**Clinical Chemistry** 64:4
735–742 (2018)



# Resolution of Spurious Immunonephelometric IgG Subclass Measurement Discrepancies by LC-MS/MS

Grace van der Gugten,[1] Mari L. DeMarco,[1,2†] Luke Y.C. Chen,[2,3] Alex Chin,[4,5] Mollie Carruthers,[2,6] Daniel T. Holmes,[1,2] and Andre Mattman[1,2*]

**BACKGROUND:** The Binding Site immunonephelometric (IN) IgG subclass reagents (IgG1, IgG2, IgG3, IgG, BSIN) are used for assessment of both immunodeficiency and IgG4-related disease (IgG4-RD). In our laboratory, suspected analytic errors were noted in patients with increases in IgG4: The sum of the individual IgG subclasses was substantially greater than the measured total IgG concentrations (unlike samples with normal IgG4), and the IgG4 concentration was always less than the IgG2 concentration.

**METHODS:** We developed a tryptic digest LC-MS/MS method to quantify IgG1, IgG2, IgG3, and IgG4 in serum. Samples with IgG4 concentrations ranging from <0.03 g/L to 32 g/L were reanalyzed by LC-MS/MS, and a subset was also reanalyzed by Siemens IN (SIN) subclass measurements.

**RESULTS:** Multivariate linear regression identified 3 subclass tests with multiple predictors of the measured subclass concentration. For these 3 subclasses, the predominant predictors were (in terms of LC-MS/MS IgG subclass measurement coefficients) BSIN IgG1 = 0.89·IgG1 + 0.4·IgG4; BSIN IgG2 = 0.94·IgG4 + 0.89·IgG2; and SIN IgG2 = 0.72·IgG2 + 0.24·IgG4.

**CONCLUSIONS:** There is apparent IgG4 cross-reactivity with select IN subclass measurements affecting tests from both vendors tested. These findings can be explained either by direct cross-reactivity of the IN reagents with the IgG4 subclass or unique physicochemical properties of IgG4 that permit nonspecific binding of IgG4 heavy chain to other IgG immunoglobulin heavy chains. Irrespective of the mechanism, the observed intermethod discrepancies support the use of LC-MS/MS as the preferred method for measurement of IgG subclasses when testing patients with suspected IgG4-RD.



IgG subclasses are measured with increasing frequency for the diagnosis and monitoring of IgG4-related disease (IgG4-RD), a recently recognized rare condition that can affect many organ systems and has serious morbidity. Importantly, IgG4-RD may act as a mimic of other rheumatologic and malignant conditions; therefore, its recognition has important management implications *(1–3)*. The IgG subclass panel is also used for the diagnosis and monitoring of humoral immunodeficiency disorders.

The most common method of measuring IgG subclasses (IgG1–4) is immunonephelometry (IN)[7], of which there are 2 major vendors in North America: Binding Site™ IN (BSIN) and Siemens™ IN (SIN). Although IN is robust and widespread, it is vulnerable to analytical performance issues in the setting of IgG4-RD, including hook effect *(4)* and calibration biases between the BSIN and SIN *(5)*. Further, the sum of the individual IgG subclasses (SumIgG) diverges markedly from the total IgG measurement in many patients with increased IgG4 concentrations *(6)*. When we previously noted this IgG4 concentration-dependent discordance between BSIN SumIgG and the Siemens total IgG, we hypothesized incorrectly that it was attributable to deficiency in the Siemens total IgG method. Our subsequent comparisons of BSIN IgG subclass and total IgG measurements with serum protein electrophoresis suggested the error was instead associated with the BSIN IgG subclass measurements. In this study, we investigated the matter further to determine whether mass spectrometric measurement of the IgG subclasses would corroborate the respective BSIN measurements, and whether, in the setting of an increased IgG4, the mass spectrometric SumIgG would

[1] Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Vancouver, BC, Canada; [2] Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada; [3] Division of Hematology, Vancouver General Hospital, Vancouver, BC, Canada; [4] Department of Pathology and Laboratory Medicine, Calgary Laboratory Services, Calgary, AB, Canada; [5] Cumming School of Medicine, University of Calgary, Calgary, AB, Canada; [6] Division of Rheumatology, Vancouver General Hospital, Vancouver, BC, Canada.
† Member of the Society for Young Clinical Laboratorians (SYCL) (http://www.aacc.org/community/sycl).
* Address correspondence to this author at: St. Paul's Hospital, 1081 Burrard St., Vancouver, BC V6Z 1Y6, Canada. Fax 604-806-8815; e-mail amattman@providencehealth.bc.ca.
Received September 23, 2017; accepted January 5, 2018.
Previously published online at DOI: 10.1373/clinchem.2017.282319

[7] Nonstandard abbreviations: IN, immunonephelometric; BSIN, Binding Site immunonephelometry; SIN, Siemens immunonephelometry; SumIgG, sum of the 4 individual IgG subclasses; MRM, multiple reaction monitoring.

equal the total IgG measurement, as opposed to exhibiting a positive bias as observed with the BSIN SumIgG.

