



Altered Fc galactosylation in IgG₄ is a potential serum marker for chronic lung disease

Tina Heyder^{1,2,6}, Emil Wiklundh^{1,6}, Anders Eklund¹, Anna James^{3,4}, Sven-Erik Dahlén^{3,4} on behalf of the BIOAIR study group⁵, Johan Grunewald¹, Roman A. Zubarev^{2,7} and Susanna L. Lundström^{2,7} 

Affiliations: ¹Respiratory Medicine Unit, Dept of Medicine Solna and Centre for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden. ²Division of Physiological Chemistry I, Dept of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden. ³Centre for Allergy Research, Karolinska Institute, Stockholm, Sweden. ⁴Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. ⁵For a list of the BIOAIR study group members and their affiliations see the Acknowledgements section. ⁶Joint first authors. ⁷Joint last authors.

Correspondence: Susanna L. Lundström, Dept of Medical Biochemistry and Biophysics, Chemistry I, Solnavägen 9, 171 77 Stockholm, Sweden. E-mail: susanna.lundstrom@ki.se

ABSTRACT Characterising chronic lung diseases is challenging. New, less invasive diagnostics are needed to decipher disease pathologies and subphenotypes. Fc galactosylation is known to affect IgG function, and is altered in autoimmune disorders and under other pathological conditions. We tested how well Fc glycans in IgG from bronchoalveolar lavage fluid (BALF) and serum correlated, and if the Fc glycan profile could reveal pulmonary inflammation.

A shotgun proteomics approach was used to profile Fc glycans in serum and BALF of controls (n=12) and sarcoidosis phenotypes (Löfgren's syndrome (LS), n=11; and non-LS, n=12). Results were further validated in severe asthma (SA) (n=20) and published rheumatoid arthritis (RA) patient data (n=13) including clinical information.

Intra-individually, Fc-galactosylation status of IgG₁ ($R^2=0.87$) and IgG₄ ($R^2=0.95$) correlated well between matrixes. Following GlycoAge-index correction, the ratio between agalactosylated and digalactosylated Fc glycans of IgG₄ could distinguish sarcoidosis and SA from healthy and RA subjects with a mean±SE area under the curve (AUC) of 78±6%. The AUC increased to 83±6% using the more chronic lung disease types (non-LS and SA) and most strikingly, to 87±6% for the SA subgroup.

The results indicate that the Fc galactosylation status of IgG₄ is a potential blood test marker for chronic lung inflammation.



@ERSpublications

IgG₄ Fc galactosylation correlates between serum and BALF ($R^2=0.95$) and is a potential blood marker for chronic lung inflammation <http://ow.ly/XaNd30k35wg>

Cite this article as: Heyder T, Wiklundh E, Eklund A, *et al.* Altered Fc galactosylation in IgG₄ is a potential serum marker for chronic lung disease. *ERJ Open Res* 2018; 4: 00033-2018 [<https://doi.org/10.1183/23120541.00033-2018>].



Introduction

Chronic inflammatory lung diseases such as sarcoidosis and asthma are characterised by altered airway function and continuous inflammation. Assessing disease activity and predicting prognosis is pivotal to decide on adequate treatment. Reliable noninvasive markers to target ongoing inflammation in the lungs are needed. We tested whether isotype-specific IgG Fc glycosylation (figure 1) could be used as such a marker. Fc glycans of IgG have strong impact on interactions with Fc receptors, activation of complement cascade and effector functions [2, 3]. Furthermore, the IgG Fc glycosylation profile is known to be skewed in autoimmune and inflammatory disorders compared to controls [4–6]. The more complex the Fc glycan (particularly if the glycan is substituted with galactose and sialic acid), the less likely it is that the IgG has proinflammatory effects [3, 7–9]. For this reason, the amount of galactosylated and/or sialylated Fc glycans (or the ratio between agalactosylated and galactosylated glycans) has been suggested as a marker for inflammation and/or immune system activation [10].

Given that IgGs are circulating in the body, we expected intra-individual similarities in the Fc glycan profile of paired bronchoalveolar lavage fluid (BALF) and serum samples. Furthermore, we hypothesised that specific features within the serum Fc glycan profile could serve as markers for inflammation in the lung, and/or, ideally, for disease-specific activity. To the authors' knowledge, no prior studies have described how well the Fc glycosylation patterns in serum and in BALF IgG correlate intra-individually or how similar or different Fc glycan profiles of different IgG isotypes are in inflammatory lung disease compared to controls and to other chronic inflammatory disorders. Thus, this study investigates an unexplored area of lung inflammation and may shed light on disease aetiology.

To test how well serum and BALF Fc glycans correlate intra-individually, we used matched samples from healthy controls and sarcoidosis patients suffering from Löfgren's syndrome (LS) or non-LS patients [11]. Sarcoidosis presents as a systemic inflammatory disease characterised by non-caseating granulomas and predominately involving lung parenchyma and intra-thoracic lymph nodes. Of the sarcoidosis phenotypes, LS has an acute disease onset, usually with a favourable outcome compared to non-LS. A favourable disease course in LS is associated with human leukocyte antigen (HLA) type HLA-DRB1*03 [12].

For validation, results were confirmed in another type of complex pulmonary disease (severe asthma (SA)) and compared to published data from another type of chronic inflammatory disorder sometimes affecting

