Poor correlation between pneumococcal IgG and IgM titers and opsonophagocytic activity in vaccinated patients with multiple myeloma and Waldenstrom’s macroglobulinemia

Johanna Karlsson\textsuperscript{abc,}\textsuperscript{#}, Lucy Roalf\textsuperscript{c}, Harriet Hogevik\textsuperscript{b}, Marta Zancolli\textsuperscript{d}, Björn Andréasson\textsuperscript{c}, David Goldblatt\textsuperscript{d}, and Christine Wennerås\textsuperscript{b}

\textsuperscript{a}Department of Infectious Diseases, NU Hospital Group, Trollhättan/Uddevalla, Sweden, \textsuperscript{b}Department of Infectious Diseases, Sahlgrenska Academy, University of Gothenburg, Göteborg, Sweden, \textsuperscript{c}Department of Hematology and Coagulation, Sahlgrenska Academy, University of Gothenburg, Göteborg, Sweden, \textsuperscript{d}Immunobiology Section, Institute of Child Health, University College London, London, UK, \textsuperscript{e}Department of Hematology/Internal Medicine, NU Hospital Group, Trollhättan/Uddevalla, Sweden

Running title: Pneumococcal ELISA and OPA in B cell disorders

\textsuperscript{#}Corresponding author: Johanna Karlsson, Department of Infectious Diseases, Sahlgrenska Academy, University of Gothenburg, Guldhedsgatan 10, SE 413 46 Göteborg, Sweden. Phone: 46 31 3424623. E-mail: johanna.karlsson@vgregion.se
ABSTRACT

Patients with multiple myeloma and other B cell disorders respond poorly to pneumococcal vaccination. Vaccine responsiveness is commonly determined by measuring pneumococcal serotype-specific antibodies by enzyme-linked immunosorbent assay (ELISA), a functional opsonophagocytosis assay (OPA) or by both assays. We compared the two methods in vaccinated elderly patients with multiple myeloma, Waldenstrom’s macroglobulinemia, and monoclonal gammopathy of undetermined significance (MGUS). Post-vaccination sera from 45 patients (n = 15 from each patient group) and 15 control subjects were analyzed by multiplexed OPA for pneumococcal serotypes 4, 6B, 14, and 23F and compared to IgG and IgM antibody titers measured by ELISA. While there were significant correlations between pneumococcal OPA and IgG titers for all serotypes among the control subjects (correlation coefficients (r) between 0.51 and 0.85), no significant correlations were seen for any of the investigated serotypes in the myeloma group (r = -0.18-0.21), or in the group with Waldenstrom’s macroglobulinemia (borderline significant correlations for 2 out of 4 serotypes). The MGUS group resembled the control group by having good agreement between the two test methods for 3 out of 4 serotypes (r = 0.53-0.80). Pneumococcal post-vaccination IgM titers were very low in the myeloma patients compared to the other groups, and did not correlate with the OPA results. To summarize, our data indicate that ELISA measurements may over-estimate anti-pneumococcal immunity in elderly subjects with B cell malignancies, and that a functional antibody test should be used particularly for myeloma and Waldenstrom’s macroglobulinemia patients.
INTRODUCTION

Patients with multiple myeloma (MM) and other clonal B cell disorders such as Waldenstrom’s macroglobulinemia (WM) and monoclonal gammopathy of undetermined significance (MGUS) are at increased risk of contracting and succumbing to pneumococcal infections (1-4). In a study of invasive pneumococcal disease the attack rate in MM patients was 674 cases/100,000 per year compared to 11 cases/100,000 per year in an adult population ≥ 18 years (OR 62.8) (5). A recent Swedish study of MM and infection showed an increased risk of pneumonia with a HR of 7.7 compared to matched population-based controls (6). Kristinsson et al. found an increased risk of pneumonia in MGUS patients with a HR of 2.4 (4).