## Methods

### PATIENT SAMPLES

We obtained a series of discarded anonymized serum samples from consecutive patients who had been investigated over a 3-month period in select hematology and rheumatology clinics in Vancouver, where IgG4-RD was included in the differential diagnosis. In each case, the patient's investigation included serum IgG subclasses. Seventy-five samples were obtained for the comparison study of BSIN and LC-MS/MS out of 453 clinical samples. Forty-two samples were subselected for the additional SIN measurements. Institutional ethics review was obtained to review IgG subclass measurements in these cohorts.

### IgG SUBCLASS AND TOTAL IgG ANALYSIS

IgG subclass measurements were performed by BSIN using the Siemens BNII Nephelometer and, in a subset, also by SIN using the Siemens BN ProSpec Nephelometer. In all cases, the individual subclasses were also measured by LC-MS/MS, and the total IgG was measured separately by IN (Siemens BNII). The BSIN and SIN methods were performed using protocols designed to prevent hook effect errors as previously described *(5)*. The samples were first analyzed by BSIN and then stored for 6 to 12 months postanalysis at −80 °C, before retesting with the LC-MS/MS method. After an additional month of freezing at −80 °C, the samples were sent to a second laboratory for SIN measurement, resulting in 2 additional freeze–thaw cycles as compared with the original BSIN measurements.

The IgG subclasses LC-MS/MS method was developed by means of substantial modification of a previously reported method *(7)*. In brief, serum was subjected to denaturation, reduction, and alkylation, followed by tryptic digest and subsequent LC-MS/MS analysis. Multiple reaction monitoring (MRM) transitions were monitored for peptides unique to each individual subclass, peptides common to all subclasses, and corresponding stable labeled internal standard peptides. The peptides were selected based on the publication by Ladwig et al. *(7)*.

The LC-MS/MS method was calibrated to the international reference material ERM-DA470K to allow for direct comparison of results with those from the Binding Site method *(5)*. Specifically, Binding Site calibrators were diluted into fetal bovine serum to create a 5-point calibration curve, which was used for quantification of the LC-MS/MS method. The 5 respective calibrator values were reassigned using the ERM-DA470K certified reference material to enable harmonization. The calibrator values were reassigned using data from 6 calibration curves prepared over 3 separate runs of the LC-MS/MS method. The reassigned values for the binding site calibrators were calculated using a single-point calibration (linear through zero) using the ERM-DA470K. In each batch, at least 3 replicates of the ERM were analyzed (total n = 12).

Details of the method including digest conditions, chromatography, MRM transitions, and calibration are provided in Table 1 and in the Materials file of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue4.

### STATISTICAL ANALYSIS

Univariate and multivariate linear regression of the respective BSIN and SIN IgG subclass measurements against the LC-MS/MS measurements was performed using R version 3.4.1. For multivariate analyses, the concentration of the *ith* subclass ($IgG_{IN-i}$), as measured by IN, was modeled as a function of mass spectrometric results by:

$$IgG_{IN-i} = \beta_{1i} \times IgG_{MS-1} + \beta_{2i} \times IgG_{MS-2} + \beta_{3i} \times IgG_{MS-3} + \beta_{4i} \times IgG_{MS-4} + \alpha_i$$

Robust multivariate linear regression was performed using the robust package *(8)*.

For the univariate and multivariate regression statistical analyses, the statistical significance of the slope and *y* intercept correlation coefficients were determined based on a $P$ value $<0.05$, calculated using the statistical program R version 3.4.1.

## Results

### LC-MS/MS METHOD PERFORMANCE

The LC-MS/MS IgG subclasses performance was assessed by determination of the analytic performance of each of the individual subclasses. Typical chromatograms (see Fig. 1 in the online Data Supplement) and calibration curves (see Fig. 2 in the online Data Supplement) are shown. The method precision (see Table 1 in the online Data Supplement) and limit of quantification (see Table 2 in the online Data Supplement) are presented in detail within the Materials file found in the online Data Supplement. In brief, the total CV for low, medium, and high serum pools for each of the subclasses ranged from 4.0% to 6.3%. With respect to the limit of quantification, the CV was <10% for IgG1 of 0.37 g/L, IgG2 of 0.16 g/L, IgG3 of 0.040 g/L, and IgG4 of 0.032 g/L. This precision performance of the LC-MS/MS method was either equivalent or improved, as compared with that listed in the BSIN package insert. Additional informa-