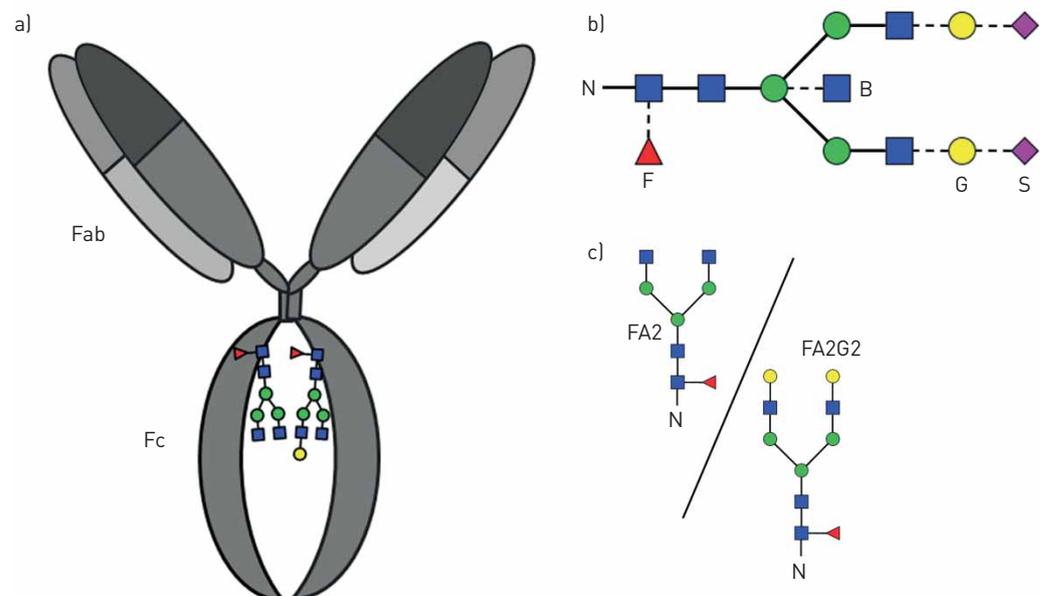


FIGURE 1 a) Schematic of the IgG molecule and location of the Fc glycans. b) Glycan structures with nomenclature according to ROYLE *et al.* [1]. The core oligosaccharide structure comprises a biantennary heptasaccharide moiety (A2). Usually, the first sugar unit (an *N*-acetylglucosamine [GlcNAc]) is additionally core fucosylated (e.g. FA2). The biantennary structure can also be bisected by an additional GlcNAc (FA2B). Furthermore, the outer glucosamine units can be elongated with galactoses (FA2G_n, where *n*=1 or 2) and the galactoses can be further extended with sialic acids (FA2G_nS_n, where *n*=1 or 2). Blue squares: GlcNAc; red triangles: fucose (F); green circles: mannose; yellow circles: galactose (G); purple diamonds: sialic acid (S). c) Galactosylation status, *i.e.* the ratio between the main agalactosylated form (FA2) and the main digalactosylated form (FA2G2). B: bisected.

the lungs (rheumatoid arthritis (RA)) [13]. Asthma is characterised by airway hyperreactivity and activation of smooth muscle cells. In contrast to sarcoidosis, the inflammatory processes in asthma are located to the intima rather than to the interstitium, and while sarcoidosis has both Th1 and Th17 immune responses, asthma is preliminarily considered as a Th2 disease. However, in SA, Th17 responses can also be present [14].

Material and methods

Patients

Biobanked material from cohort studies of sarcoidosis and asthma were used. RA patient information and Fc glycan data were acquired from previously published data accessible online [13]. The respective substudies of sarcoidosis (2005/1031–31/2) and asthma (KS 01–329) had obtained written informed consent for investigations approved by the regional ethical review board. Clinical characteristics of subjects (n=68) are described in table 1 and supplementary table 1.

Sarcoidosis patients and controls

Sarcoidosis patients (LS, n=11; non-LS, n=12) and healthy controls (n=12) underwent bronchoscopy with BAL as previously described [15]. LS and non-LS were age matched (LS, 42±7 years; non-LS, 43±9 years) while controls were younger (27±3 years). Sarcoidosis patients were lavaged within 3 months of referral to the lung clinic (*i.e.* from time of suspected sarcoidosis due to symptoms compatible with the disease such as fatigue, fever, cough, effort dyspnoea and weight reduction). LS was defined by acute onset, usually with fever, chest radiographic findings with bilateral hilar lymphadenopathy, sometimes with pulmonary infiltrates, and with erythema nodosum and/or bilateral ankle arthritis. Patients were diagnosed as defined by the World Association of Sarcoidosis and Other Granulomatous Disorders, the American Thoracic Society and the European Respiratory Society [16]. Non-LS patients were diagnosed by pulmonary specialists by positive lung biopsies, chest radiography, BALF cell counts and HLA typing. Controls had no signs of infection for 4 weeks prior to bronchoalveolar lavage, and had normal dynamic spirometry and blood screen results. Subjects were nonsmoking males of northern European decent. One LS patient was sampled twice with 1 year between sampling dates. The median value of these two occasions was used.

SA patients: validation cohort

SA patients (n=20) were recruited as part of BIOAIR, a European multicentre study [17]. Subjects' (19 females and one male) mean age was 49±11 years. Diagnosis was confirmed by pulmonary specialists according to published criteria [18]. Patients had minimum of 1 year of specialist treatment and had experienced exacerbations (one or more) in the past year. Exacerbations were defined by initiation of oral corticosteroid (OCS) therapy from regular inhaled corticosteroid treatment, or for those on OCS therapy, a significant increase in their dose of OCS for an acute deterioration in their disease control. For details, see the supplementary material.

TABLE 1 Patient characteristics

Information	H	RA [#]	LS	Non-LS	SA
BALF/serum/plasma	12/12/0	0/8/6	12 ^f /12/0	12/12/0	0/20/0
Age years	27±3	55±16	42±7	43±9	49±11
Never-smokers/ex-smokers	12/0	NA	8/3	9/3	17/3
Males/females	12/0	7/6	12/0	12/0	1/19
Radiographic stage I/II/III/IV [¶]	NA	NA	5/6/0/0	3/9/0/0	NA
FVC [*] % of predicted	114±11	NA	84±16	90±14	102±23
FEV ₁ % of predicted	107±9	NA	86±16	86±15	82±20
FEV ₁ /FVC [§]	79±7	NA	78±7	70±6	69±9

Data are presented as n or mean±sd. For additional information, see supplementary table 1. H: healthy controls; RA: rheumatoid arthritis; LS: Löfgren's syndrome; SA: severe asthma; BALF: bronchoalveolar lavage fluid; FVC: forced vital capacity; FEV₁: forced expiratory volume in 1 s; NA: not available. [#]: information obtained from published data [13]. [¶]: stage I indicates granuloma formation in the hilar lymph nodes; stage II also has, in addition to the hilar lymphadenopathy, granuloma formation in the lung shown as diffuse infiltrates on the radiograph; stage III has parenchymal infiltrates on the radiograph but absence of hilar adenopathy; stage IV indicates irreversible pulmonary scarring. ^{*}: LS group is missing one value. [§]: LS group is missing one value; non-LS group is missing three values. ^f: LS group contains two different BALF samples from the same patient.

RA data: positive control cohort

IgG₁ and IgG₂ Fc galactosylation is low in RA, particularly for anti-citrullinated protein antibody (ACPA)-positive patients [13, 19]. Thus, as positive controls, previously published data on IgG Fc glycans enriched from serum/plasma from ACPA-positive RA patients (n=13, six females; 55±16 years) [13] were used. Eight patients were sampled following short (0.5–4 years) and six patients following long (24–47 years) symptom duration. Patients were receiving healthcare and treatment according to clinical guidelines and practice. For details, see the supplementary material.

