0.0002	CL	0.84	1	IL10d7				−0.85			2.21
44	0.58	0.0002	StFP	0.88	2	IL10d7	IL10d1			−1.07	0.53		1.67
44	0.56	0.0003	L	0.87	2	IL10d7	TNFD1			−0.89	−0.37		2.83
44	0.66	0.0002	OMP	0.91	3	IL10d7	TNFD1	IL10d1		−1.10	−0.37	0.52	2.29

CL = ConeLasso.

L = Lasso.

StFP = Stepwise Forward Procedure.

OMP = Orthogonal Matching Pursuit.

classification of cytokine samples in baseline and post-baseline. The computed pattern variables demonstrated also statistically significant baseline to post-baseline changes.

4.3. Summary of the results of the secondary efficacy variables symptom severity and safety

Both routes of administration demonstrate a statistically significant reduction in SAR symptom severity, with larger effects of the subcutaneous route of administration. In the Citrus/Cydonia group a statistically significant SAR symptom reduction was measured already after one week and two weeks of treatment, in the morning and the evening, respectively. In the Gencydo group a statistically significant SAR symptom reduction was measured already after two weeks of treatment in the evening. TSS reduction of the subcutaneous route of administration was larger in the morning, but not in the evening. Cohen's delta effect sizes were larger for the subcutaneous route of administration (= large) than the nasal spray route of administration (= medium), both in the morning and the evening. During the treatment period, a total of 9 adverse events (AEs) were observed with none of the AEs classified as serious. Also the in vitro analyses demonstrated acceptable cell survival, with no signs of toxicity. The overall conclusion of the safety analysis in this study is that both routes of administration of Gencydo and Citrus/Cydonia comp. are safe for use by SAR patients (for more detailed information about the secondary efficacy variables, see: Baars et al., 2011).

5. Discussion

In this study at first we confirmed the problem of high inter-individual variability of cytokine production among humans for the use of separate cytokines as biomarkers. Even in this study with a controlled, homogeneous group of SAR patients, the individual production of cytokines was highly variable (five of nine cytokines data demonstrated

skewness, Tables 2 and 3). This wide individual biological variability in cytokine production levels may arise from factors such as circadian rhythm, sex, age, and smoking status (de Roos & McArdle, 2008; Duramad et al., 2004). Alternatively, the observed inter-individual variability might be partly due to an extensive degree of polymorphism in the cytokine (receptor) genes as a potential, but likely, explanation (Kubistova et al., 2006). Therefore three robust, systems biology-oriented, pattern variable biomarkers for the monitoring of SAR were computed, that demonstrated small to moderate improvement in the classification of cytokine samples in baseline and post-baseline compared to the monitoring of the separate cytokine (IL-10 (day 7)) (CV correct improvement: 0.07 (total group), 0.04 (Citrus/Cydonia group), and 0.16 (Gencydo group)). This study therefore provides a new and solid strategy in the field of immunology for the development of objectively quantifiable, high-quality biomarkers that are relevant to the analysis of potential activity of new therapeutics for the treatment of allergic diseases. The procedure that is successfully used in other new scientific areas like for example proteomics (Kim et al., 2010), is expected to be widely applicable in immune-mediated diseases.

Currently, in immunological research practice, multiple cytokines in particular are often measured in relation to immune responses under diseased conditions or in response to treatments. However, the interpretation of these observed data is most often subjective and based on the relative importance of some, but not all, different cytokines. This obscures the validity of the observed immune responses and compromises the comparison of different studies in literature. The presented approach in this study provides an improvement for this situation by offering an objectively handling of the *complete* cytokine response and making the data available for objective and standardized analysis in patients.

The presented approach is also an improvement for statistical procedures that analyze several predictive variables at

Table 5

The computation of an optimal pattern variable model by means of a permuted stepwise regression (PStR) on all cytokines for the Citrus/Cydonia group.