**Table 1. Method parameters for IgG subclass measurements by LC-MS/MS utilizing stable labeled peptide internal standards.**

| Sample parameters | Specification |
|---|---|
| Specimen volume, type, and batch size | 20 µL of serum in a 96-well plate |
| Reducing and denaturing reagents added for 30 min at 55°C | 100 µL of 50 mmol/L $(NH_4)_2HCO_3$ |
| | 100 µL of 2,2,2-trifluoroethanol |
| | 25 µL of 100 mmol/L DL-dithiothreitol |
| Acetylating reagent added for 60 min at room temperature | 30 µL of 200 mmol/L iodoacetamide |
| Treated sample volume for digestion | 50 µL |
| Digestion reagents added with sonication and subsequent heating at 37°C for 1 h (stopped by 20 µL of formic acid) | 200 µL of deionized water |
| | 50 µL of 50 mmol/L $(NH_4)_2HCO_3$ |
| | 30 µL of 1 g/L trypsin |
| Digested sample volume for injection after addition of IgG subclass peptide internal standard mixture | 10 µL |
| **Liquid chromatography parameters** | **Specification** |
| Mobile phase A | 0.1% formic acid in water |
| Mobile phase B | 0.1% formic acid in 75:25 ACN/IPA |
| Column | Phenomenex Aeris PEPTIDE 3.6 µm XDB-C18 50 × 2.1 mm, with an in-line filter |
| Column flow rate | 0.4 mL/min |
| Gradient (total run time 11 min) | 0% B for 1 min |
| | 0% to 50% B over 6.5 min |
| | 50% to 98% B in 0.1 min |
| | 98% B for 1.4 min |
| | Return to 0% B in 0.1 min, hold for 2 min |
| **MS/MS parameters (SCIEX 5500 QTrap)** | **Specification** |
| Ionization type | ESI+ |
| Curtain gas (CUR) | 40.0 |
| Collision gas (CAD) | High |
| Ion spray voltage (IS) | 5500.0 |
| Temperature (TEM) | 600.0 |
| Ion source gas 1 (GS1) | 35.0 |
| Ion source gas 2 (GS2) | 30.0 |
| Entrance potential | 10 |

**MRM quantifier transitions and compound-specific parameters**

| ID | MRM transition, *m/z* | Declustering potential, V | Collision energy, V | Cell exit potential, V |
|---|---|---|---|---|
| IgG1 | 593.8/418.2 | 110 | 18 | 22 |
| IgG1_SIS | 602.0/699.2 | 50 | 29 | 14 |
| IgG2 | 412.7/486.3 | 60 | 40 | 30 |
| IgG2_SIS | 419.7/493.0 | 60 | 26 | 30 |
| IgG3 | 593.7/626.3 | 70 | 35 | 20 |
| IgG3_SIS | 599.7/735.0 | 70 | 30 | 20 |
| IgG4 | 951.6/850.6 | 80 | 55 | 35 |
| IgG4_SIS | 960.2/859.2 | 120 | 42 | 18 |
| Total IgG | 418.2/619.4 | 67 | 8 | 38 |
| Total IgG_SIS | 428.1/513.4 | 80 | 22 | 11 |

**Table 2.** LC-MS/MS univariate and multivariate linear regression coefficients, intercepts, and coefficients of determination for each IgG subclass with statistical parameters displayed separately for the respective IN IgG subclass methods.

| | BSIN subclass | | | | SIN subclass | | | |
|---|---|---|---|---|---|---|---|---|
| Univariate regression | IgG1 | IgG2 | IgG3 | IgG4 | IgG1 | IgG2 | IgG3 | IgG4 |
| N | 73 | 73 | 73 | 73 | 40 | 38 | 38 | 40 |
| Slope | 0.92 | 0.92 | 1.08 | 0.94 | 0.95 | 0.65 | 0.53 | 1.64 |
| Intercept, g/L | 1.2[a,b] | 3.2[c,d] | −0.02 | 0.02 | 0.42 | 2.4[c,e] | 0.11 | 0.13 |
| $R^2$ | 0.92 | 0.18 | 0.96 | 0.96 | 0.95 | 0.46 | 0.87 | 0.96 |
| **LC-MS/MS multivariate linear regression coefficients by immunonephelometric method** | | | | | | | | |
| | IgG1 | IgG2 | IgG3 | IgG4 | IgG1 | IgG2 | IgG3 | IgG4 |
| IgG1 coefficient | 0.89[f,g] | 0.06 | 0.00 | 0.03[a,h] | 1.00[f,i] | −0.02 | 0.00 | 0.07[a,j] |
| IgG2 coefficient | 0.01 | 0.89[f,k] | −0.02 | 0.03 | −0.10 | 0.72[f,l] | 0.00 | −0.02 |
| IgG3 coefficient | −0.39 | 0.17 | 1.07[f,m] | −0.30 | −1.39[a,n] | 0.01 | 0.53[f,o] | −1.01[a,p] |
| IgG4 coefficient | 0.40[f,q] | 0.94[f,r] | −0.01 | 0.94[f,s] | 0.11 | 0.24[f,t] | 0.00 | 1.65[f,u] |
| Intercept, g/L | 0.33 | −0.35 | 0.03 | 0.03 | 0.94 | 1.21[a,v] | 0.11 | 0.30 |
| $R^2$ | 0.97 | 0.87 | 0.97 | 0.96 | 0.96 | 0.74 | 0.87 | 0.98 |