Melon Gel IgG enrichment

Serum (40 µL per sample) and BALF (500 µL per sample) of patients and controls were aliquoted in triplicate. Polyclonal IgGs were enriched using the Melon Gel IgG Spin Purification Kit (Thermo Fisher Scientific, Gothenburg, Sweden) [20]. For details, see the supplementary material.

IgG sample trypsin digestion

Samples were treated similarly to what has previously been described [20]. For serum, 10 µg of IgG per sample and for BALF, total IgG content per sample <10 µg was digested. For details, see the supplementary material.

Liquid chromatography–tandem mass spectrometry analysis

~1 µg of digest per sample was analysed using an EASY-nLC system connected to a Fusion Orbitrap mass spectrometer (both Thermo Fisher Scientific). For details, see the supplementary material.

Fc glycopeptide identification and quantification

As previously described [13, 21], 63 glycopeptide variants of glycans N-linked to tryptic peptides EEQYNSTYR and TKPREEQYNSTYR (IgG₁), EEQFNSTFR and TKPREEQFNSTFR (IgG₂ or IgG₃), and EEQFNSTYR and TKPREEQFNSTYR (IgG₄) were screened (supplementary table 2). Note that IgG₃ also occurs as EEQYNSTFR, but less frequently in Caucasians [13, 22, 23]. Glycopeptide ion abundances were integrated over respective chromatographic peaks of monoisotopic ions (<10 ppm deviation from theoretical mass value) and within a ±2-min interval around expected retention times. Glycan abundances were normalised to total content of Fc glycosylated IgG₁ or IgG_{2/(3)} and IgG₄ peptides.

Statistical analysis

Univariate analyses were performed using Student's t-test (with equal or unequal variance depending on F-test). p-values were false discovery rate (FDR) corrected (n=506). Receiver operating characteristic (ROC) curve and correlation analyses (linear regression and two-tailed Pearson or Spearman regression tests) were performed using Prism version 5.02 for Windows (GraphPad Software, La Jolla, CA, USA). Multivariate modelling using principal component analysis (PCA) and orthogonal projections to latent structures discrimination analysis (OPLS-DA) was performed using SIMCA 14.0 (Umetrics, Umeå, Sweden) following mean centring, log transformation and unit variance scaling. For details, see the supplementary material.

Results**BALF and serum Fc glycan profiles**

21 Fc glycans from IgG₁, 20 from IgG_{2/(3)} and 18 from IgG₄ were quantified (supplementary table 3). Multivariate PCA was performed to investigate intra-individual correlation of BALF and serum subject profiles (R²=0.46, Q²=0.35, two components) (figure 2). From the plot, it is evident that the main variations in the Fc glycan profiles are attributed to two factors: 1) matrix (*i.e.* BALF versus serum) and 2) intra-individual differences (according to cohort and/or age). To more accurately identify which Fc glycans contributed to the separation between 1) matrixes and 2) cohorts, OPLS-DA models were constructed.

BALF and serum differences

A robust OPLS-DA model (R²=0.90, Q²=0.85, p=1.33×10⁻²⁶ by cross-validated (CV)-ANOVA) was obtained when distinguishing BALF and serum Fc glycan profiles. It is noteworthy that the majority of glycans (n=31) correlated with 95% confidence with serum compared to BALF (n=5) (supplementary table 4). This might indicate that serum IgGs are more heterogeneous in their Fc glycan composition than BALF IgGs. However, the dissimilarities could also be due to differences in IgG enrichment procedures. For details, see the supplementary material.

Differences between healthy controls, LS and non-LS

One OPLS-DA predictive axis was obtained when investigating differences between patient subgroups and controls (R²=0.28, Q²=0.24, p=4.4×10⁻¹²). Along the predictive-scores axis, controls (mean±SD

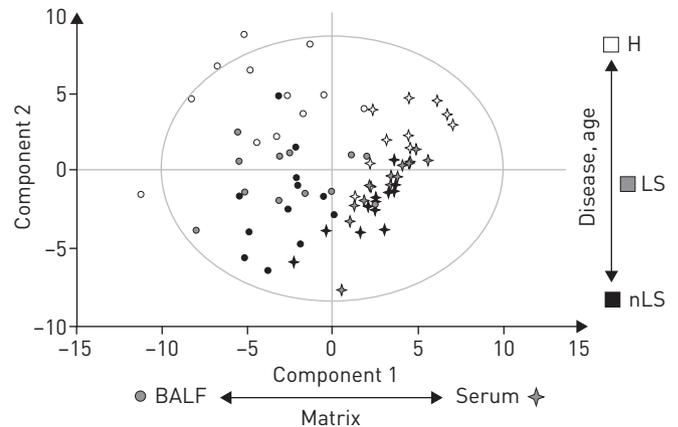


FIGURE 2 Principal component analysis scores plot of the Fc glycan profiles measured in serum and bronchoalveolar lavage fluid (BALF) samples of the healthy controls (H) and sarcoidosis patients. Two general trends are observed in the plot. First, the Fc glycan serum and BALF profiles separate along component 1, and second, the H, Löfgren's syndrome (LS) and non-LS (nLS) patients separate along component 2. The separation along the first component can be attributed to a higher abundance of low-abundance glycoforms in serum and the separation along the second component can be attributed to a lower abundance of galactosylated species in the sarcoidosis patients.

tcv[1]=2.9±2.4) and non-LS patients (tcv[1]=−2.1±2.1) separated most prominently with LS patients placed in between (tcv[1]=−0.9±2.1). This indicates that Fc glycan profiles of non-LS patients are more different from controls compared to LS. Nine galactosylated glycans correlated with controls with 95% confidence (supplementary table 5). It is noteworthy that the top three of these were the same digalactosylated (FA2G2) form (from the respective IgG isotype) and glycans ranked four to six were the monosialylated counterpart of FA2G2 (*i.e.* FA2G2S1). In contrast, a majority (13 out of 16) of glycans correlating with sarcoidosis patients were truncated forms without galactose and sialic acid. For details, see supplementary table 5. Note that differences in galactosylation between patients and controls are a combination of age and disease [10, 24]. To investigate how much of the differences are attributed to disease, an age normalisation was performed using the GlycoAge index (see later) [24].