The susceptibility to pneumococcal infection among patients with B cell disorders is primarily due to an overproduction of non-functional immunoglobulins called M-protein and, as a result, varying degrees of hypogammaglobulinemia (1, 2). Antibody-mediated opsonisation of bacteria followed by phagocytosis is considered to be a critical immune defense mechanism against pneumococci. However, the immune deficiency in patients with B cell disorders encompasses not only B cell dysfunction but also defective cellular immunity with reduced numbers and function of T cells, NK cells, and dendritic cells (2). Decreased numbers of CD4+ T cells is a feature of MM as well as WM and MGUS (7, 8). This is also seen in HIV patients, another risk group of pneumococcal infection (9).

Pneumococcal vaccination is recommended for myeloma patients even though they are known to be poor responders as defined by poor IgG responses post vaccination measured by ELISA (1, 10-13). In a previous study, we reported that pneumococcal vaccination not only evoked poor responses in MM patients, but also in elderly patients with WM and MGUS compared to a control group with subjects within the same age range but without hematological disorders (14).

Pneumococcal vaccine studies in high risk populations are generally small and thus preclude any clinical endpoints and measures of vaccine effectiveness. Instead, such studies are designed with immunogenicity endpoints, and serotype-specific IgG is often used as a surrogate marker for assessment of likely vaccine effectiveness. A correlate of protection of 0.35 μg/ml serotype-specific IgG (measured by ELISA) was derived as a population-based correlate in infants and has been used for comparing and licensing new pneumococcal conjugate vaccines (15, 16).
aggregate correlate for all serotypes has recently been refined and new serotype-specific correlates for both ELISA and functional antibodies in infants have been proposed (17). The pneumococcal serotype-specific IgG ELISA has been the standard method due to its relative simplicity and cost-effectiveness but it has its drawbacks, not least the capture of potentially non-functional, cross-reacting antibodies, although this has largely been overcome by adsorption of cell wall polysaccharide and heterologous pneumococcal polysaccharide (22F) (18). The assessment of the functionality of pneumococcal antibodies by a biological opsonophagocytosis assay (OPA) is more complicated and time-consuming but measures the functional antibody titers. It encompasses mixing various dilutions of patient sera with phagocytic HL60 cells, complement, and live pneumococci, and has the actual number of surviving bacteria as the read-out. It has been multiplexed for several serotypes and is regarded as the gold standard for the evaluation of pneumococcal vaccine responses (19).

Determination of anti-pneumococcal IgG levels by means of later generations of serotype-specific ELISA has been shown to correlate well with opsonic activity in children but more poorly in adults, in particular in the elderly population (19, 20). The knowledge regarding the performance of the ELISA for determination of pneumococcal vaccine efficacy in high-risk groups such as immunocompromised patients is limited. Patients with B cell malignancies have not been investigated in this aspect. In our previous study, we found unexpectedly high anti-pneumococcal antibody levels among some myeloma patients, possibly indicative of cross-reactive, non-functional antibodies (14). We therefore wished to assess the reliability of the serotype-specific pneumococcal ELISA in patients with multiple myeloma and related B cell disorders, who, due to their susceptibility to pneumococcal infections, constitute a target group for vaccination. To this end, we compared the results from serotype-specific IgG ELISA with those of a standardized OPA. Since the antibodies that mediate killing-type OPA may be of any isotype, we also investigated the post-vaccination serum levels of IgM and their correlation to OPA titers.
MATERIALS AND METHODS

Study population. In an earlier study we investigated pneumococcal vaccine responses in 56 patients aged ≥ 60 years with MM (n = 24), WM (n = 15) or MGUS (n = 17), and 20 control subjects without hematological disorders but within the same age range, recruited at the Department of Hematology, Uddevalla Hospital, Uddevalla, Sweden, between May 2008 and March 2009 (14). The study persons were randomized to receive a single dose (0.5 ml) of either the 7-valent conjugated pneumococcal vaccine (PCV7; Prevenar; Pfizer) or the 23-valent polysaccharide pneumococcal vaccine (PPV; Pneumo 23; Sanofi Pasteur). Serum samples were collected before and 4-8 weeks after vaccination, and were stored at -20°C until analyzed. The study was approved by the Regional Ethics Committee in Göteborg, and written informed consent was obtained from all patients.

