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Pneumovax[®] Response when on Intravenous Immunoglobulin

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An Enzyme-Linked ImmunoSpot Assay (ELISPOT) to Assess Pneumovax[®] Response when on Intravenous Immunoglobulin

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Abbreviations	Definition
ASC	Antibody-secreting cells
BSA	Bovine serum albumin
CVID	Common variable immunodeficiency
DPBS	Dulbecco's Phosphate-Buffered Saline
ELISPOT	Enzyme-linked ImmunoSpot assay
ESID	European Society for Immunodeficiencies
ICON	International Consensus Document
IGRT	Immunoglobulin replacement therapy
IVIG	Intravenous immunoglobulin
KW	Kruskal-Wallis
NJH	National Jewish Health
P23	23-valent polysaccharide Pneumovax [®] vaccine
PBMCs	Peripheral blood mononuclear cells
PCV13	13-valent pneumococcal conjugate Prevnar [®] vaccine
PID	Primary immunodeficiency disease
PVDF	Polyvinylidene fluoride
WRS	Wilcoxon rank-sum

Background: Testing for common variable immunodeficiency (CVID) requires evaluation of specific antibody responses to vaccines. Current practice of assessing pneumococcal serotype-specific IgG levels after Pneumovax® (P23) has several limitations and is not accurate for patients already on immunoglobulin replacement. In contrast, the Enzyme-Linked ImmunoSpot (ELISPOT) assay can be interpreted in patients on immunoglobulin replacement as ex vivo measurement of specific antibody secreting cells (ASC).

Objective: Optimize and test an ELISPOT assay to assess vaccination response to P23 and compare to P23 serotype-specific IgG for patients on IVIG.

Methods: We prospectively enrolled 15 adults: normal controls (n=8), patients with CVID on IVIG replacement (n=2), patients on IVIG replacement for recurrent infections who did not meet diagnostic criteria for CVID, considered IgG deficiency (n=2), and patients without immunodeficiency on high-dose IVIG for other diagnosis (n=3). We measured P23 serotype-specific IgG before and 4 weeks after P23 and ELISPOT ASC before and 1 week after P23 (with B-cell subpopulation analysis by flow cytometry in patients on IVIG).

Results: Normal controls showed significant vaccination response by P23 serotypespecific IgG while patients on IVIG did not. Except for true CVID patients on IVIG, a P23 ELISPOT ASC response was demonstrated in normal controls (highest) and most patients on IVIG for recurrent infections or other diagnosis.

Conclusion: Our pilot study suggests an optimized ELISPOT protocol has utility to assess the P23-specific antibody response after vaccination. Our ELISPOT assay seemed reliable for patients on IVIG and may help differentiate true CVID patients from those with a less stringent diagnosis while on IVIG.

Introduction:

Common variable immunodeficiency (CVID) is the most frequent serious primary immunodeficiency disease (PID) with a prevalence estimated between 1:10,000 and 1:100,000 of the population ¹. The diagnosis of CVID is difficult as the onset of the varied clinical phenotypes and laboratory abnormalities may not coincide and can occur at almost any age. The most recent consensus document for CVID diagnosis, the International Consensus Document (ICON) published in 2016, outlines the following core criteria: CVID is a diagnosis of exclusion that is best conferred after the age of 4 years to account for transient hypogammaglobulinemia of infancy. Patients should have at least one clinical manifestation (infection, autoimmunity, or lymphoproliferation). Age-adjusted hypogammaglobulinemia should be demonstrated on at least 2 separate occasions unless the IgG level is very low (<100-300 mg/dL depending on age). IgA and/or IgM levels must also be low. Patients with IgG levels of more than 200 mg/dL should be evaluated for their vaccine response to T-dependent and T-independent antigens ¹. Other published criteria, including those originally proposed by the European Society for Immunodeficiencies (ESID) in 1999 ², criteria proposed by Ameratunga et al. in 2013 ³, and the revised ESID criteria in 2014 ⁴, have been extensively compared ^{4, 5}. While differences exist between criteria, they all consider a poor response to vaccines as a key component to CVID diagnosis ^{4, 5}.

Vaccination response is measured by obtaining pre-vaccination and 4 weeks post-vaccination antibody levels to evaluate for specific IgG to the vaccine antigens. For efficiency, the response to readily available vaccine antigens such as tetanus, diphtheria, *Haemophilus influenzae* type b, and pneumococcal polysaccharides are commonly measured. Of these readily

available vaccine antigens, the only pure polysaccharide, or T-cell independent antigens, are in the 23-valent polysaccharide Pneumovax[®] vaccine (P23). Pure polysaccharide vaccines are also available for meningococcal disease and *Salmonella typhi*, but these vaccines and their serologic assays are far less accessible. P23 consists of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. Given its widespread use to prevent pneumococcal infection and the sheer number of serotypes within the vaccine to evaluate, P23 is often used in immunodeficiency workup. Unfortunately, pneumococcal serotype-specific IgG levels, often colloquially referred to as “pneumococcal titers”, are difficult to interpret because they can vary considerably based on the age of the patient, the particular testing methodology used, and differences in assay calibration between laboratories^{4, 6, 7}. Even commercial multiplex assays have been shown to have significant interlaboratory variability as pointed out in a recent study⁸. Furthermore, it is still unknown what defines a protective antibody level against each pneumococcal serotype, whether the protective level is different for each serotype, if antibodies to each serotype are necessary for an adequate immune response, and what constitutes a sufficient response to vaccination⁹⁻¹¹.

Despite difficulties with pneumococcal serotype-specific IgG level interpretation, many patients are subjected to immunoglobulin replacement therapy (IGRT) because of a P23 response considered inadequate. In fact, many CVID patients are started on IGRT before their vaccination response is adequately evaluated given the potential risk for serious infections¹². Measurement of serum antibody levels cannot differentiate between the specific IgG made by the patient's B cells in response to the vaccine and the specific IgG they are potentially receiving in IGRT, which creates a diagnostic dilemma. In contrast, the use of an *ex vivo* functional measurement

that enumerates antigen-specific antibody-secreting cells (ASC) by means of the Enzyme-linked ImmunoSpot assay (ELISPOT) mitigates this problem since results are independent of IGRT¹².

The validity of using the ELISPOT to assess the functional B-cell response to vaccination in CVID patients on IGRT has been established for P23 by Chovancova et al.¹². Thirty-seven patients with CVID were compared to fifty normal individuals, and only the CVID group showed a markedly lower percentage of specific pneumococcal antibody secreting B cells after P23. In the same study, B-cell specific antibody production by ELISPOT was correlated with B-cell subpopulations, primarily plasmablasts. Plasmablasts are best evaluated on day 7 after vaccination by flow cytometry, and their formation has been widely used to analyze early antibody production of B cells in vaccinated individuals¹². Plasmablasts were increased after vaccination for normal individuals but not those with CVID. This observed lack of plasmablasts after P23 in CVID patients indirectly represents poor specific antibody production to the vaccine antigens. These results, however, have not been correlated with serotype-specific IgG levels to P23, which despite its diagnostic difficulties, have been used for several years as a cornerstone of immunologic workup. Furthermore, patients on IGRT without immunodeficiency, given at immunomodulatory doses for different indications, have not been evaluated.

In this exploratory pilot study with limited participants, we first optimized an ELISPOT protocol to assess pre- and post-P23 vaccination response and compared the results with P23 serotype-specific IgG levels. We then prospectively compared the P23 vaccination response by ELIPOST and P23 serotype-specific IgG levels in normal controls and participants on IVIG with and without immunodeficiency.