[a] Significantly different from 0 ($P < 0.05$) but insignificant with robust linear regression; [b] 0.032; [c] significantly different from 0 ($P < 0.05$); [d] 0.0041; [e] $1.2 \times 10^{-5}$; [f] significantly different from 0 ($P < 0.00001$); [g] $8.6 \times 10^{-47}$; [h] 0.044; [i] $3.3 \times 10^{-25}$; [j] 0.0057; [k] $5.5 \times 10^{-10}$; [l] $1.2 \times 10^{-9}$; [m] $3.2 \times 10^{-47}$; [n] 0.0026; [o] $4.2 \times 10^{-15}$; [p] 0.0011; [q] $2.0 \times 10^{-15}$; [r] $4.9 \times 10^{-26}$; [s] $1.1 \times 10^{-48}$; [t] $1.6 \times 10^{-6}$; [u] $1.6 \times 10^{-29}$; [v] 0.020.

tion on method linearity, sample stability, and sample extract stability is presented in the Materials file of the online Data Supplement (see Tables 3–5 in the online Data Supplement). Method bias, as compared with IN methods, is subsequently described.

#### IgG SUBCLASSES: BSIN VS LC-MS/MS

Of the 75 samples analyzed using the BSIN method, 2 had IgG4 measurements greater than the analytic measuring range of the LC-MS/MS method (20 g/L) and, therefore, were not included in subsequent statistical regression analyses. For the remaining 73 samples, the median and interquartile range for LC-MS/MS IgG4 was 2.02 (0.33–7.21 g/L). The linear regression statistics for the 4 subclass comparisons are shown in Table 2. For each subclass, the slope and *y* intercept values approximated 1 and 0, respectively, with a high $R^2$ value (≥0.92), except for the IgG2 subclass, which had an $R^2$ value of 0.18 and an intercept of 3.2 g/L.

#### SIN VS LC-MS/MS

After review of the low coefficient of determination of the BSIN vs LC-MS/MS intermethod comparison for the IgG2 subclass, additional samples were sent for analysis by the other major vendor of IgG subclass IN, SIN, to determine whether the apparent IN errors were vendor-specific. The subset of samples was selected to include those samples with the largest IgG2 intermethod discrepancies as observed in the BSIN vs LC-MS/MS comparison. This subset of samples had a median IgG4 concentration (by LC-MS/MS) of 4.2 g/L with an interquartile range of 1.7 to 8.5 g/L, which was relatively higher than the previous group of 73 samples. The remainder of the subclass distributions were similar between the 2 sets of samples (see Table 6 in the online Data Supplement). Included in this second subset was 1 sample with an IgG4 > 20 g/L and another sample with low SIN values for each of the 4 subclasses. These 2 samples were not included in further SIN vs LC-MS/MS regression analyses. Two additional samples had suppressed SIN IgG2 concentrations (but normal IgG2 as measured by both LC-MS/MS and BSIN), whereas 2 others had SIN IgG3 concentrations that were positive outliers as compared with both LC-MS/MS and BSIN. These outliers were not further investigated and were not included in subsequent SIN vs LC-MS/MS statistical regression analyses. For the remaining 38 to 40 samples, the linear regression coefficients, intercept, and coefficient of determination for each of the 4 subclass comparisons are shown in Table 2.

The LC-MS/MS method for the IgG subclasses was calibrated to a different standard (ERM-DA470K) than the SIN methods (WHO 67/97). This calibration difference is reflected in the respective slopes seen in Table 2, which are similar to those seen in other recent studies of SIN vs ERM-DA470K calibrated methods *(9, 10)*. Of