From the above mentioned study population, 15 patients from each disease group and 15 control subjects were randomly selected for re-analysis of their post-vaccination sera by four-fold multiplexed OPA and pneumococcal serotype-specific IgM ELISA. Both assays were performed at the World Health Organization (WHO) reference laboratory for pneumococcal serology at the Institute of Child Health, University College of London, UK. Similar proportions in each study group were immunized with conjugate or polysaccharide vaccines.

IgG pneumococcal ELISA. The serum concentrations of IgG antibodies to the pneumococcal serotypes 4, 6B, 14, and 23F were analyzed in our previous study (14) at Statens Serum Institut, Copenhagen, Denmark by an ELISA employing cell wall polysaccharide adsorption and described in detail elsewhere (21). A local reference serum calibrated to the US standard reference serum 89-SF was used. Antibody concentrations were expressed as μg/ml. The investigated pneumococcal serotypes are included in both vaccine types employed in the study.

Opsonophagocytosis assay (OPA). Sera were shipped frozen to the University College London, Institute of Child Health (UCL ICH) laboratory and analyzed for functional antibodies to pneumococcal serotypes 4, 6B, 14, and 23F by the multiplexed OPA as previously described (22). Each serum was tested in duplicate.

Twelve OPA results out of a total of 240 analyses (5%) did not pass the assay acceptance criteria despite retesting (three analyses of serotype 4 and 6B, respectively, one analysis of serotype 14,
and five of serotype 23F), and were excluded. The samples failed due to incomplete killing of the bacteria (between 40 and 70%). In the assay in use, samples are only accepted if they do not kill, i.e., show killing ≤ 40% (negative samples), or if they display killing ≥ 70%. Samples with values between 40 and 70% are usually “low killing” and tend to give inconsistent results and are therefore not reported.

Four study patients were treated with antibiotics (two MM patients and one WM patient had cotrimoxazole in prophylactic dosage, one MM patient had cloxacillin) when post-vaccination sera were collected. To exclude the possibility that residual concentrations of antibiotics had interfered with the OPA analysis, the bactericidal effect of their serum samples were tested (test wells containing bacteria, patient serum, and buffer) and compared to the complement controls of the standard assay (test wells containing bacteria, HL-60 effector cells, complement, and buffer). Since no significant titer differences were found, these samples were not excluded from the study.

**IgM pneumococcal ELISA.** Serum was assayed for IgM antibodies to pneumococcal serotypes 4, 6B, 14 and 23F at the UCL ICH laboratory by adapting the previously described IgG ELISA ([http://www.vaccine.uab.edu/ELISA%20Protocol.pdf](http://www.vaccine.uab.edu/ELISA%20Protocol.pdf)) (23). Each serum was tested in duplicate.

**Statistical analyses.** Geometric means with 95% confidence intervals were calculated for ELISA and OPA titers. OPA titers below the detection limit of 8 were set to 4. Comparisons between study groups were done using the Mann-Whitney U test. Correlations between ELISA and OPA titers were calculated using the Spearman rank correlation test. P values were two-sided. A significance level of P < 0.05 was used. Graph Pad Prism 5.0 software was used for all statistical analyses (GraphPad, San Diego, CA).
RESULTS

Study population. Patient characteristics are presented in Table 1. Half of the study persons were female (55%). The median age was significantly higher in the MM and WM groups (80 and 75 years, respectively) than in the MGUS and the control study groups (71 and 68 years, respectively; \( P < 0.05 \)). Eleven of the MM patients (73%) were on active cancer treatment during the study, only one of the WM patients (Table 1).

Serology. Since no significant differences in either OPA, IgG or IgM antibody titers/concentrations for any of the four serotypes were found between the subgroups of patients vaccinated with PCV7 or PPV the data was pooled for the correlation analyses (data not shown).