Methods:

Participants: After obtaining informed written consent, we prospectively enrolled 15 adult participants (8 healthy controls and 7 participants on IVIG) for this study (11 females, 4 males, age range 24-74 years (mean age 45 ± 17 SD) who were seen at National Jewish Health (NJH) in Denver, Colorado from 2017-2018. Access to medical records provided information about previous test results and concurrent medications for participants on IVIG. The 8 healthy controls were labeled Normal Controls (N 1-8). Seven participants were on IVIG replacement: 3 without immunodeficiency on high dose IVIG for other disorders (IC 1-3) and 4 on IVIG replacement for CVID. Two of these participants were labeled with CVID by MD diagnosis only and did not meet ICON criteria for CVID and more likely represent IgG Deficiency (IgG Def-1, IgG Def-2), and 2 participants had CVID based on stringent ICON criteria (CVID-1, CVID-2)¹. Normal controls and CVID-1 were naïve to both P23 and the 13-valent pneumococcal conjugate Prevnar[®] vaccine (PCV13), IC 1-3 had P23 6, 2, and 5 years prior respectively, IgG Def-1 had PCV13 5 years prior and P23 2 years prior, IgG Def-2 had PCV13 2 years prior, and CVID-2 had P23 6 years prior. Detailed information including immunoglobulin replacement product and infusion interval, diagnosis for immunoglobulin replacement, and pneumococcal vaccination status prior to the study were obtained for participants on IVIG (Table 1). This study was approved by the NJH IRB (HS-3069).

IgG Def-1 had frequent reported pneumonias, recurrent HSV infections, and chronic hives with angioedema. IgG Def-1 did not meet ICON criteria for CVID given low total IgG, but normal IgM, normal IgA and protective antibodies to pneumococcal antigens. IgG Def-2 had recurrent pneumonias (annually and chest x-ray confirmed) in the setting of reported recurrent sinusitis about 6 times per year. IgG Def-2 did not meet ICON criteria for CVID given normal IgG, IgM, and IgA. CVID-1 had chronic arthralgias and was hospitalized with pneumonia.

CVID-1 met ICON criteria given low IgG, low IgM, undetectable IgA and no protective antibodies to pneumococcal antigens. Participant CVID-2 had mild cylindrical bronchiectasis, chronic sinusitis confirmed by sinus computed tomography, arthritis, and mild hypothyroidism. CVID-2 met ICON criteria for CVID given undetectable IgG, normal IgM, undetectable IgA, and poor specific antibody response to vaccination (Table 2).

Research Specimens: Serum and heparinized blood specimens were collected before P23 vaccination from all participants and 7-10 days after P23 vaccination from participants on IVIG. An additional serum sample was collected at approximately 4 weeks after P23 vaccination. For participants on IVIG, samples were drawn immediately prior to their next IVIG infusion (trough) for all samples collected both pre- and 4 weeks post-P23 vaccination.

Measurement of P23 Serotype-specific IgG Levels in Serum: P23-serotype-specific IgG levels were measured in serum before and 4 weeks after P23 vaccination. All samples were analyzed simultaneously at the end of the study using a quantitative multiplex bead assay (ARUP Laboratories®, Salt Lake City, UT)¹³⁻¹⁵.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs): Heparinized blood was processed within 1-2 hours after collection using density gradient centrifugation. In brief, diluted whole blood was layered over Histopaque (Sigma-Aldrich, St. Louis, MO) in a 50 mL Leucosep centrifuge tube (Greiner Bio-one, Monroe, NC). The samples were centrifuged to separate mononuclear cells. The mononuclear cell layer was collected and washed twice with Dulbecco's phosphate buffered saline (DPBS). Pelleted cells were resuspended in warm serum-free media (AIM V, Thermo Fisher Scientific, Waltham, MA). PBMC count was obtained using an ACT 10 Hematology Blood Analyzer (Beckman Coulter, Brea, CA).

ELISPOT Assay

Coating of Plates with Antigen: Polyvinylidene fluoride (PVDF) ELISpot plates (Millipore, Burlington, MA) were coated with 10 mcg/mL of the P23 vaccine (Merck, Kenilworth, NJ), diluted in DPBS. P23-coating concentration was determined through several optimization experiments (data not shown). For coating, ELISpot plates were treated with 15 mL of 35% ethanol per well for a maximum of 1 minute and immediately washed 5 times with 200 mL of sterile water per well using a multichannel pipette. After washing, 100 mL per well of P23 at 10 mcg/mL was added to 4 wells. One remaining well was coated with DPBS alone for a no antigen negative control. Coated plates were stored for up to 4 weeks at 4°C.

Blocking, Cell Plating, and Detection of spots: ELISpot plates coated with P23 as described above were washed 5 times with 200 mL per well of DPBS. Wells were then blocked using 200 mL per well of 2% Bovine Serum Albumin (BSA) in DPBS and incubated at room temperature for 1-2 hours. The blocking solution was removed carefully using a multichannel pipette to prevent bubble formation on the well filter. PBMCs were plated at 200,000 cells per 100 mL of AIMV media for 3 of the 4 wells per sample including the no antigen well. For each sample, a well coated with P23 received 100mL of AIMV alone and served as a 'no cells' negative control. Plates were then placed in a humidified incubator at 37°C with 5% CO₂ for 5 hours. Plates were removed from incubator, washed 5 times with 200 mL per well of DPBS and 1 mcg/mL enzyme-conjugated anti-human IgG secondary antibody (MT78-ALP, MabTech) was added at 100 mL per well. The plate was incubated for 2 hours at room temperature, washed 5 times with 200 mL per well of DPBS and 100 mL of BCIP/NBT-plus (MabTech) was added to each well. The plate was placed in the dark for 10 minutes at room temperature and then extensively washed with distilled water to stop color development. Plates were kept in

the dark, allowed to dry overnight and stored at room temperature in aluminum foil. All plates were analyzed at the end of the study using the automated reader system by ZellNet consulting (Fort Lee, NJ).

B-cell Population Analysis: Flow cytometry for plasmablasts was performed on PBMCs from samples collected prior to P23 vaccination and 7-10 days following vaccination¹² for participants on IVIG. In brief, PBMCs were incubated for 30 minutes at room temperature with CD19-PerCP (clone 4G7), CD38-FITC (clone HIT2) and IgM-APC (clone G20-27) (all antibodies were purchased from BD Biosciences, San Jose, CA). Following two washes with DPBS, cells were fixed with 1% methanol-free formaldehyde in DPBS. Data was acquired on a FACS Canto II flow cytometer and analyzed using FACS Diva software (BD Biosciences). Plasmablasts were identified by their expression of CD19 and CD38, and lack of IgM expression.

Statistical Analysis: As there were only two participants in the IgG Def group and two participants in the CVID group, these groups were combined into a Low IgG group when necessary for statistical analysis. Hierarchical cluster analysis was performed using average linkage on log-transformed data [pre and one-week post-vaccination spot forming units per one million cells (SFU/10⁶) for ELISPOT; one month fold change for P23 antibody levels]. Kruskal-Wallis (KW) tests were performed to compare distributions of fold changes for ELISPOT SFU/10⁶ or pneumococcal antibody levels between different combinations of groups (this reduces to the Wilcoxon Rank-Sum (WRS) test for 2 groups); these tests were chosen primarily due to the limited sample sizes available. In all analyses, fold change variables were natural log transformed before analysis. For ELISPOT SFU/10⁶, due to 0 values in data, the pre, post and fold-change variables used in analysis were $\log(\text{pre}+1)$, $\log(\text{post}+1)$ and $\log[(\text{post}+1)/(\text{pre}+1)]$,

respectively. A lattice-ordered test¹⁶ using a Kendall-type statistic was used to test for order of distributions among groups N, IC, and Low IgG participants (IgG Def, CVID), specifically that N participants had the largest distribution of fold changes and that the Low IgG participants (IgG Def, CVID) had the smallest distribution of fold changes, but no other order among groups was specified. Like the WRS test, the lattice-order test is nonparametric and uses ranks of the data instead of actual values.