Intra-individual similarities between Fc glycan BALF and serum

Both uni- and multivariate data indicated that the ratio between galactosylated and agalactosylated Fc glycans is significantly skewed in sarcoidosis compared to controls. By using the logarithmically transformed ratio between the main agalactosylated form FA2 and FA2G2, we tested how well $\log(\text{FA2}/\text{FA2G2})$ would correlate intra-individually between BALF and serum (figure 3). Particularly, $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ ($R^2_{\text{all}}=0.95$, $R^2_{\text{healthy}}=0.83$, $R^2_{\text{non-LS}}=0.90$ and $R^2_{\text{LS}}=0.96$) and IgG₁ ($R^2_{\text{all}}=0.87$, $R^2_{\text{healthy}}=0.93$, $R^2_{\text{non-LS}}=0.79$ and $R^2_{\text{LS}}=0.68$) correlated well between matrices. For IgG_{2/(3)}}, correlation was slightly weaker ($R^2_{\text{all}}=0.76$, $R^2_{\text{healthy}}=0.80$, $R^2_{\text{non-LS}}=0.69$ and $R^2_{\text{LS}}=0.70$).

Age correction of the galactosylation status

Due to a limited amount of BALF samples, our subgroups were age skewed (non-LS, 43±9 years; LS, 42±7 years; H, 27±3 years). Hence, we tested whether $\log(\text{FA2}/\text{FA2G2})$ would remain significant following age normalisation. Since the change in galactosylation according to age is not linear, we used age normalisation based on the GlycoAge index [24]. This expected GlycoAge index is based on changes in $\log(\text{FA2}/\text{FA2G2})$ according to age in a large, predominantly healthy population. Thus, for normalisation, we used $\log([\text{FA2}/\text{FA2G2}]_{\text{measured}}/[\text{FA2}/\text{FA2G2}]_{\text{expected}})$ with expected values based on a GlycoAge index curve (from 2298 individuals) [24] (supplementary table 6). In supplementary table 7, $\log(\text{FA2}/\text{FA2G2})$, age-normalised $\log(\text{FA2}/\text{FA2G2})$ and FDR-corrected p-values are given for sarcoidosis patients and controls. The majority of measured $\log(\text{FA2}/\text{FA2G2})$ values are significant between controls and patients. Following GlycoAge index correction, $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ remain significant both in serum ($p=1.6\times 10^{-2}$) and in BALF ($p=1.1\times 10^{-2}$). Thus, low abundance of galactosylated IgG₄ Fc-glycans in sarcoidosis is an effect caused not only by age but also by inflammation or immune regulation.

Age-corrected $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ is a potential blood marker for chronic inflammatory lung disease

To evaluate how $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ in sarcoidosis compares to other lung disorders and chronic inflammatory disease, we further analysed IgG-enriched serum samples from SA patients and compared

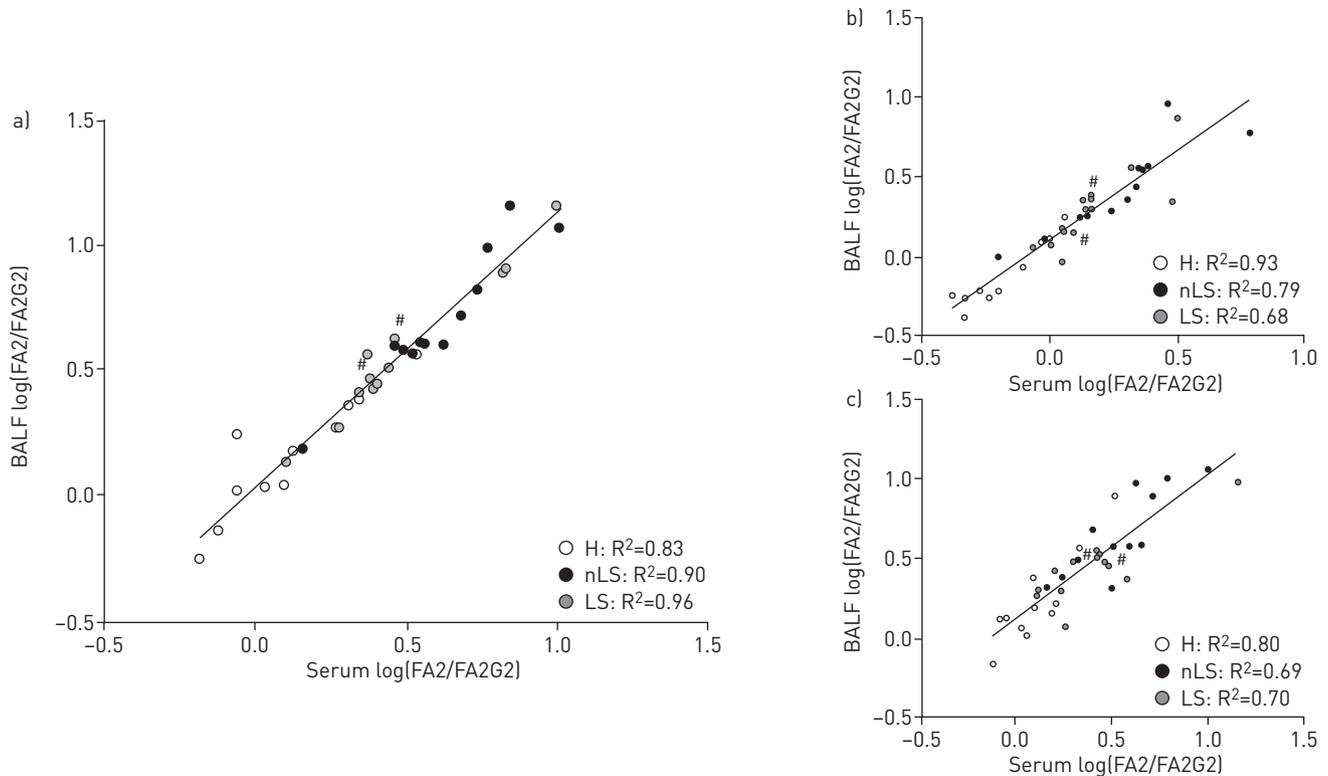


FIGURE 3 Intra-individual correlation between the logarithmically transformed ratio between the main agalactosylated form [FA2] and the main digalactosylated form [FA2G2] in serum *versus* bronchoalveolar lavage fluid (BALF) in a) IgG₄ (R²=0.95), b) IgG₁ (R²=0.87) and c) IgG_{2/3} (R²=0.76). H: healthy controls; LS: Löfgren's syndrome; nLS: non-LS. #: for the correlation analysis we used both intra-individual data time-points for the LS patient that was sampled twice with a year between the sampling dates.