OPA titers. All three patient groups had lower anti-pneumococcal OPA titers than the control subjects (Figure 1). This was significant for all four pneumococcal serotypes in the group of WM patients, for three serotypes (4, 6B, 14) in the MM group, and for two serotypes (6B, 14) among the MGUS patients (Figure 1). The distribution between the study groups of the un-reportable OPA results was as follows: 2 results from the MM group, 5 results from the WM group, 3 results from the MGUS group, and 2 results from control subjects. Eight out of the 12 un-reportable results were from individual samples and individual serotypes, 4 results were from 2 WM patients, each with two serotypes affected (6B and 23F, 4 and 23F, respectively). There was no obvious skew regarding the unreported OPA results in favor of certain serotypes or patient groups.

Correlations between ELISA IgG and OPA titers. The correlations between anti-pneumococcal IgG concentrations and functional antibody titers as measured by OPA are presented in Figure 2. While there were significant correlations between ELISA concentrations and OPA titers for all four pneumococcal serotypes among the control subjects (correlation coefficients \( r \) between 0.51 and 0.85), no correlations were found for any of the investigated serotypes in the myeloma group (\( r \) between -0.18 and 0.21). Correlations were significant for three serotypes (4, 14, 23F) among the MGUS patients (\( r \) between 0.53 and 0.80) and almost reached significance for serotype 4 and 23F in the WM group (\( r = 0.54 \) and 0.53; \( P = 0.06 \)), Figure 2.
ELISA IgM concentrations to pneumococci. IgM antibody titers were low among the MM patients for all four pneumococcal serotypes, both in relation to the vaccine-induced IgG titers and in comparison with the IgM responses seen in the other three study groups ($P < 0.001$ for MM patients vs. control subjects for serotypes 4, 6B, and 14; $P < 0.05$ for serotype 23F) (Figure 3). The control subjects, patients with WM, and MGUS patients had comparable IgM titers after vaccination (Figure 3).

Correlations between ELISA IgM and OPA titers. The correlations between ELISA IgM and OPA titers were generally poor and inconsistent. Significant correlations were found only for WM patients regarding serotype 6B, and for the control subjects regarding serotypes 4 and 14 (Table 2). No significant correlation coefficients were seen for either MM or MGUS patients (Table 2).
DISCUSSION

In a previous study of pneumococcal vaccine responses in elderly patients with multiple myeloma, Waldenstrom’s macroglobulinemia and monoclonal gammopathy of undetermined significance, we suspected falsely high ELISA IgG titers, in particular among the myeloma patients (14). We therefore wished to assess the ELISA results by comparison with OPA findings. Excellent correlation between IgG concentrations determined by ELISA and OPA titers were seen for the control subjects for all four serotypes. In contrast, multiple myeloma patients showed no positive correlation between IgG and OPA titers for the four serotypes studied. High ELISA IgG titers with no functional activity were noted in some MM patients. Of the remaining two groups of patients studied, the MGUS patients resembled the control subjects by having good correlations between their IgG and OPA titers for 3 out of 4 of the serotypes while the Waldenstrom’s macroglobulinemia patients had intermediate correlations.

Discrepancies in the outcome of pneumococcal ELISA and OPA such as those described in this study have previously been noted for other immunocompromised patients with increased risk of pneumococcal infections, for example HIV patients (9) and renal transplant patients (24). In contrast, our results differ from a study of allogeneic stem cell transplanted patients with hematological malignancies, where significant correlations between anti-pneumococcal IgG and OPA titers were found for all seven investigated pneumococcal serotypes (25). Of note is that the patients in that study were younger than ours (mean age 37 years), had different hematological diagnoses and thus may have been able to mount better quality IgG responses. Also, correlations were modest with $r$ values ranging from 0.48 to 0.76.