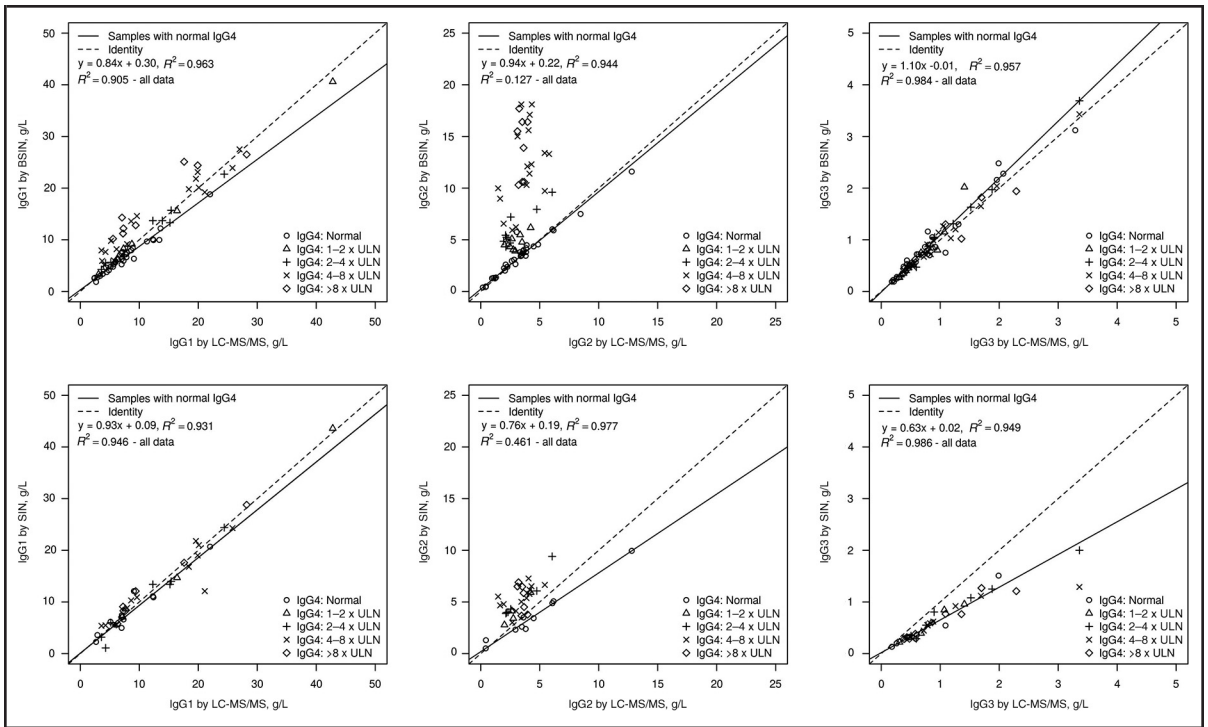


**Fig. 1. Linear regression of IN IgG1, IgG2, and IgG3 subclass measurements against the corresponding LC-MS/MS methods.**

Each plot shows a differential display of data points for each of 5 categories of samples demarcated by IgG4 concentration: within the normal reference interval, 1 to 2 times the upper limit of normal (1–2 × ULN), 2 to 4 × ULN, 4 to 8 × ULN, and >8 × ULN. The trend line and regression equation are plotted only for those data points that are within the normal IgG4 reference interval. The coefficient of determination is given for the entire set of data and for the data points within the normal IgG4 reference interval.

the 4 intermethod comparisons, the IgG2 $R^2$ value was disproportionately low compared with the other subclass $R^2$ values. This finding was similar to that observed for BSIN IgG2 vs LC-MS/MS IgG2 (discussed below). Further, the 2 IN IgG2 methods also had a low inter-IN method $R^2$ value ($R^2 = 0.19$; see Fig. 3 in the online Data Supplement).

### EFFECT OF LC-MS/MS IgG4 CONCENTRATION ON IN IgG SUBCLASS

Because we had previously noted an IgG4-dependent discrepancy between SumIgG vs IgG total IN measurements *(6)*, we reviewed the effect of IgG4 concentration on the linear regression analyses for both the BSIN vs LC-MS/MS and the SIN vs LC-MS/MS comparisons. The method comparisons are shown in Fig. 1 for IgG1, IgG2, and IgG3. In each case, the regression trend line reflects data points derived only from the cohort of patients with an IgG4 concentration less than the upper limit of normal (<1.25 g/L by LC-MS/MS). On the same graphs, the data points for 4 other cohorts, with progressively higher IgG4 concentrations by mass spectrometry, are plotted with different symbols to demonstrate the effect of IgG4 concentration on the respective method comparison studies. There was a clear IgG4-dependent positive bias on the IN IgG2 measurement compared with LC-MS/MS that was present for both BSIN IgG2 and SIN IgG2. Notably, there was also a clear positive bias for the BSIN IgG1 method but no clear bias for the SIN IgG1 or for either of the IgG3 IN methods. This relationship, between IgG4 concentration and an IN positive bias relative to LC-MS/MS, for select subclass measurements, is further depicted in bias plots included in Fig. 4 of the online Data Supplement.

To further clarify the magnitude of the apparent IgG4 interference on the IgG1 and IgG2 methods, and to identify other potentially relevant intersubclass effects on IN IgG subclass measurements, multivariate regression of each of the individual IN IgG subclass measurements was performed against all 4 LC-MS/MS IgG subclass measurements. The data for standard multivariate regression are summarized in Table 2, whereas the data for robust linear regression are included in Table 6 of the online Data Supplement.

#### IN SUBCLASS MULTIVARIATE LINEAR REGRESSION ON LC-MS/MS IgG SUBCLASS MEASUREMENTS

*IgG2: effect of IgG4 concentration on BSIN vs LC-MS/MS.* Multivariate linear regression of the BSIN IgG2 measurements with the 4 LC-MS/MS subclasses (Table 2) showed that the LC-MS/MS IgG4 concentration coefficient (0.94) was equal to or greater than the corresponding LC-MS/MS IgG2 coefficient (0.89). Further, in the multivariate model, the coefficient of determination improved from 0.18 to 0.87 when both LC-MS/MS measurements (IgG2 and IgG4) were used compared with LC-MS/MS IgG2 alone. The results of these analyses were consistent with a nearly direct (100%) cross-reactivity between the BSIN IgG2 method and the serum IgG4 concentration.