N	R ²	P-value	Method	CV correct	N of var.	Variables in the model				Regression weights of variables				Intercept
22	0.58	0.0064	L	0.86	1	IL10d7				−0.86				2.23
22	0.66	0.0083	L	0.85	2	IL10d7	IL10d1			−1.05	0.50			1.76
22	0.74	0.0063	L	0.87	2	IL10d7	IL10d1	IL17d7		−1.22	0.60	0.23		1.49
22	0.78	0.0085	L	0.9	3	IL10d7	IL10d1	IL17d7	IFN-Gd7	−1.22	0.76	0.26	−0.21	1.75

L = Lasso.

Table 6

The computation of an optimal pattern variable model by means of a permuted stepwise regression (PStR) on all cytokines for the Gencydo group.

N	R ²	P-value	Method	CV correct	N of var.	Variables in the model			Regression weights of variables			Intercept
22	0.37	0.0133	StFP	0.79	1	IL10d7			−0.85			2.19
22	0.59	0.0012	StFP	0.86	2	IL10d7	TNfd1		−0.85	−0.60		3.06
22	0.79	0.0018	StFP	0.95	3	IL10d7	TNfd1	IL10d1	−1.21	−0.64	0.80	2.26

CL = ConeLasso.

L = Lasso.

StFP = Stepwise Forward Procedure.

OMP = Orthogonal Matching Pursuit.

the same time and by doing that, provide more information than by analyzing separate variables. Like in proteomics, genomics, metabolomics, etcetera, extracting useful multivariate relations from measured data is not so straightforward and an ongoing learning process. We think that this relative new methodology, combining stepwise regressions with permutation tests, can offer valuable insights in immunological multivariate relations. With respect to the stepwise regressions relationships among multiple variables predicting a response can be modeled additive in a linear or multiplicative way. The resulting regression weights define the optimal balance between the predictive variables for obtaining the most accurate prediction. Imagine an example with two predictors where the difference between the variables or the product of the variables (the interaction) is related to the response. The specific additive or multiplicative relation can be found by applying respectively linear or loglinear regression with the two predictors. One can intuitively understand that the relations found in regression models can compensate for the high variability in the predictors by realizing that the predicted values are proportional to the weighted mean of the predictors with weights fixed to the

regression weights. As long as there is no overfitting the weighted mean will have less variability than the separate predictors. In the methods presented here the permutation tests monitor the robustness of the stepwise regressions and prevent overfitting.

The results provide also relevant information for the comparison of the immunological effects of the nasal (Gencydo) and the subcutaneous (Citrus/Cydonia comp.) route of administration of these effective treatments for hay fever. For both groups the most important effects come from IL-10 (day 7), which reflect modulation of differentiated Treg activity. However, for the nasal spray route of administration in addition TNF- α (day 1) and IL-10 (day 1) contribute to the treatment effect (CV correct improvement of 0.16). The production level of monocyte-derived TNF- α and IL-10 reflects the local chronic SAR-related inflammatory activity between baseline and post-baseline. In addition, the observed kinetics at day 7 after allergen-specific stimulation, reflects the activation state of the immune system due to the activity of monocytes, which are the largest producers of IL-10 in the PBMC, and induced already by day 1 after allergen exposure. Subsequently, also the gradual and delayed induction of regulatory T cell subset (Treg) by day 7 will be inhibited as these cells use the IL-10 as a selective autocrine growth factor (Akdis et al., 2004; Akdis and Akdis, 2009; Kant, 2010). The observed kinetics can be interpreted as a decrease in the activation state of the immune system due to a decrease in the activity of monocytes and a reduction of the chronic inflammation (TNF- α (day 1)). This additional effect can be attributed to the acute local effect of the nasal spray route (for more detailed information, see: Baars et al., 2011).