Age is an important factor when evaluating pneumococcal vaccine responses. Previous studies have shown poorer functional antibody responses after vaccination with PPV among elderly adults compared to younger adults (20, 26, 27). The 13-valent conjugate pneumococcal vaccine (PCV13), which was launched after the initiation of this study, has been shown to prevent invasive pneumococcal disease and community-acquired pneumonia in adults > 65 years (28); however, vaccine efficacy declined with increasing age (29). Since the control subjects and the MGUS patients in this study were significantly younger than the MM and WM patients, this might have influenced our results with poorer OPA concentrations in the more diseased (and older) study groups (MM, WM).
The weak correlations between ELISA-determined IgG concentrations and OPA titers among MM and WM patients in the present study could also be affected by the advanced age of these study persons, which is in accordance with previous studies (19, 20). Romero-Steiner et al. found poorer correlations between post-vaccination IgG antibody concentrations and OPA titers in a group of elderly adults (mean age 85 years) compared to younger adults (mean age 37 years). This was explained by lower antibody avidity to capsular polysaccharides among the elderly (20) and corroborated by showing that transfer of human serum with a high OPA titer and high avidity protected mice in an experimental model of pneumococcal infection, whereas serum with low avidity and low OPA titer did not despite containing the same concentration of anti-pneumococcal IgG (20).

In general, higher anti-pneumococcal IgG levels are required to achieve the same killing capacity in elderly adults compared to younger (20, 26, 27, 30). Besides differences in avidity (20), high age has been linked to loss of oligoclonality in response to pneumococcal antigens, reduced function of phagocytic cells, and decreased levels of anti-pneumococcal IgM as a consequence of deficient IgM memory B cells (26, 31, 32). In the present study, IgM concentrations were markedly reduced in the myeloma patients compared to the other study groups. In contrast, serum IgM levels were relatively high among the WM patients; however, a significant correlation with OPA titers was only seen for 1 out of 4 tested serotypes. Our results showing poor or no correlation between IgM and OPA titers for all study groups indicate that lack of IgM was not the main reason for poor OPA function among our study subjects.

A possible reason for the poor correlations between the ELISA and OPA analyses among our disease groups might be interference of the M-proteins with the ELISA analysis. This has been shown for various laboratory tests such as blood counts and electrolytes but also for immunological tests such as anti-streptococcal antibodies (33). Unfortunately we had too little serum left to measure the M-protein levels in the post-vaccination samples and could not correctly test this hypothesis. However, this should not be the sole explanation since the correlations between IgG and OPA titers were next-to-normal among the MGUS patients, who had pre-vaccination levels of M-protein that were almost as high as in the WM group (median 11 g/L, range 0.5 – 28 g/L compared to a median of 15 g/L, range 3 – 28 g/L among the WM...
patients). The OPA responses might also be affected by deficient cellular immunity, which has been shown to be more pronounced in MM and WM patients compared to MGUS patients (8).

The major limitation of this study was the small series of patients. The age difference between 2 out of 3 patient groups (MM, WM) and the control subjects may also have affected the results. Nevertheless, an evaluation of methods for measuring pneumococcal vaccine responses has to our knowledge not previously been done for patients with B cell malignancies and related hematological disorders. Since patients with these diagnoses have a high incidence of pneumococcal infections and thus are target groups of pneumococcal vaccination, it should be of interest to find a sufficiently reliable method of measuring their vaccine responses.

Our main finding was that pneumococcal antibody measurement by ELISA in vaccinated elderly patients with multiple myeloma and Waldenström’s macroglobulinemia did not correlate with antibody titers determined by the multiplex OPA. We suggest that ELISA measurements may over-estimate anti-pneumococcal immunity in patients with B cell malignancies and propose the use of a functional antibody assay, in particular for multiple myeloma and Waldenström’s macroglobulinemia patients.

**FUNDING INFORMATION**

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REFERENCES


FIGURE LEGENDS

FIG. 1. Opsonophagocytic antibody (OPA) titers after pneumococcal vaccination. (A), serotype 4; (B), serotype 6B; (C) serotype 14; (D) serotype 23F. Each triangle represents one study person. Horizontal bars denote the geometric mean value for each study group. Statistical comparisons have been performed for the patient groups, respectively, vs. the control group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Mann-Whitney U test).

FIG. 2. Correlations between anti-pneumococcal IgG titers (ELISA) and opsonophagocytic antibody titers (OPA) after pneumococcal vaccination. (A), serotype 4; (B), serotype 6B; (C) serotype 14; (D) serotype 23F. MM, multiple myeloma; WM, Waldenstrom’s macroglobulinemia; MGUS, monoclonal gammopathy of unknown significance. Diamonds indicate persons vaccinated with 7-valent conjugated pneumococcal vaccine (PCV7), circles indicate persons vaccinated with 23-valent pneumococcal polysaccharide vaccine (PPV). Note that the values on the X and Y axes vary between the different pneumococcal serotypes (A-D). r and P values were calculated by Spearman rank sum test.