This hypothesis of BSIN IgG2 reagent cross-reactivity with IgG4 was further borne out by the observation that the SumIgG, when measured by BSIN, was frequently (13 of 75) >20% higher than the SIN total IgG. Further, the likelihood of BSIN SumIgG overestimation of total IgG (by SIN or LC-MS/MS) was proportional to the IgG4 concentration (see Fig. 5, A and B, in the online Data Supplement). In contrast, the SumIgG by LC-MS/MS was a more accurate representation of the SIN total IgG measurement, being within 20% in 70 of 75 cases. Further, the magnitude of the bias (between LC-MS/MS SumIgG and total IgG by either method) was minimally related to the IgG4 concentration (see Fig. 5, C and D, in the online Data Supplement).

*IgG2: effect of IgG4 concentration on SIN vs LC-MS/MS.* For the SIN IgG2 subclass, the coefficient of determination with the LC-MS/MS IgG subclass measurements was also improved when multivariate linear regression incorporating both IgG2 and IgG4 ($R^2 = 0.74$) was used compared with using LC-MS/MS IgG2 alone ($R^2 = 0.46$; Table 2). The SIN IgG2 multivariate linear regression showed a significant contribution from LC-MS/MS IgG4 with a coefficient (0.24) that was approximately 33% of the corresponding LC-MS/MS IgG2 coefficient (0.72).

*IgG1: effect of IgG4 concentration on IN vs LC-MS/MS.* The BSIN IgG1 multivariate linear regression on the LC-MS/MS IgG subclasses also showed a significant contribution from LC-MS/MS IgG4 with a coefficient (0.40) that was slightly <50% of the corresponding LC-MS/MS IgG1 coefficient (0.89). In contrast, there was no significant relationship between the LC-MS/MS IgG4 concentration and the SIN IgG1 (Table 2).

*IgG3: effect of IgG4 concentration on IN vs LC-MS/MS.* The multivariate linear regression of either IN IgG3 measurements with the respective LC-MS/MS measurements showed no significant relationship with LC-MS/MS IgG4 (Table 2).

#### EFFECT OF LC-MS/MS IgG1, IgG2, AND IgG3 CONCENTRATIONS ON IN IgG SUBCLASS MULTIVARIATE LINEAR REGRESSION

Statistically significant associations between LC-MS/MS IgG1 measurements and IN IgG4 measurements (both SIN and BSIN) were observed (Table 2). The degree of cross-reactivity was estimated to be <5% in each case, and was insignificant in the case of BSIN IgG4 after robust linear regression (see Table 6 in the online Data Supplement).

There were no statistically significant associations between LC-MS/MS IgG2 measurements and IN measurements aside from the IgG2 measurements themselves.

Statistically significant negative associations between LC-MS/MS IgG3 measurements and both SIN IgG1 and SIN IgG4 were present (Table 2). The derived coefficients had a small impact on the respective SIN IgG1 and IgG4 concentrations because of the relatively small magnitude of the IgG3 concentration. Further, all LC-MS/MS IgG3 coefficients were deemed insignificant after robust linear regression (see Table 6 in the online Data Supplement), aside from those associated with the IN IgG3 measurements themselves.

#### EFFECT OF LC-MS/MS IgG4 CONCENTRATION ON SIN TOTAL IgG MEASUREMENT

The SIN total IgG measurement was not affected by LC-MS/MS IgG4 concentration (see Fig. 6 in the online Data Supplement).

## Discussion

Although IN is a convenient and reliable methodology, it is subject to errors related to nonspecificity common to all antibody-based approaches. In this study, we have shown that there is discordance between IgG subclass measurement by mass spectrometric and IN methods. The discordance was most evident for the IgG2 subclass with the magnitude of errors being substantially larger using BSIN reagents, but also present when using SIN reagents. The mass spectrometric measurements are considered reliable because the SumIgG equaled the total IgG measurement (within 20% in 70 of 75 samples) when SumIgG was measured with LC-MS/MS. In contrast, the BSIN SumIgG overestimated total IgG to a degree directly proportional to the IgG4 concentration.

The errors with the IN measurement of IgG subclasses are noteworthy in samples with substantially increased IgG4 concentrations commonly observed in the more severe forms of IgG4-RD. The mean IgG4 concentration in a recent multiethnic IgG4-RD cohort study was 11.2 g/L in Asian patients and 2.9 g/L in non-Asian

patients *(11)*. In non-IgG4-RD patients or controls, the small percentage of IgG4 immunoglobulins (<5%) would result in relatively small IgG4-dependent errors. It is notable that, in the absence of samples from patients with substantial increases in IgG4, these errors would escape many quality control checks of test reagent antibody fidelity and intermethod bias *(9)*. Similarly, without these high IgG4 samples, the previously described issue of falsely decreased IgG4 results owing to antigen excess *(4, 12)* would also go undetected in a method validation study.