There are two major theoretical arguments for an increasing focus on a pattern-oriented or a systems biology approach for the development of valid immune system biomarkers. First, focusing on the pattern of relationships overcomes the large inter-individual variations in cytokine production. Since these relationships, other than the calculated mean scores of separate immunological components, are independent of the absolute magnitude of the component's production, individual changes can be compared more precisely. Second, studying the systems-level changes in the relationships among separate immunological components accounts for the observation that changes in the production of one immunological component can lead to compensatory changes in others (Atkinson et al., 2001).

The systems biology-oriented approach for developing biomarkers may also innovate outcome studies in SAR treatment (Kitano, 2002). Until now, in designing SAR outcome studies, clinical symptoms have been regarded as primary

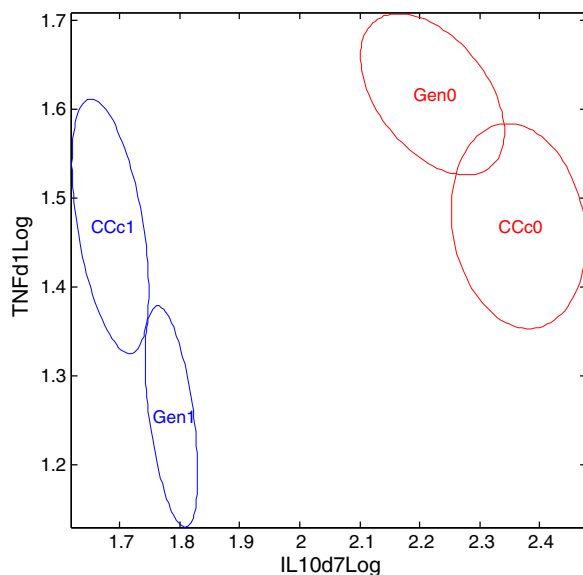


Fig. 1. The additional effect of TNF- α for the Gencydo group but not for the Citrus/Cydonia comp. group with regard to the classification of cytokine samples in baseline and post-baseline as compared to the effect of IL-10 for both groups; CCc = Citrus/Cydonia comp.; Gen = Gencydo; 0 = baseline; 1 = post-baseline.

efficacy parameters, and immunological parameters are relegated to a secondary status (European Medicines Agency, EMA, 2004); indeed, measurements of separate immunological parameters are often unreliable. The systems biology-oriented pattern variables proposed in this study offer a first step toward reliable biomarkers of immune activity that could be used in SAR outcome research. Similar approaches could result in the development of reliable immune system biomarkers for use in different studies and different diseases. Additionally, our results support a holistic, systems-biology view of the immune system in terms of practical application.

The major limitations of this study are the small group sizes (11 patients in each group) and the absence of data from healthy persons and from categories of SAR patients with varying symptom severities. The small group sizes may have resulted in imprecise computation of the pattern variables, since larger samples provide more precise calculations. The absence of data from healthy persons and categories of SAR patients prevented study in a more controlled setting the differences between health and (levels of) disease. We expect that computation with a larger and better dataset will provide larger differences between results based on computing pattern variables and results based on analyzing only separate variables. The presented study must therefore be regarded as a first study exploring the possible contribution of permuted stepwise regression approaches to the development of pattern variables that reflect immunological activity on the system level, with promising results.

Future (replication) studies on the applicability of this PStR procedure to SAR are indicated with larger populations, with categories of SAR patients with varying symptom severities, and with a control group of healthy persons. These study design changes will allow the computation of pattern variables that are able to distinguish among disease severities and between disease and health more precisely. This PStR procedure may also be applicable to other immune-mediated diseases; datasets describing rich cytokine sets will determine the critical parameters that can be combined in the various pattern variables. In addition, a future literature study should demonstrate the contribution of the here presented method, its advantages and disadvantages, to the use of multivariate analytical approaches in immunology by positioning it within the approaches that have been used in the identification of immunological biomarkers in recent years.

Acknowledgments

We would like to thank the Weleda AG for financial support and Elslien Kingma for constructive comments on the manuscript.

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