the results with published data from RA patients [13]. The results clearly indicated that age-corrected IgG₄ log(FA2/FA2G2) is not sarcoidosis specific. A majority of SA patients had abnormally high log(FA2/FA2G2) IgG₄ values (0.73±32) (table 2 and figure 4). RA patients' log(FA2/FA2G2) IgG₄ values (0.37±26), were generally lower than, particularly, SA and non-LS patients' (0.52±0.22) (table 2 and figure 4). ROC curve analysis of log(FA2/FA2G2) IgG₄ values generated a mean±SE AUC of 78±6% when comparing individuals with lung disorder (LS, non-LS and SA) to negative and positive controls (*i.e.* healthy controls and RA, respectively). The AUC went up to 83±6% when comparing individuals with severe chronic lung disorders (non-LS and SA) to healthy controls and RA (table 2 and figure 4c). As expected, the AUCs went up using controls only (85±6% and 90±5%, respectively) and down compared to RA patients only (72±7% and 77±7%, respectively) (table 2 and figure 4c). From these results (figure 4a), we suggest that age-corrected log(FA2/FA2G2) of IgG₄ can be divided into low- (<0.3), medium- (0.3–0.6) and high-range (≥0.6) values. Particularly high-range values suggest high inflammatory lung activity (figure 4a and b).

Correlation analysis of log(FA2/FA2G2) with other clinical factors

The log(FA2/FA2G2) of IgG₁, IgG_{2/3} and IgG₄ were correlated with other available information listed in table 1 and in supplementary table 1. For SA, periostin (p=0.04, R²=0.35) correlated positively with log(FA2/FA2G2) of IgG₄. Chitinase 3-like protein (CHI3L) did reach significance using Pearson correlation (p=0.02, R²=0.15) but the data were not normally distributed (Spearman correlation, p=0.20, R²=0.22). Periostin, RAGE, galectin-3 and sputum eosinophils correlated positively with log(FA2/FA2G2) of IgG_{2/3}, and sputum macrophages correlated negatively with IgG₁ and IgG_{2/3} log(FA2/FA2G2). For more details, see supplementary figure 1.

For non-LS patients, the best correlation between log(FA2/FA2G2) and chest radiographic stage at time of blood sampling and diagnosed disease was for IgG_{2/3} (R²=0.53). This correlation increased at follow-up 2–4 years later (R²=0.63, p=0.04), where patients ranged from radiographic stage I to IV.

Discussion

We showed that Fc galactosylation status of IgG in serum and BALF are well correlated and following age correction, remain significantly skewed (particularly for IgG₄) in patients with chronic lung disorders

TABLE 2 Summary of the age-normalised galactosylation status (logarithmically transformed ratio between the main agalactosylated form (FA2) and the main digalactosylated form (FA2G2)) for each subgroup as well as false discovery rate-corrected p-values (n=506) and area under the curve (AUC) values when comparing the individuals with and without lung disease

	IgG ₄	IgG ₁	IgG _{2/3}
log(FA2/FA2G2) mean±sd			
H	0.22±0.24	-0.08±0.19	0.19±0.21
RA [#]	0.37±0.26	0.18±0.26	0.33±0.21
LS	0.42±0.33	0.10±0.24	0.31±0.35
Non-LS	0.52±0.22	0.17±0.28	0.44±0.26
SA	0.73±0.32	0.41±0.35	0.73±0.31
p-value			
<i>H versus</i>			
LS+non-LS+SA	3.6×10 ⁻⁴	5.3×10 ⁻⁴	1.1×10 ⁻³
Non-LS+SA	2.7×10 ⁻⁴	2.8×10 ⁻⁴	9.8×10 ⁻⁴
LS	1.3×10 ⁻¹	1.4×10 ⁻¹	4.7×10 ⁻¹
Non-LS	6.2×10 ⁻³	5.3×10 ⁻²	5.6×10 ⁻²
SA	2.7×10 ⁻⁴	2.6×10 ⁻⁴	2.6×10 ⁻⁴
<i>H+RA versus</i>			
LS+non-LS+SA	9.2×10 ⁻⁴	2.9×10 ⁻²	1.2×10 ⁻³
Non-LS+SA	2.6×10 ⁻⁴	9.2×10 ⁻³	2.6×10 ⁻⁴
LS	3.2×10 ⁻¹	7.1×10 ⁻¹	7.0×10 ⁻¹
Non-LS	3.1×10 ⁻²	3.6×10 ⁻¹	1.0×10 ⁻¹
SA	2.0×10 ⁻⁴	1.9×10 ⁻³	5.9×10 ⁻⁵
<i>RA versus</i>			
LS+non-LS+SA	7.9×10 ⁻²	5.2×10 ⁻¹	1.2×10 ⁻¹
Non-LS+SA	2.2×10 ⁻²	3.3×10 ⁻¹	2.0×10 ⁻²
LS	7.5×10 ⁻¹	5.6×10 ⁻¹	9.1×10 ⁻¹
Non-LS	2.6×10 ⁻¹	9.6×10 ⁻¹	4.2×10 ⁻¹
SA	1.0×10 ⁻²	1.2×10 ⁻¹	2.4×10 ⁻³
AUC mean±se			
<i>H versus</i>			
LS+non-LS+SA	85±6%	82±6%	80±6%
Non-LS+SA	90±5%	85±6%	88±5%
LS	70±11%	70±11%	58±12%
Non-LS	87±7%	80±9%	78±10%
SA	91±5%	88±6%	94±4%
<i>H+RA versus</i>			
LS+non-LS+SA	78±6%	70±6%	75±6%
Non-LS+SA	83±6%	73±7%	83±5%
LS	60±10%	58±10%	51±11%
Non-LS	76±8%	62±10%	70±9%
SA	87±6%	80±7%	90±5%
<i>RA versus</i>			
LS+non-LS+SA	72±7%	59±9%	69±7%
Non-LS+SA	77±7%	62±9%	78±7%
LS	50±12%	53±12%	59±12%
Non-LS	67±11%	55±12%	62±12%
SA	83±7%	73±9%	87±6%

For the Löfgren's syndrome (LS) patient measured twice (1 year in between sampling dates), the mean was used as data point. H: healthy controls; RA: rheumatoid arthritis; SA: severe asthma. [#]: information obtained from published data [13].

compared to controls and RA patients (for whom particularly the IgG₁ Fc glycans are less galactosylated [5, 8, 13]). Interestingly, the Fc galactosylation status of the SA validation cohort differed the most from both healthy controls and the positive RA controls (table 2). The SA patients required continuous treatment with high doses of inhaled steroids. The effect of corticosteroids on protein glycosylation has been associated with more complex (sialylated and galactosylated) forms [25–27]. However, follow-up/increased doses with OCS indicated no significant changes in Fc galactosylation status for the patients. Thus, the low amounts of galactosylated (and sialylated) epitopes in SA is not due to treatment and