Results:

Participant Characteristics: We enrolled a total of 15 participants for this study (Table 1). The majority were female (73%) and IC, IgG Def, and CVID participants were Caucasian. N consisted of 4 Caucasians (50%), 1 African American (12.5%), 2 Mixed Hispanic/Caucasian (25%), and 1 Mixed Asian/Caucasian (12.5%). IC were on daily oral corticosteroids and received higher immunomodulatory doses of IVIG (2030 ± 244 mg/kg total per month) compared to the replacement doses of the IgG Def and CVID participants (407 ± 84 mg/kg total per month).

Pneumococcal 23 Serotype-Specific IgG Levels: One-month fold change in P23 antibody levels after P23 vaccination was calculated for all serotypes (Figure 1, eTable 1-2). N demonstrated P23 response for all serotypes ($p < 0.05$) and patients on IVIG including IVIG Controls (IC, IgG Def, CVID) did not ($p > 0.1$ except serotypes 2, 11A, and 33F [$p = 0.06$]). Comparing N to participants on IVIG (IC, IgG Def, CVID) using WRS tests, P23 antibody levels were significantly different for all variables ($p < 0.05$) except serotypes S3 ($p = 0.28$), S19A ($p = 0.08$) and S20 ($p = 0.054$). Comparing Low IgG participants (IgG Def, CVID) and IC, no 2-

sided test demonstrated statistical significance. Cluster analysis clearly formed two main clusters based on membership in N or not (eFigure 1).

ELISPOT Assay Spot Forming Units: ELISPOT response one-week post P23 vaccination was performed for all participants (Figure 2). N had a significant ELISPOT ASC response to P23 ($p=0.008$); ASC post-vaccination were significantly higher for N compared to other groups ($p=0.006$). ELISPOT ASC responses of IC and Low IgG participants (IgG Def, CVID) were not statistically different ($p=0.54$, WRS). However, when analyzing IgG Def and CVID as their own groups, there was a significant difference between all groups ($p=0.001$, KW). Notably, CVID participants did not demonstrate an ELISPOT ASC response. When evaluating IC and IgG Def together, they had a substantial ELISPOT ASC response to P23 ($p=0.06$, the smallest possible 2-sided p-value for $n=5$).

Considering 4 groups (N, IC, IgG Def, and CVID), a lattice-order test¹⁶ demonstrated that N participants had the largest distribution of values among all groups, and CVID participants had the smallest distribution of values ($p=0.0004$). Cluster analysis for ELISPOT response to P23 formed three notable clusters: one cluster with all N barring one; a second cluster with IC, IgG Def and the remaining N participant; and a third cluster with CVID participants (Figure 3). The last cluster containing CVID participants was separated furthest from all others.

Plasmablast analysis: Analysis of B-cell subpopulations performed for participants on IVIG (IC, IgG Def, and CVID) indicated that the plasmablast response to P23 (eTable 3) generally correlated with ELISPOT results. CVID participants showed the lowest frequency of plasmablasts post-P23 (eFigure 2).

Discussion:

Here we describe the clinical utility of an optimized ELISPOT protocol to assess P23-specific antibody responses in comparison to the current standard of measuring pre- and post-vaccination P23 serotype specific IgG levels¹⁷. For P23 serotype-specific IgG response to vaccination, we chose the most common method of clinical interpretation, which is to evaluate the fold change 1-month post vaccination to each serotype individually¹⁷. P23 serotype-specific IgG vaccine response was significantly higher in N compared to other groups for almost all serotypes. Cluster analysis easily split all participants into two groups, N or not, the latter including all patients on IVIG. Our findings are in line with the consensus that P23 serotype-specific IgG levels are not reliable in patients on IVIG¹⁷. IVIG is pooled from the general population and should therefore contain specific antibodies to P23 antigens either from prior vaccination or exposure. IC, IgG Def, and CVID participants had higher baseline values presumably due to P23 IgG they received in their IVIG and likely therefore did not demonstrate a significant fold change in response to P23. The ELISPOT assay, best performed one-week post-vaccination¹², quantifies ASC and is independent of circulating antibodies. Therefore, the ELISPOT assay should be reliable in patients on IVIG. Our study showed a robust vaccine response per ELISPOT ASC one week after P23 in N, with a much lower response in IC, IgG Def, and CVID participants. Although the ELISPOT response was lower in Low IgG participants (IgG Def and CVID), this difference was not significantly different compared to IC.

We included IC to help differentiate between patients with the inability to make specific antibody with immunocompetent patients subjected to the immunomodulatory effects of IVIG itself (IC)¹⁸. Replacement dosing for the IgG Def and CVID groups (~400-600mg/kg per month) was lower than immunomodulatory dosing (~1000-2000mg/kg per month) used in IC. IC were also older and on daily oral corticosteroids. Taken together these differences in IC may

have depressed the ELISPOT ASC response enough to make them similar to IgG Def and CVID participants. Another possibility is that the IgG Def participants were able to make specific antibody and therefore could be considered more IC than CVID. In fact, when IC and IgG Def were combined, they had a substantial ELISPOT ASC response to P23 ($p=0.06$, the smallest possible 2-sided p -value for $n=5$) while CVID participants had no ELISPOT ASC response at all. This was further supported by cluster analysis, which could not differentiate IC from IgG Def participants, while N barring one, and CVID participants formed their own clusters. A lattice-order test also demonstrated a striking difference between N and CVID participants. Plasmablasts generally correlated with ELISPOT results, but future studies are needed to determine whether plasmablast formation could serve as an initial screen for pneumococcal vaccine response.

Recent evidence suggests that patients who do not meet strict criteria for CVID, such as IgG Def-1 and IgG Def-2 in our study, can be considered “IgG-deficient” rather than having true CVID. Patients with CVID and IgG deficiency do not seem to share the same disease manifestations: CVID patients have lower IgG levels, lower responsiveness to vaccines, and lower percentages of memory and isotype switched memory B cells¹⁹. The distinction of true CVID patients from those with IgG deficiency or CVID not meeting criteria, is supported by our data on assessment of the P23 vaccine response by ELISPOT and plasmablast formation. However, more research is needed to corroborate the findings of this exploratory pilot study.

To our knowledge this is the first study to assess an optimized ELISPOT for P23 response in comparison with the current standard of measuring P23 serotypes specific IgG levels. We prospectively compared these assays in normal controls, participants with Low IgG (IgG deficiency and CVID) on replacement dose IVIG and participants without immunodeficiency on

high dose IVIG, a group not yet studied for ELISPOT response. As this was an exploratory pilot study, the main limitation was the low number of participants. We were unable to control for demographics and participants were mostly Caucasian females of different ages with varying vaccination histories. Additionally, the ELISPOT is a functional assay and requires fresh whole blood, making the assay less accessible. However, the ability to assess B-cell function in patients on IVIG may be worth the extra effort in some clinical cases, especially when the indication for continued IVIG is primarily based on the ability to produce specific antibodies to pneumococcal antigens. Further studies, with age- and sex-matched controls are therefore needed to evaluate the utility of the ELISPOT assay in evaluation of CVID patients on IVIG in response to P23. Finally, Janoff et al showed that IgA plays an important role in mediating protection against *Streptococcus pneumoniae* infection²⁰. Therefore, comparison of our results with P23-specific IgA secreting cells would be interesting as these analyses have previously shown promise¹².

Our exploratory pilot study suggests that an optimized ELISPOT protocol has utility to assess the P23-specific antibody response after vaccination. When compared to the current standard of measuring P23-serotype-specific IgG levels or “pneumococcal titers,” the ELISPOT assay seemed reliable for patients on IGRT and may help differentiate true CVID patients from those with a less stringent diagnosis while on IGRT.