In this study, in which we included samples from patients with IgG4-RD who had increased IgG4 concentrations, we noted unexpectedly increased IgG2 results using IN methods. These apparently erroneous increased IgG2 IN measurements may be attributable to >1 source of error. In the case of BSIN vs LC-MS/MS, the BSIN IgG2 measurements had a substantial positive bias that, on average, was of the same magnitude of the IgG4 concentration, suggesting 100% cross-reactivity of the reagent antisera. This observation strongly suggests that the BSIN IgG2 reagents have similar affinity for both IgG4 and IgG2.

In the case of both the SIN IgG2 reagents and the BSIN IgG1 reagents, the respective positive interferences, while also proportional to IgG4 concentration, were of smaller magnitude and less predictable than the IgG4-mediated interference on the BSIN IgG2 measurement. These smaller and more variable positive IgG4-related biases associated with the SIN IgG2 and the BSIN IgG1 methods may be related to a lack of reagent antibody specificity, but they may also be because of the unique physicochemical properties of IgG4 immunoglobulins *(13)*. One such property is that IgG4 can act as a "novel rheumatoid factor" in which IgG4 heavy chains can bind with the heavy chains of the 3 other IgG subclasses *(13, 14)*. This effect has been noted in vitro, with solid-phase bound antisera binding nonspecifically with serum IgG4, to predictably bias test results *(13–15)*, but its relevance to in vivo IgG4 activity is unknown. If a fraction of the IgG4 immunoglobulins were associated with IgG1 (or IgG2) heavy chains in vivo, then IN methods may inaccurately record positively biased IgG1 (or IgG2) concentrations because of the greater size of the antibody antigen aggregates created by the adherent IgG4 immunoglobulins. Similarly, the hypothesized nonspecific serum IgG4 interactions may be occurring in vitro, via binding of IgG4 to reagent antisera, to generate larger immune complexes. If so, the binding of serum IgG4 with reagent antisera is affected by numerous factors, including reagent antisera species, subclass type, and the presence of a solid phase *(14)*. Because these factors are distinct for each of the reagent methods studied (BSIN, polyclonal sheep antisera using latex-enhanced IN for IgG3 only; SIN, monoclonal antisera using latex-enhanced IN for IgG3, and polyclonal rabbit for SIN total IgG), the degree of interference by IgG4 would also be expected to differ between the respective tests.

The possibility of nonspecific in vivo IgG4/IgG1 immune complexes influencing IN IgG1 results is tentatively supported by the reciprocal observation that the IgG1 concentration (as measured by LC-MS/MS) influences IN IgG4 measurement (Table 2). However, the magnitude of the bias was <5% for LC-MS/MS IgG1, and no bias was observed for LC-MS/MS IgG2 on IN IgG4 measurement. Therefore, the significance of these small to nonexistent IgG1-dependent biases on IgG4 IN measurements requires clarification.

Whether in vivo or in vitro nonspecific IgG4 immune complexes or IN antisera cross-reactivity explains the observed IgG4-dependent effects on IN measurements, LC-MS/MS subclass measurements, which rely on analysis of tryptic digests, would be unaffected by IgG4 concentration. This lack of interference is supported by the observation that the SumIgG by LC-MS/MS matched the total IgG measurement (Siemens total IgG or the LC-MS/MS total IgG method; see Fig. 5 in the online Data Supplement) largely irrespective of the IgG4 concentration.

Although further studies are required to obtain direct evidence to support these hypothesized mechanisms for the analytic discordances, we can conclude that the discordances are present and attributable to error in the IN methods, as the sum of the LC-MS/MS consistently agrees with the total IgG measurement. Therefore, to enable progress in the understanding of the IgG subclass biomarker pathophysiology associated with IgG4-RD, as well as other immune disorders with increased IgG4 concentrations, methods free from IgG4-associated IN errors are required.

Specifically, previous publications may have reported misleading results in the setting of IgG4-RD because of the IN interferences noted in this study. For example, previous reports that found IgG2 to be a biomarker of IgG4-RD *(16, 17)* should be reevaluated in light of these observations. Similarly, the utility of IgG4/IgG1 (or IgG4/IgG2) ratios as biomarkers of IgG4-RD may be superior to IgG4 concentration alone, if evaluated using a method that is free from the IN interferences on IgG1 or IgG2. From a clinical perspective, these IgG4-mediated IN interferences may obscure recognition of a clear pattern of a selective isolation of IgG4, suggestive of IgG4-RD, by shifting the differential diagnosis to include conditions associated with elevations in IgG1 or IgG2 *(18)*.