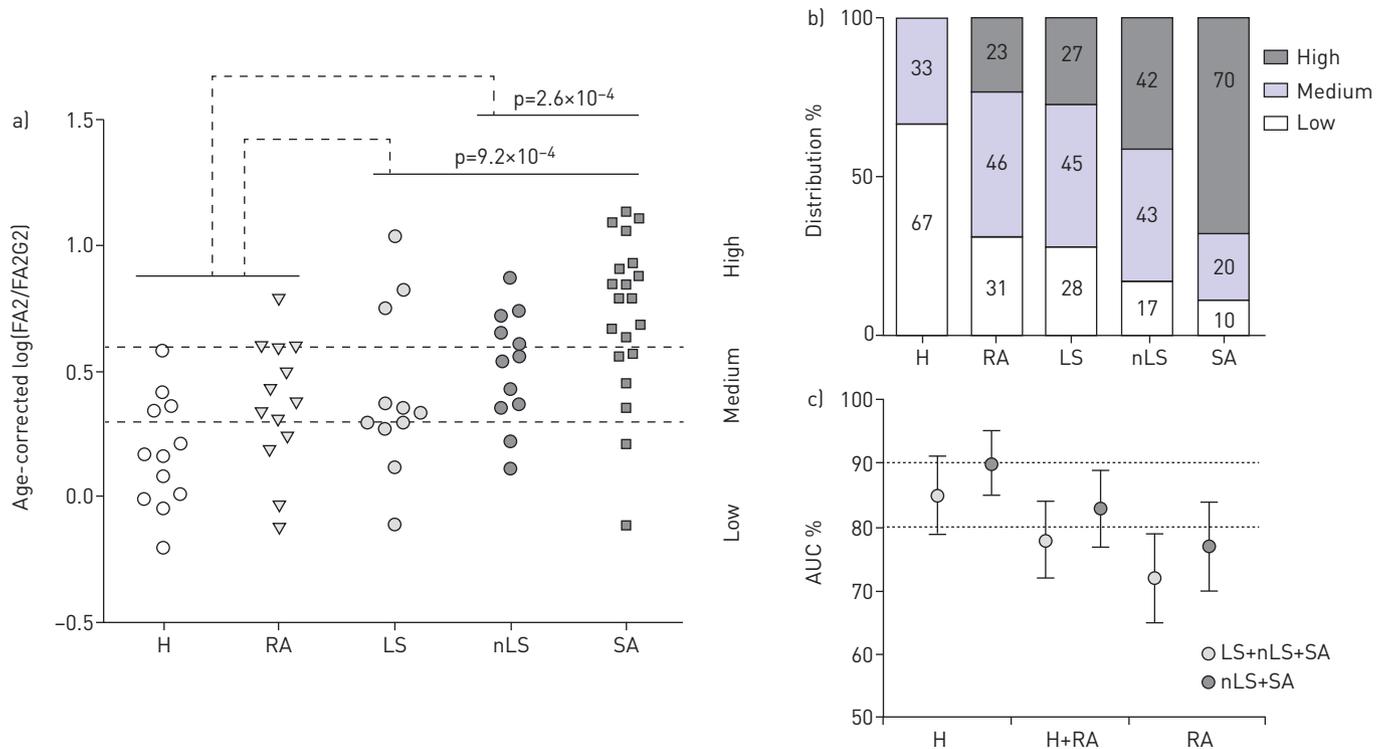


FIGURE 4 The selectivity of the age-corrected serum galactosylation status (logarithmically transformed ratio between the main agalactosylated form [FA2] and the main digalactosylated form [FA2G2]) of IgG₄ as a potential marker for chronic lung disease. **a)** Age-corrected serum galactosylation status of IgG₄ for healthy controls (H) and patients suffering from rheumatoid arthritis (RA), Löfgren's syndrome (LS), non-LS (nLS) sarcoidosis and severe asthma (SA). Low- (≤ 0.3), medium- (0.3–0.6) and high-range values (≥ 0.6) are indicated. **b)** The distribution of the different groups according to low, medium and high range values. **c)** Area under the curve (AUC) values from receiver operating characteristic curve analyses obtained by comparing the individuals with and without lung disorder. The error bars represent the standard error.

potentially are even an indicator of a lack of response to treatment. Even though sarcoidosis and asthma are clinically vastly different diseases, both are inflammatory disorders that yield symptoms primarily in the airways and lungs. To facilitate diagnosis, a marker that reveals inflammatory airway activity and is easily accessible through blood sampling would be of clinical significance.

The data show distinct intra-individual similarities in Fc galactosylation status of BALF and serum. This was particularly evident for the galactosylation status of IgG₁ and IgG₄ (figure 3). The $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ was remarkably well correlating for non-LS ($R^2=0.90$) and LS patients ($R^2=0.96$) (figure 3). It is possible that other IgG isoform variants (and subsequently their corresponding Fc glycans) are more affected by immune status in different microenvironments, while IgG₄ remains more unchanged. Alternatively, IgG₄ has a specific function and is expressed at higher levels in the lungs of sarcoidosis patients, which is reflected in circulating blood. For example, control $\log(\text{FA2}/\text{FA2G2})$ had a weaker correlation for IgG₄ ($R^2=0.83$) compared to IgG₁ ($R^2=0.93$).

Strikingly, age-corrected $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ distinguished chronic lung inflammatory disorder (particularly non-LS and SA) from controls and RA. Our results indicate that IgG₄ $\log(\text{FA2}/\text{FA2G2})$ can be divided into low-, medium- and high-range values (figure 4b), with high values significantly associated with inflammatory lung disorder. For example, eight out of nine sarcoidosis patients with high values were diagnosed with chest radiographic stage II. The $\log(\text{FA2}/\text{FA2G2})$ of IgG₄ of the SA patients was also intra-individually significantly correlated with periostin and weakly correlated with CHI3L. Periostin is a potential diagnostic target in asthma [28], and CHI3L has been suggested as an airway inflammation and remodelling marker in both SA and pulmonary sarcoidosis [17, 29, 30]. Interestingly, CHI3L is produced by Th17 cells [31], which can be active both in sarcoidosis and SA. Furthermore, Th17 cells have been shown to regulate IgG sialylation in antibody-producing plasma cells [32]. Compared to other IgG isotypes, IgG₄ is only weakly interacting with Fc γ receptors [33], and linked to anti-inflammatory processes *via* Fab-arm exchange [34] and clearance [33, 35]. It is likely that the decrease in galactosylated Fc glycans of IgG₄ in sarcoidosis and SA is linked or a response to lung remodelling. However, if the reduction in IgG₄ galactosylated Fc glycans result in an active pro-inflammatory effect similar to what would be expected from IgG₁ [3, 7–9, 36] needs to be explored further.