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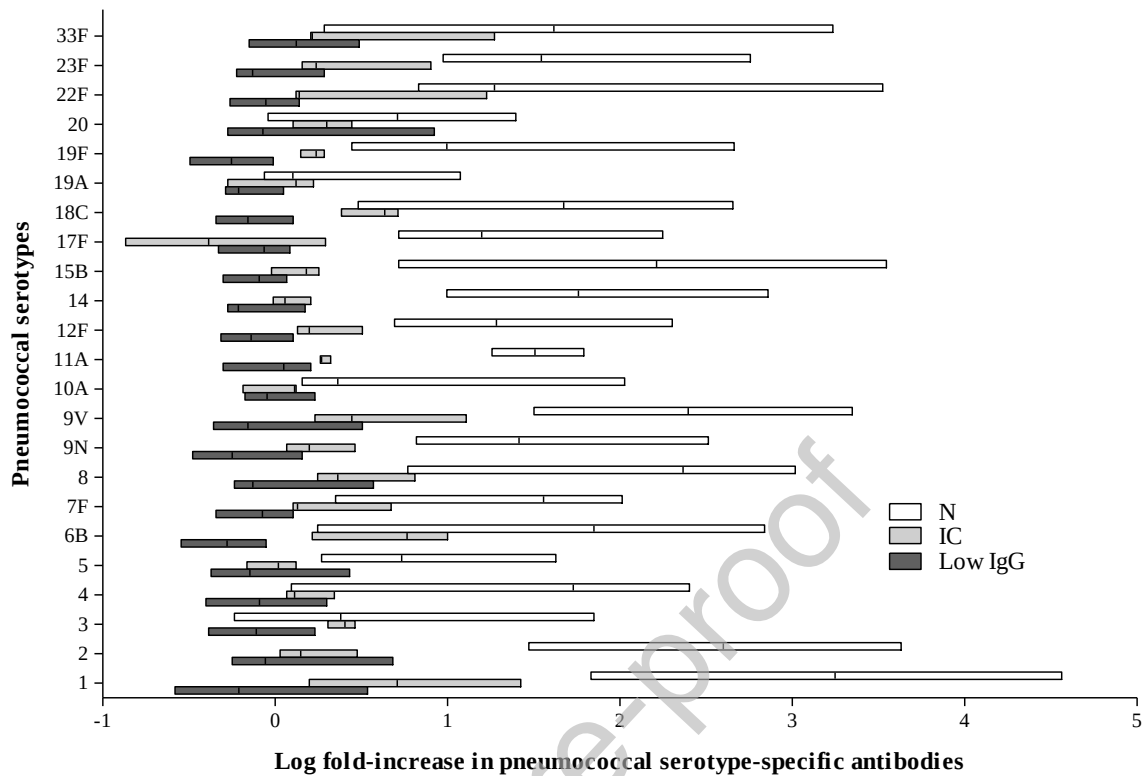


Figure 1. Pneumococcal serotype-specific IgG response 4 weeks after Pneumovax (P23). Normal controls (N) demonstrated P23 response for all serotypes ($p < 0.05$) and patients on IVIG including IVIG Controls (IC), and Low IgG (CVID and IgG deficiency) did not ($p > 0.1$ except serotypes 2, 11A, and 33F [$p = 0.06$]). Fold change natural log transformed prior to analysis. Bars represent minimum and maximum with median as dividing line.

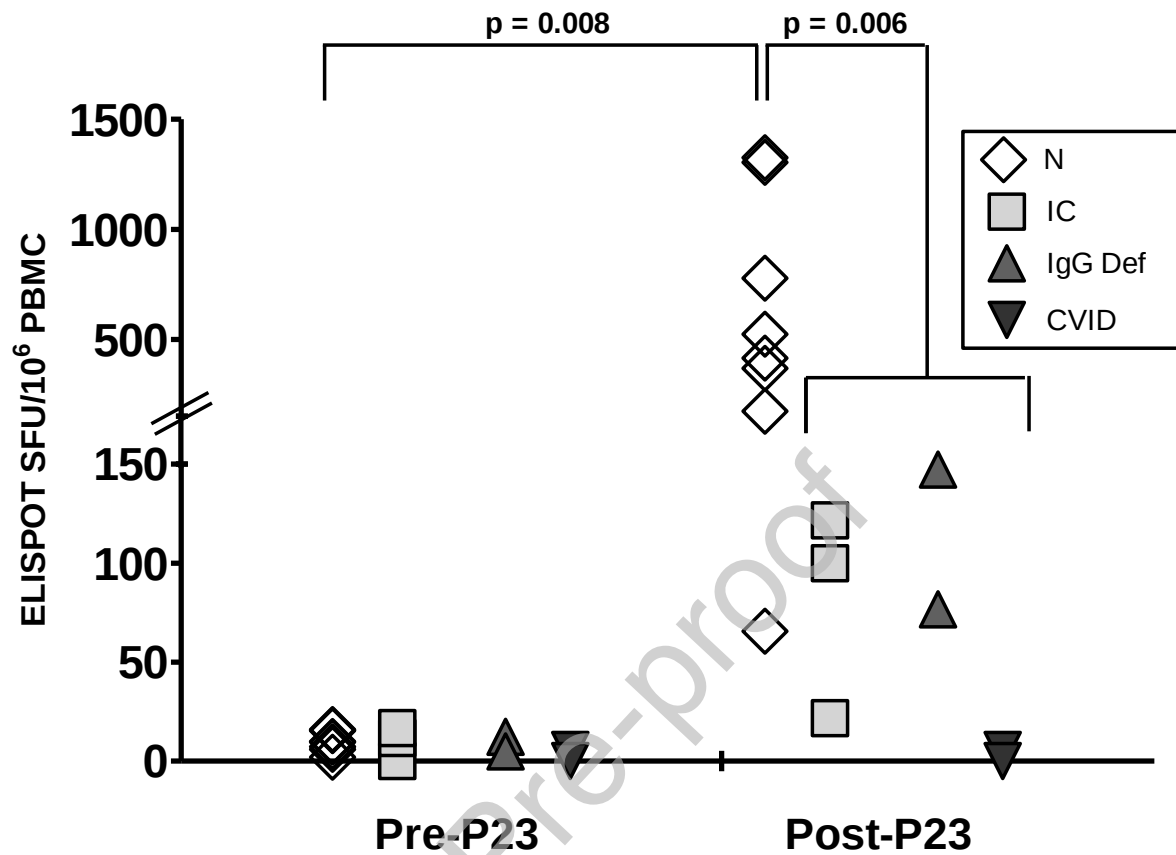


Figure 2. ELISPOT response after Pneumovax (P23). Normal controls (N) had a significant ELISPOT antibody secreting cell (ASC) response one-week post-P23 ($p=0.008$), and fold changes were significantly higher for N compared to other groups ($p=0.006$). CVID participants did not show any ELISPOT ASC response.