In summary, we observed biases of the IN IgG subclass measurements compared with the corresponding LC-MS/MS measurements, which potentially reflect 2 analytical phenomena: (*a*) cross-reactivity of sample IgG4 with the BSIN IgG2 reagents and (*b*) IN measurement of IgG1 and IgG2 immunoglobulins that represent an aggregate of the target immunoglobulin and nonspe-

cifically bound IgG4 (IgG4 bound to either the target immunoglobulin or the reagent immunoglobulin). In each case, these proposed phenomena would explain the observations of IgG4-dependent positive biases with IN IgG1 and IgG2 concentration measurements, as compared with the corresponding LC-MS/MS measurements, and IgG4-dependent positive bias in the sum of the BSIN subclasses as compared with the total IgG method. Irrespective of the mechanism, the observed intermethod discrepancies support the use of LC-MS/MS as the preferred method for measurement of IgG subclasses when testing patients with suspected IgG4-RD. This conclusion can be supported solely by the analytic improvements described, without considering any ancillary benefits, which may also accompany such a change.

**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

**Authors' Disclosures or Potential Conflicts of Interest:** *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

**Employment or Leadership:** None declared.
**Consultant or Advisory Role:** None declared.
**Stock Ownership:** None declared.
**Honoraria:** D.T. Holmes, MSACL.org.
**Research Funding:** None declared.
**Expert Testimony:** None declared.
**Patents:** None declared.

**Role of Sponsor:** No sponsor was declared.

## References

1. Wallace ZS, Deshpande V, Mattoo H, Mahajan VS, Kulikova M, Pillai S, et al. IgG4-related disease: clinical and laboratory features in one hundred twenty-five patients. Arthritis Rheumatol 2015;67:2466–75.
2. Kamisawa T, Zen Y, Pillai S, Stone JH. IgG4-related disease. Lancet 2015;385:1460–71.
3. Mahajan VS, Mattoo H, Deshpande V, Pillai SS, Stone JH. IgG4-related disease. Annu Rev Pathol 2014;9:315–47.
4. Khosroshahi A, Cheryk LA, Carruthers MN, Edwards JA, Bloch DB, Stone JH. Brief report: spuriously low serum IgG4 concentrations caused by the prozone phenomenon in patients with IgG4-related disease. Arthritis Rheumatol 2014;66:213–7.
5. Parker AR, Carr-Smith HD. Calibration differences and the prozone phenomenon in IgG4-related disease: comment on the article by Khosroshahi et al. Arthritis Rheumatol 2014;66:1964–5.
6. Mattman A, Wong S, Urquhart N, Wong P. Total IgG method insensitivity to the presence of IgG4 immunoglobulins. Clin Biochem 2014;47:1124–7.
7. Ladwig PM, Barnidge DR, Snyder MR, Katzmann JA, Murray DL. Quantification of serum IgG subclasses by use of subclass-specific tryptic peptides and liquid chromatography–tandem mass spectrometry. Clin Chem 2014;60:1080–8.
8. Wang J, Zamar R, Marazzi A, Yohai V, Salibian-Barrera M, Maronna R, et al. Port of the S+ "robust library" version 0.4-18; https://CRAN.R-project.org/package=robust. Accessed on September, 2017.
9. Sarnago A, Pascual RM, Moreno MJ, Laiz B, Fuster O. IgG subclasses quantitation: analytical performance of the binding site SPAPLUS(R) human assay and comparison with Siemens BNII(R) assay. Clin Biochem 2018;51:85–89.
10. Ludwig-Kraus B, Kraus FB. Similar but not consistent: revisiting the pitfalls of measuring IgG subclasses with different assays. J Clin Lab Anal 2017;31:e22146. 10.1002/jcla.22146.
11. Qi R, Chen LY, Park S, Irvine R, Seidman M, Kelsall JT, et al. Utility of serum IgG4 levels in a multiethnic population. Am J Med Sci 2018;355:61–6.
12. Urquhart N, Mattman A. Corroboration of the prozone phenomenon in IgG4-related disease: comment on the article by Khosroshahi et al. Arthritis Rheumatol 2014;66:1963–4.
13. Lighaam LC, Rispens T. The immunobiology of immunoglobulin G4. Semin Liver Dis 2016;36:200–15.
14. Ito T, Kitahara K, Umemura T, Ota M, Shimozuru Y, Kawa S, et al. A novel heterophilic antibody interaction involves IgG4. Scand J Immunol 2010;71:109–14.
15. Rispens T, Leeuwen A, Vennegoor A, Killestein J, Aalberse RC, Wolbink GJ, et al. Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody. Anal Biochem 2011;411:271–6.
16. Dunkley L, Mudhar HS. IgG4-related disease presenting with raised serum IgG2-real timeline of IgG4-RD? Rheumatology (Oxford) 2018;57:197–9.
17. Chan ASY, Mudhar H, Shen SY, Lang SS, Fernando M, Hilmy MH, et al. Serum IgG2 and tissue IgG2 plasma cell elevation in orbital IgG4-related disease (IgG4-RD): potential use in IgG4-RD assessment. Br J Ophthalmol 2017;101:1576–82.
18. Engelhart S, Glynn RJ, Schur PH. Disease associations with isolated elevations of each of the four IgG subclasses. Semin Arthritis Rheum 2017;47:276–80.