Note that Fc glycopeptides derived from IgG₃ (EEQFNSTFR) in a non-European population (*i.e.* different from the individuals in this study) will frequently occur as EEQYNSTFR. This is important since EEQYNSTFR glycopeptides have identical molecular masses and similar liquid chromatography retention times to IgG₄ Fc glycopeptides (EEQFNSTYR) [13, 22, 23]. Prospectively, to avoid interference of IgG₃ Fc glycans, these should be removed prior to analysis (*via* protein A column enrichment *etc.*). Additionally, the pilot cohorts in this study, which initially was not designed as one cohesive study group, are skewed according to sex (table 1). Prospectively, the results need to be validated in larger patient cohorts. In terms of sex, RA males and females were not different according to log(FA2/FA2G2), and the male SA patient was in the high range (≥ 0.6) of log(FA2/FA2G2) for IgG₄ (supplementary table 6).

Even though the galactosylation status of IgG₄ had the best intra-individual correlation between blood and lung, and was the factor that best distinguished chronic lung disease from RA and controls, the other IgG isotypes' log(FA2/FA2G2) should not be neglected. Particularly, log(FA2/FA2G2) of IgG_{2/(3)} correlated positively not only with periostin but also with other factors (sputum eosinophils, RAGE and galectin-3) that have been linked to asthma [17, 37, 38]. Both log(FA2/FA2G2) of IgG₁ and IgG_{2/(3)} also correlated negatively with sputum macrophages. Furthermore, and as shown in table 2, despite the abnormally high log(FA2/FA2G2) of IgG₄ for SA, the log(FA2/FA2G2) of IgG_{2/(3)} compared to the other subgroups was more SA specific. Additionally, for non-LS, log(FA2/FA2G2) of IgG_{2/(3)} correlated better with chest radiographic stage at time of diagnosis and follow-up than log(FA2/FA2G2) of IgG₁ and IgG₄.

In summary, we have identified a candidate marker for chronic inflammatory airway activity that correlates well between BALF and serum ($R^2=0.95$), and is easily accessible through blood sampling. This marker has potential use in clinical applications (*e.g.* in diagnosis and prognosis), and may improve the specificity and accuracy of evaluating lung disease status. Furthermore, our data, not only for log(FA2/FA2G2) of IgG₄, but also for the other IgG isotypes, show correlation with clinically relevant factors such as periostin (asthma) and follow up chest radiograph stage (non-LS). Thus, it is of importance to target all IgG isotypes to elucidate similarities and differences in detail, and investigate how Fc glycans affect underlying lung disease pathologies.

Acknowledgements: We thank the research nurses Helene Blomqvist, Margitha Dahl and Gunnel de Forest, as well as biomedical analyst Benita Dahlberg for sample collection and preparation (all Respiratory Medicine Unit, Dept of Medicine Solna and Centre for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden). We also thank the volunteers for making the study possible.

Author contributions: J. Grunewald, R.A. Zubarev, T. Heyder, E. Wiklundh and S.L. Lundström designed the study; E. Wiklundh characterised the sarcoid, and A. James and S-E. Dahlén the asthma subjects; A. Eklund performed bronchoscopies and generated clinical data; J. Grunewald, S-E. Dahlén and R.A. Zubarev provided financial support and essential infrastructure; T. Heyder, E. Wiklundh and S.L. Lundström performed IgG purifications; S.L. Lundström performed mass spectrometric analysis; T. Heyder, E. Wiklundh, A. James and S.L. Lundström performed data analyses; and T. Heyder, E. Wiklundh and S.L. Lundström interpreted the data and drafted the manuscript. All authors read, edited and commented on the manuscript.

BIOAIR study group: Mina Gaga (University of Athens, Athens, Greece), Nikos M. Sifakas (University of Crete, Heraklion, Greece), Alberto Papi (University of Ferrara, Ferrara, Italy), Leonardo M. Fabbri (University of Modena, Modena, Italy), Guy Joos (University of Ghent, Ghent, Belgium), Klaus F. Rabe (The Christian Albrechts University, Kiel, Germany), Peter Sterk (Amsterdam Medical Centre, Amsterdam, The Netherlands), Elisabeth H. Bel (Amsterdam Medical Centre, Amsterdam, The Netherlands), Sebastian L. Johnston (The Imperial College of Science and Technology, London, UK), Pascal Chanez (University of Marseilles, Marseilles, France), Mark Gjomarkaj (Italian Research Council, Palermo, Italy), Peter H. Howarth (University of Southampton, Southampton, UK), Ewa Nizankowska-Mogilnicka (The Jagellonian University, Krakow, Poland) and Roelinde Middelveld (Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden).

Conflict of interest: S-E. Dahlén reports receiving personal fees from AstraZeneca, GlaxoSmithKline, Novartis, Merck, RSPR Pharma, Regeneron and Teva for service on scientific advisory boards, outside the submitted work.

Support statement: This study was supported by The Swedish Heart Lung Foundation; The Swedish Research Council; King Gustaf V's and Queen Victoria's Freemasons' Foundation; The Stockholm County Council; The Center for Inflammatory Diseases; the ChAMP (Centre for Allergy Research Highlights Asthma Markers of Phenotype) consortium, which is funded by the Swedish Foundation for Strategic Research, Karolinska Institutet, the AstraZeneca and Science for Life Laboratory Joint Research Collaboration, and the Vårdal Foundation; and Karolinska Institutet. Funding information for this article has been deposited with the Crossref Funder Registry.

References

- 1 Royle L, Campbell MP, Radcliffe CM, *et al.* HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal Biochem* 2008; 376: 1–12.
- 2 Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. *Adv Immunol* 2007; 96: 179–204.
- 3 Malhotra R, Wormald MR, Rudd PM, *et al.* Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement *via* the mannose-binding protein. *Nat Med* 1995; 1: 237–243.