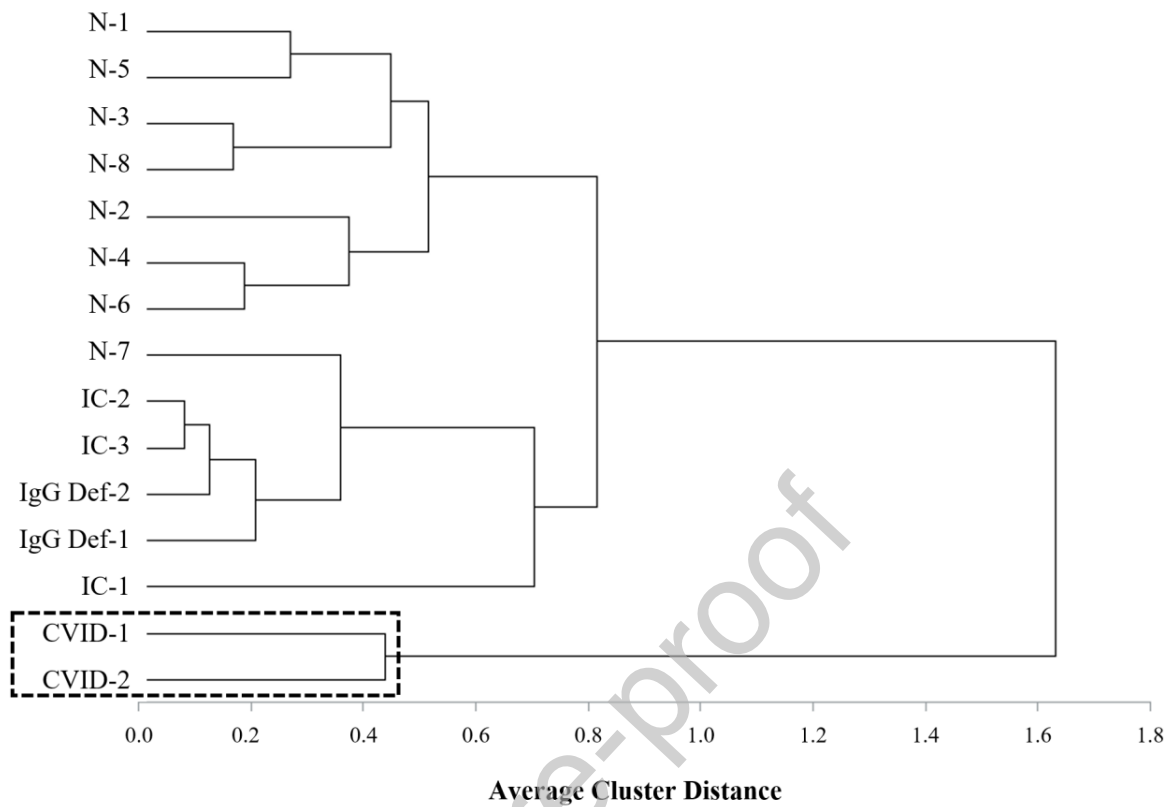


Figure 3. Cluster analysis of ELISPOT response after Pneumovax (P23) vaccination. CVID participants separated furthest from all other groups. Cluster analysis performed on natural log of ELISPOT spot forming units per million cells.

Table 1. Participant characteristics

Participant	Not on IVIG	Participants on IVIG						
	N-1-8	IC-1	IC-2	IC-3	IgG Def-1	IgG Def-2	CVID-1	CVID-2
Age (y)	Range 24-46 Mean 32 ± 8 SD	63	62	46	74	71	53	57
Gender	3 M, 5 F	F	M	F	F	F	F	F
Indication for IVIG	*	Asthma	Small fiber neuropathy	Demyelinating neuropathy	CVID	CVID	CVID	CVID
IVIG total dose per month	*	Gamunex-C® 2272mg/kg	Gamunex-C® 1785mg/kg	Privigen® 2034mg/kg	Privigen® 332mg/kg	Privigen® 395mg/kg	Privigen® 373mg/kg	Gamunex-C® 526mg/kg
IVIG dose interval	*	q2weeks	Split over 5 days, q4weeks	Split over 5 days, q4weeks	q4weeks	q4weeks	q4weeks	q4weeks
OCs	*	Methyl-prednisolone 64mg daily	Prednisone 10mg daily	Prednisone 17mg daily	*	*	*	*
PCV13 prior to study	*	*	*	*	2013	2016	*	*
P23 prior to study	*	2012	2016	2013	2016	*	*	2012

* Did not receive

CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59]; IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; N = Normal controls; OCs = oral corticosteroids; PCV13 = 13-valent pneumococcal conjugate vaccine Prevnar® 13; P23 = 23-valent polysaccharide Pneumovax® vaccine; SD = Standard deviation

Table 2. Laboratory workup for participants with a CVID prior to study

ICON Criteria for CVID met	Reference Range	Participants			
		IgG Def-1 No	IgG Def-2 No	CVID-1 Yes	CVID-2 Yes
IgG (mg/dL)	700 - 1620	530	1063	173	<75
IgM (mg/dL)	44 - 266	49	86	38	56
IgA (mg/dL)	50 - 462	80	239	<0.7	<0.7
Tetanus IgG pre/post (IU/mL)	0.1 - 13.1	3.71 / 22.78	* / *	0.24 / *	0 / 0.73
Hib IgG pre/post (IU/mL)	0.09 - 17.7	0.14 / >9.00	6.25 / *	<0.11 / *	<0.11 / <0.11
Pneumo. IgG pre P23 ≥ 0.35mcg/mL	†	12 of 12 (100%)	8 of 14 (57%)	8 of 12 (67%)	0 of 12 (0%)
Pneumo. IgG post P23 ≥ 0.35mcg/mL	≥50%‡	12 of 12 (100%)	8 of 14 (57%)	*	0 or 12 (0%)
Pneumo. IgG pre P23 ≥ 1.3mcg/mL	≥ 70%§	10 of 12 (83%)	5 of 14 (36%)	0 of 12 (0%)	0 of 12 (0%)
Pneumo. IgG post P23 ≥ 1.3mcg/mL	≥ 70%§	12 of 12 (100%)	7 of 14 (50%)	*	0 of 12 (0%)
Pneumo. IgG post P23 ≥ 2-fold response	≥ 70%§	7 of 12 (58%)	0 of 14 (0%)	*	0 of 12 (0%)
CD3 T cells/mcL (%)	678 - 2504 (62-88%)	1017 (74%)	1101 (63%)	781 (75%)	903 (56%)
CD4 T cells/mcL (%)	414 - 1679 (35-61%)	778 (56%)	848 (48%)	569 (55%)	622 (39%)
CD8 T cells/mcL (%)	162 - 1038 (11-37%)	222 (16%)	270 (15%)	207 (20%)	244 (15%)
CD19 B cells/mcL (%)	96 - 515 (6-20%)	258 (19%)	390 (22%)	147 (14%)	515 (32%)
Naïve B cells (IgD ⁺ /CD27 ⁻)	30-70%	78.4%	93.3%	77.4%	*
Unswitched memory B cells (IgD ⁺ /CD27 ⁺)	8-35%	15.9%	2.8%	17.8%	*
Switched memory B cells (IgD ⁻ /CD27 ⁺)	8-42%	4.4%	2.8%	3.6%	*
Immature B cells (CD38 ^{low} /CD21 ^{low})	0.5-8.0%	6.2%	0.8%	10.3%	6.2%
Transitional B cells (CD38 ^{high} /IgM ^{high})	0.3-9.2%	8.1%	5.3%	4.5%	4.7%
Plasmablasts (CD38 ^{high} /IgM ⁻)	0.1-4.7%	0.0%	0.3%	1.0%	2.8%

* Test not performed

†World Health Organization found a serotype-specific IgG level of ≥0.35 mcg/mL protective based on studies of invasive pneumococcal disease in infants who had received the pneumococcal conjugate vaccine [WHO Tech Rep Ser. 2005;927(2):64-98]

‡ Adequate response to Pneumovax has been considered when ≥50% of serotype-specific IgG levels above 0.35 mcg/mL after vaccination [Front Immunol. 2017;8doi:10.3389]

§For patients > 6 years of age, serotype-specific IgG ≥1.3mcg/mL can be considered protective. Pre-vaccination of ≥70% protective serotypes or post-vaccination 2-fold increase of ≥70% serotypes can be considered adequate [JACI. Sep 2012;130(3 Suppl):S1-24. doi:10.1016]

CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59]; IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; Hib = *Haemophilus influenzae* type b; Pneumo. = Pneumococcal Serotypes; P23 = 23-valent polysaccharide Pneumovax[®] vaccine

eTable 1. Pre-P23 Pneumococcal serotype-specific IgG

Participant	Pneumococcal serotypes (mcg/mL)																						
	1	2	3	4	5	6B	7F	8	9N	9V	10A	11A	12F	14	15B	17F	18C	19A	19F	20	22F	23F	33F
N1	0.2	0.3	2.4	0.1	2.3	0.2	14.1	0.2	0.2	0.3	3.3	2.0	0.2	0.4	0.3	1.4	2.6	4.5	0.6	1.0	1.1	0.0	0.3
N2	3.1	5.7	2.6	0.3	15.6	0.7	6.7	2.2	4.4	0.6	12.7	3.6	0.8	15.7	9.5	7.4	7.0	29.7	10.2	6.4	1.1	0.6	7.6
N3	1.2	4.0	5.3	0.4	12.1	0.7	1.4	0.5	4.2	0.5	29.4	0.5	1.1	4.6	3.3	10.6	1.7	37.8	3.1	9.6	7.2	0.4	2.8
N4	0.2	0.2	0.9	0.1	6.2	0.3	1.9	0.2	0.3	0.1	4.9	2.3	0.2	0.5	7.0	2.9	7.0	4.3	1.8	1.8	7.5	1.2	0.2
N5	1.2	0.2	0.1	2.3	1.2	2.0	1.0	0.1	1.2	0.1	3.2	2.5	0.5	5.7	1.9	4.6	0.8	15.0	1.1	1.8	3.0	0.6	1.5
N6	0.3	0.4	3.0	0.1	2.3	0.2	0.6	0.9	0.2	0.2	5.7	2.4	0.1	0.5	0.2	1.2	0.9	20.0	0.7	1.1	0.5	0.3	0.5
N7	0.1	0.3	1.0	0.5	1.3	0.2	0.6	0.2	0.3	0.3	3.2	0.1	0.2	0.7	0.3	1.0	1.0	3.1	0.5	1.1	3.1	0.5	0.2
N8	0.2	0.7	2.8	0.6	3.4	0.6	0.9	7.2	1.2	0.6	4.1	1.8	0.4	1.1	0.9	1.3	3.7	11.2	7.9	3.5	3.4	0.7	2.6
IC1	3.1	5.3	10.9	0.9	8.2	2.9	22.2	1.8	4.6	1.6	11.7	5.0	4.3	17.9	13.2	7.9	5.1	19.3	7.4	8.0	4.4	2.2	3.1
IC2	9.0	7.3	3.4	0.9	8.9	3.0	6.1	2.6	3.9	4.4	10.7	5.7	0.9	16.3	9.2	10.0	4.9	19.3	8.3	8.4	4.9	7.5	3.7
IC3	3.6	2.8	2.1	0.6	6.0	1.8	17.2	1.6	2.3	1.0	9.0	3.7	0.7	12.1	6.2	6.1	3.2	14.6	14.3	3.2	3.7	1.7	3.4
IgG Def-1	5.6	3.7	11.3	1.8	5.2	15.9	17.0	2.0	9.0	2.7	4.5	3.6	0.6	12.9	5.5	12.3	9.7	22.8	9.9	3.1	8.2	2.7	3.8
IgG Def-2	4.9	5.2	5.5	0.8	11.9	2.7	20.2	2.0	4.1	1.9	8.7	3.3	1.0	13.1	10.6	12.9	12.1	23.0	10.9	6.2	5.0	2.4	6.2
CVID-1	1.9	1.9	1.9	0.4	4.7	1.6	2.9	1.1	1.6	0.9	5.0	1.9	0.5	9.9	5.0	4.2	2.8	12.3	4.3	2.8	2.4	1.3	1.7
CVID-2	1.7	1.9	1.6	0.4	4.4	1.5	2.8	1.2	1.5	0.8	5.7	2.1	0.5	10.0	5.0	4.6	2.8	12.1	4.3	2.9	2.6	1.5	1.9

CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59]; IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; N = Normal controls; P23 = 23-valent polysaccharide Pneumovax[®] vaccine

eTable 2. One Month Post-P23 Pneumococcal serotype-specific IgG

Participant	Pneumococcal serotypes (mcg/mL)																						
	1	2	3	4	5	6B	7F	8	9N	9V	10A	11A	12F	14	15B	17F	18C	19A	19F	20	22F	23F	33F
N-1	8.5	10.2	1.9	0.8	5.0	0.8	20.1	3.7	1.2	2.1	9.7	10.6	0.4	2.5	2.9	4.6	11.2	13.3	1.2	1.8	3.8	0.6	1.9
N-2	19.2	24.6	4.8	1.6	20.6	1.3	29.8	16.5	13.8	2.9	19.0	14.6	3.9	42.4	20.6	24.5	45.2	34.6	16.0	18.2	2.6	2.3	10.1
N-3	19.2	17.6	16.4	2.5	31.2	1.0	8.3	5.6	16.7	13.4	40.1	1.8	2.6	26.7	7.7	21.7	18.2	35.6	5.3	9.2	18.3	1.3	4.5
N-4	19.2	3.2	1.1	0.3	17.6	2.1	3.0	4.5	1.8	1.3	11.1	13.6	0.6	8.4	14.4	18.6	11.3	7.4	25.1	3.7	20.4	14.6	4.6
N-5	11.5	2.1	0.9	2.6	6.3	19.8	5.1	1.4	2.6	1.4	24.4	14.0	5.3	33.6	17.0	15.4	11.1	26.8	8.2	4.2	13.6	3.1	10.9
N-6	9.7	4.8	16.4	1.6	4.5	3.3	2.9	10.3	0.9	3.6	7.3	10.2	1.0	5.3	5.6	2.7	6.3	20.2	2.9	2.2	17.3	0.7	1.5
N-7	4.2	11.7	1.2	2.5	1.7	0.9	2.4	2.2	1.2	3.5	3.8	0.6	0.3	4.4	9.4	3.5	3.1	3.1	1.3	1.5	11.2	2.6	1.4
N-8	5.3	13.5	3.2	6.0	6.2	8.6	6.7	15.6	14.3	2.7	5.6	6.9	1.6	3.2	17.3	12.3	15.5	11.7	21.7	14.0	12.3	2.8	11.0
IC-1	3.8	8.5	16.4	1.0	8.4	6.1	25.3	2.3	4.9	2.0	13.1	6.6	5.0	17.7	16.9	5.4	9.6	21.9	8.6	10.7	5.0	2.6	3.9
IC-2	18.2	8.4	4.6	1.2	10.1	3.7	6.8	3.7	6.2	13.4	12.1	7.4	1.0	20.0	11.1	13.3	7.2	24.0	10.6	13.2	5.5	9.6	4.6
IC-3	14.9	2.9	3.3	0.7	5.1	5.0	33.7	3.7	2.8	1.6	7.5	5.1	1.2	12.8	6.1	2.5	6.6	11.1	19.0	3.5	12.7	4.2	12.2
IgG Def-1	9.6	3.9	14.2	2.4	8.0	15.0	16.6	3.5	10.6	4.4	4.7	4.4	0.6	15.3	5.9	13.4	10.8	24.1	9.8	3.4	8.7	3.6	4.7
IgG Def-2	4.8	10.2	3.7	0.6	10.6	1.9	22.4	1.7	2.8	1.6	7.3	3.7	0.9	10.0	8.9	12.7	11.6	17.6	9.3	4.9	4.2	1.9	10.1
CVID-1	1.0	1.6	1.8	0.4	3.3	0.9	2.0	1.1	1.0	0.6	4.3	1.4	0.4	7.5	3.7	3.0	2.0	9.2	2.6	2.2	2.7	1.0	1.4
CVID-2	1.2	1.5	1.4	0.3	3.7	1.2	2.5	1.0	1.3	0.7	7.3	2.0	0.5	8.6	4.9	4.1	2.2	10.2	3.1	7.4	2.0	1.4	2.0

CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59]; IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; N = Normal controls; P23 = 23-valent polysaccharide Pneumovax[®] vaccine

eTable 3. Pneumococcal serotype-specific IgG by participant group

Group	Pre/Post P23	Pneumococcal serotypes (mcg/mL; median [25 th - 75 th percentile])																						
		1	2	3	4	5	6B	7F	8	9N	9V	10A	11A	12F	14	15B	17F	18C	19A	19F	20	22F	23F	33F
N-1-8	Pre	0.3 [0.2-1.2]	0.3 [0.3-1.5]	2.5 [1.0-2.9]	0.3 [0.1-0.5]	2.8 [2.0-7.7]	0.4 [0.2-0.7]	1.2 [0.8-3.1]	0.4 [0.2-1.2]	0.7 [0.3-1.9]	0.3 [0.2-0.5]	4.5 [3.2-7.4]	2.1 [1.5-2.4]	0.3 [0.2-0.6]	0.9 [0.5-4.9]	1.4 [0.3-4.2]	2.2 [1.3-5.3]	2.1 [0.9-4.5]	13.1 [4.5-22.4]	1.4 [0.6-4.3]	1.8 [1.1-4.2]	3.1 [1.1-4.3]	0.5 [0.3-0.6]	1.0 [0.3-2.6]
	Post	10.6 [7.7-19.2]	11.0 [4.4-14.5]	2.6 [1.2-7.7]	2.1 [1.4-2.5]	6.3 [4.9-18.3]	1.7 [0.9-4.6]	5.9 [3.0-11.2]	5.0 [3.3-11.6]	2.2 [1.2-13.9]	2.8 [1.9-3.5]	10.4 [6.9-20.3]	10.4 [5.6-13.7]	1.3 [0.5-2.9]	6.9 [4.1-28.4]	11.9 [7.2-17.0]	13.9 [4.3-19.3]	11.2 [9.9-16.2]	16.7 [10.6-28.7]	6.7 [2.5-17.4]	4.0 [2.1-10.4]	12.9 [9.3-17.6]	2.4 [1.1-2.9]	4.6 [1.8-10.3]
IC-1-3	Pre	3.6 [3.4-6.3]	5.3 [4.0-6.3]	3.4 [2.7-7.1]	0.9 [0.7-0.9]	8.2 [7.1-8.6]	2.9 [2.4-2.9]	17.2 [11.6-9.7]	1.8 [1.7-2.2]	3.9 [3.1-4.2]	1.6 [1.3-3.0]	10.7 [9.8-11.2]	5.0 [4.3-5.4]	0.9 [0.8-2.6]	16.3 [14.2-17.1]	9.2 [7.7-11.2]	7.9 [7.0-8.9]	4.9 [4.1-5.0]	19.3 [17.0-19.3]	8.3 [7.9-11.3]	8.0 [5.6-8.2]	4.4 [4.1-4.6]	2.2 [2.0-4.9]	3.4 [3.3-3.6]
	Post	14.9 [9.4-16.6]	8.4 [5.7-8.5]	4.6 [3.9-10.5]	1.0 [0.8-1.1]	8.4 [6.7-9.2]	5.0 [4.4-5.6]	25.3 [16.0-29.5]	3.7 [3.0-3.7]	4.9 [3.8-5.5]	2.0 [1.8-7.7]	12.1 [9.8-12.6]	6.6 [5.8-7.0]	1.2 [1.1-3.1]	17.7 [15.3-18.9]	11.1 [8.6-14.0]	5.4 [3.9-9.3]	7.2 [6.9-8.4]	21.9 [16.5-23.0]	10.6 [9.6-14.8]	10.7 [7.1-12.0]	5.5 [5.3-9.1]	4.2 [3.4-6.9]	4.6 [4.2-8.4]
Low IgG	Pre	3.4 [1.8-5.1]	2.8 [1.9-4.1]	3.7 [1.8-6.9]	0.6 [0.4-1.0]	4.9 [4.6-6.9]	2.2 [1.6-6.0]	9.9 [2.9-17.8]	1.6 [1.2-2.0]	2.8 [1.6-5.3]	1.4 [0.8-2.1]	5.4 [4.8-6.5]	2.7 [2.0-3.3]	0.6 [0.5-0.7]	11.4 [10.0-12.9]	5.3 [5.0-6.8]	8.5 [4.5-12.5]	6.3 [2.8-10.3]	17.6 [12.3-22.9]	7.1 [4.3-10.2]	3.0 [2.9-3.8]	3.8 [2.6-5.8]	1.9 [1.4-2.5]	2.9 [1.9-4.4]
	Post	3.0 [1.1-6.0]	2.7 [1.6-5.5]	2.8 [1.7-6.3]	0.5 [0.4-1.1]	5.9 [3.6-8.6]	1.5 [1.1-5.2]	9.5 [2.3-18.1]	1.4 [1.0-2.1]	2.1 [1.2-4.8]	1.2 [0.7-2.3]	6.0 [4.6-7.3]	2.9 [1.8-3.9]	0.5 [0.4-0.7]	9.3 [8.3-11.3]	5.4 [4.6-6.7]	8.4 [3.9-12.8]	6.5 [2.1-11.0]	13.9 [10.0-19.2]	6.2 [2.9-9.4]	4.2 [3.1-5.6]	3.5 [2.6-5.3]	1.7 [1.3-2.3]	3.3 [1.9-6.0]

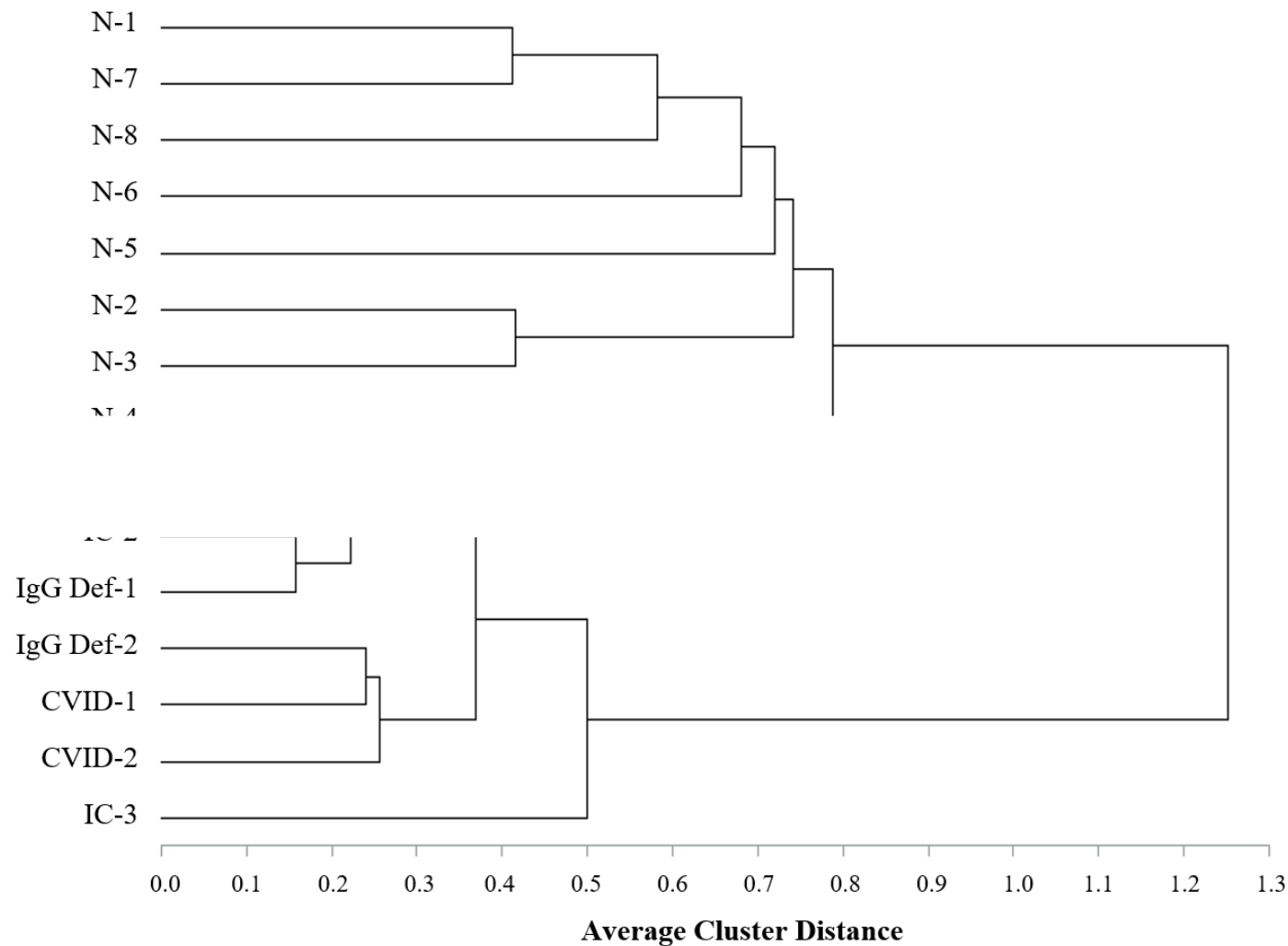
Low IgG group includes IgG Def-1-2 and CVID-1-2

CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59];

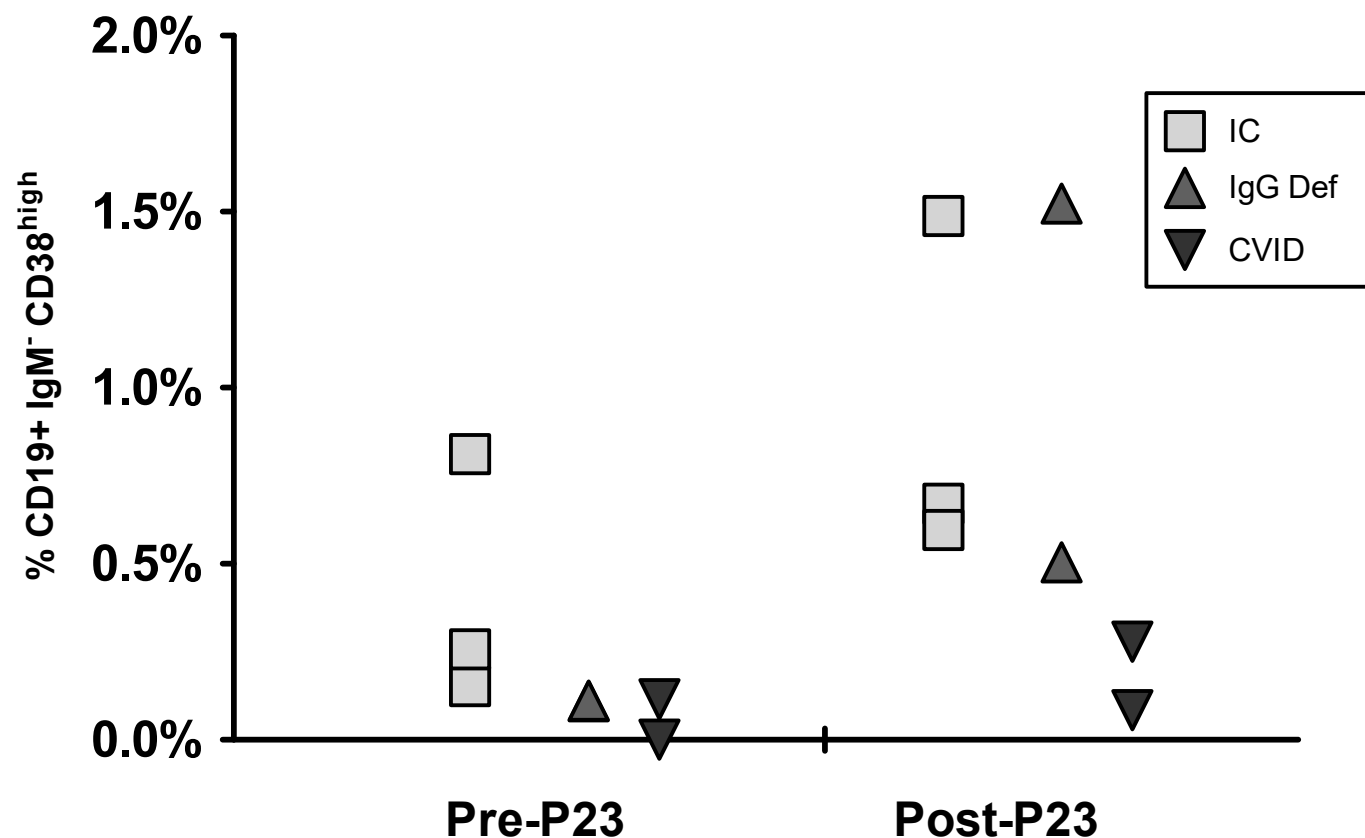
IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; N = Normal controls; P23 = 23-valent polysaccharide Pneumovax[®] vaccine

eTable 4. B-cell phenotyping by flow cytometry for participants on IVIG

Reference Range		Participant						
		IC-1	IC-2	IC-3	IgG Def-1	IgG Def-2	CVID-1	CVID-2
CVID by ICON criteria		NA	NA	NA	No	No	Yes	Yes
B cells (CD19+) pre / post %	6.3-20.0%	6.1 / 4.7	6.6 / 4.6	8.1 / 11.7	22.0 / 14.6	19.6 / 11.2	5.2 / 5.8	21.7 / 31.6
Immature B cells (CD38 ^{low} /CD21 ^{low}) pre / post %	0.5-8.0%	4.0 / 7.4	5.5 / 5.8	10.0 / 8.5	1.9 / 1.9	0.5 / 1.1	14.7 / 10.7	4.4 / 9.0
Transitional B cells (CD38 ^{high} /IgM ^{high}) pre / post %	0.3-9.2%	7.0 / 3.6	0.4 / 0.2	9.3 / 8.7	5.0 / 7.2	8.7 / 4.4	4.2 / 3.2	7.9 / 9.1
Plasmablasts (CD38 ^{high} /IgM ⁻) pre / post %	0.1-4.7%	0.2 / 0.7	0.8 / 1.5	0.3 / 0.6	0.1 / 0.5	0.1 / 1.5	0.1 / 0.3	0.0 / 0.1
CVID = participants with common variable immunodeficiency (CVID) who met International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59]; IC = IVIG Controls on IVIG for immunomodulation without immunodeficiency; IgG Def = participants with a MD diagnosis of CVID prior to the study who did not meet ICON criteria and therefore defined as IgG deficiency; NA = not applicable; P23-valent polysaccharide Pneumovax [®] vaccine								



eFigure 1. Cluster analysis of P23 serotype-specific IgG levels after Pneumovax (P23) vaccination. Natural log fold change 1 month after P23 vaccination separated normal controls (N) from all others including participants on IVIG for immunomodulation without immunodeficiency (IC), and participants with CVID meeting International Consensus Document (ICON) criteria for CVID, [JACI: In Practice 4.1 (2016): 38-59] and participants with IgG deficiency (IgG Def).



eFigure 2. Plasmablast response after Pneumovax (P23) vaccination for participants on IVIG. One-week post-P23, CVID patients who met International Consensus Document (ICON) criteria for CVID [*JACI: In Practice* 4.1 (2016): 38-59] had the lowest plasmablast response compared to participants with IgG deficiency (IgG Def) and IC (IVIG Controls on IVIG for immunomodulation without immunodeficiency). Participant numbers were too low for calculations of statistical significance. Plasmablast analysis based on enumeration of CD19⁺IgM⁻CD38^{high} cells from B cell subpopulation analysis of peripheral blood mononuclear cells.