- 4 Parekh RB, Dwek RA, Sutton BJ, *et al.* Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 1985; 316: 452–457.
- 5 Scherer HU, van der Woude D, Ioan-Facsinay A, *et al.* Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid. *Arthritis Rheum* 2010; 62: 1620–1629.
- 6 Bond A, Alavi A, Axford JS, *et al.* A detailed lectin analysis of IgG glycosylation, demonstrating disease specific changes in terminal galactose and N-acetylglucosamine. *J Autoimmun* 1997; 10: 77–85.
- 7 Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 2006; 313: 670–673.
- 8 Anthony RM, Wermeling F, Ravetch JV. Novel roles for the IgG Fc glycan. *Ann NY Acad Sci* 2012; 1253: 170–180.
- 9 Anthony RM, Wermeling F, Karlsson MC, *et al.* Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci USA* 2008; 105: 19571–19578.
- 10 de Jong SE, Selman MH, Adegnik AA, *et al.* IgG1 Fc N-glycan galactosylation as a biomarker for immune activation. *Sci Rep* 2016; 6: 28207.
- 11 Rivera NV, Ronninger M, Shchetynsky K, *et al.* High-density genetic mapping identifies new susceptibility variants in sarcoidosis phenotypes and shows genomic-driven phenotypic differences. *Am J Respir Crit Care Med* 2016; 193: 1008–1022.
- 12 Grunewald J, Eklund A. Lofgren's syndrome: human leukocyte antigen strongly influences the disease course. *Am J Respir Crit Care Med* 2009; 179: 307–312.
- 13 Lundstrom SL, Fernandes-Cerqueira C, Ytterberg AJ, *et al.* IgG antibodies to cyclic citrullinated peptides exhibit profiles specific in terms of IgG subclasses, Fc-glycans and a fab-Peptide sequence. *PLoS One* 2014; 9: e113924.
- 14 Hart KM, Choy DF, Bradding P, *et al.* Accurately measuring and modeling Th2 and Th17 endotypes in severe asthma. *Ann Transl Med* 2017; 5: 91.
- 15 Karimi R, Tornling G, Grunewald J, *et al.* Cell recovery in bronchoalveolar lavage fluid in smokers is dependent on cumulative smoking history. *PLoS One* 2012; 7: e34232.
- 16 Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999; 160: 736–755.
- 17 James AJ, Reinius LE, Verhoek M, *et al.* Increased YKL-40 and chitotriosidase in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2016; 193: 131–142.
- 18 Bel EH, Sousa A, Fleming L, *et al.* Diagnosis and definition of severe refractory asthma: an international consensus statement from the Innovative Medicine Initiative (IMI). *Thorax* 2011; 66: 910–917.
- 19 Rombouts Y, Ewing E, van de Stadt LA, *et al.* Anti-citrullinated protein antibodies acquire a pro-inflammatory Fc glycosylation phenotype prior to the onset of rheumatoid arthritis. *Ann Rheum Dis* 2015; 74: 234–241.
- 20 Lundstrom SL, Zhang B, Rutishauser D, *et al.* SpotLight Proteomics: uncovering the hidden blood proteome improves diagnostic power of proteomics. *Sci Rep* 2017; 7: 41929.
- 21 Lundstrom SL, Yang H, Lyutvinskiy Y, *et al.* Blood plasma IgG Fc glycans are significantly altered in Alzheimer's disease and progressive mild cognitive impairment. *J Alzheimers Dis* 2014; 38: 567–579.
- 22 Oxelius VA, Eibl MM. Different Gm allotype amounts in human intravenous immunoglobulin (IVIG) preparations; survival of foreign Gm allotypes in immunodeficient patients. *Clin Exp Immunol* 1996; 106: 203–207.
- 23 Balbin M, Grubb A, de Lange GG, *et al.* DNA sequences specific for Caucasian G3m(b) and (g) allotypes: allotyping at the genomic level. *Immunogenetics* 1994; 39: 187–193.
- 24 Pucic M, Knezevic A, Vidic J, *et al.* High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. *Mol Cell Proteomics* 2011; 10: M111 010090.
- 25 Rouiller Y, Perilleux A, Marsaut M, *et al.* Effect of hydrocortisone on the production and glycosylation of an Fc-fusion protein in CHO cell cultures. *Biotechnol Prog* 2012; 28: 803–813.
- 26 Maguire TM, Coughlan CM, Seckl JR, *et al.* The effect of corticosteroids on serum sialyltransferase enzyme activities in the rat. *Biochim Biophys Acta* 1998; 1379: 23–28.
- 27 Saldova R, Huffman JE, Adamczyk B, *et al.* Association of medication with the human plasma N-glycome. *J Proteome Res* 2012; 11: 1821–1831.
- 28 Li W, Gao P, Zhi Y, *et al.* Periostin: its role in asthma and its potential as a diagnostic or therapeutic target. *Respir Res* 2015; 16: 57.
- 29 Johansen JS, Milman N, Hansen M, *et al.* Increased serum YKL-40 in patients with pulmonary sarcoidosis – a potential marker of disease activity? *Respir Med* 2005; 99: 396–402.
- 30 Di Rosa M, Distefano G, Zorena K, *et al.* Chitinases and immunity: ancestral molecules with new functions. *Immunobiology* 2016; 221: 399–411.
- 31 Capone M, Maggi L, Santarlasci V, *et al.* Chitinase 3-like-1 is produced by human Th17 cells and correlates with the level of inflammation in juvenile idiopathic arthritis patients. *Clin Mol Allergy* 2016; 14: 16.
- 32 Pfeifle R, Rothe T, Ipseiz N, *et al.* Regulation of autoantibody activity by the IL-23–T_H17 axis determines the onset of autoimmune disease. *Nat Immunol* 2017; 18: 104–113.
- 33 Aalberse RC, Stapel SO, Schuurman J, *et al.* Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009; 39: 469–477.
- 34 van der Neut Kofschoten M, Schuurman J, Losen M, *et al.* Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 2007; 317: 1554–1557.
- 35 Zack DJ, Stempniak M, Wong AL, *et al.* Localization of an Fc-binding reactivity to the constant region of human IgG4. Implications for the pathogenesis of rheumatoid arthritis. *J Immunol* 1995; 155: 5057–5063.
- 36 Collin M, Ehlers M. The carbohydrate switch between pathogenic and immunosuppressive antigen-specific antibodies. *Exp Dermatol* 2013; 22: 511–514.
- 37 Oczypok EA, Perkins TN, Oury TD. All the “RAGE” in lung disease: The receptor for advanced glycation endproducts (RAGE) is a major mediator of pulmonary inflammatory responses. *Paediatr Respir Rev* 2017; 23: 40–49.
- 38 Gao P, Simpson JL, Zhang J, *et al.* Galectin-3: its role in asthma and potential as an anti-inflammatory target. *Respir Res* 2013; 14: 136.