FIG. 3. ELISA IgM titers after pneumococcal vaccination. Corresponding IgG titers are shown for comparison (B, D, F, H). Each triangle represents one study person. Horizontal bars denote the geometric mean value for each study group. (A, B), serotype 4; (C, D), serotype 6B; (E, F) serotype 14; (G, H) serotype 23F. Statistical comparisons have been performed for the patient groups, respectively, vs. the control group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Mann-Whitney U test).
TABLE 1. Study group characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Multiple myeloma</th>
<th>Waldenstrom’s macroglobulinemia</th>
<th>Monoclonal gammopathy of undetermined significance</th>
<th>Control subjects</th>
<th>All groups</th>
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<tbody>
<tr>
<td>No. Patients</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>Median age, yrs (range)</td>
<td>80 (65-88)</td>
<td>75 (62-88)</td>
<td>71 (60-77)</td>
<td>68 (61-83)</td>
<td>75 (60-88)</td>
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<tr>
<td>Female sex, n</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>M-protein median, g/L (range)(a)</td>
<td>24 (0.7-44)</td>
<td>15 (3.0-28)</td>
<td>11 (0.5-28)</td>
<td>0 (0-0)</td>
<td>-</td>
</tr>
<tr>
<td>Treatment regimens, n</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Melphalan + prednisone</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Cyclophosphamide + dexamethasone</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
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<td>1</td>
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<td>1</td>
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<tr>
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<td>1</td>
<td>0</td>
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<td>1</td>
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<td>Ongoing antibiotic treatment, n</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<td>Given pneumococcal vaccine, n</td>
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<td>PCV7(b)</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>29</td>
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<tr>
<td>PPV23(c)</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>31</td>
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</table>

\(a\) Pre-vaccination levels of monoclonal (M-)protein in serum.

\(b\) PCV7, 7-valent pneumococcal conjugate vaccine; \(c\) PPV, 23-valent pneumococcal polysaccharide vaccine.
TABLE 2. Correlation of ELISA IgM and opsonophagocytosis assay for four pneumococcal serotypes and four study groups. Each group contains 15 post-vaccination serum samples.

<table>
<thead>
<tr>
<th>Study group</th>
<th>4</th>
<th>6B</th>
<th>14</th>
<th>23F</th>
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<tbody>
<tr>
<td>MM</td>
<td>0.39</td>
<td>0.20</td>
<td>0.07</td>
<td>0.04</td>
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<tr>
<td>WM</td>
<td>0.54</td>
<td></td>
<td>0.04</td>
<td>0.58</td>
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<tr>
<td>MGUS</td>
<td>-0.16</td>
<td>0.18</td>
<td>0.44</td>
<td>0.28</td>
</tr>
<tr>
<td>Controls</td>
<td>0.56*</td>
<td>0.40</td>
<td>0.90***</td>
<td>0.09</td>
</tr>
</tbody>
</table>

MM, multiple myeloma; WM, Waldenstrom’s macroglobulinemia; MGUS, monoclonal gammopathy of unknown significance

*, P < 0.05; ***, P < 0.001 (Spearman rank sum test)
C  **Serotype 14**

**MM**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for MM.](image)

- \( r = 0.21 \)
- \( P = 0.44 \)

**WM**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for WM.](image)

- \( r = 0.36 \)
- \( P = 0.18 \)

**MGUS**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for MGUS.](image)

- \( r = 0.55 \)
- \( P = 0.04 \)

**Control**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for Control.](image)

- \( r = 0.51 \)
- \( P = 0.05 \)

D  **Serotype 23F**

**MM**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for MM.](image)

- \( r = 0.16 \)
- \( P = 0.57 \)

**WM**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for WM.](image)

- \( r = 0.53 \)
- \( P = 0.06 \)

**MGUS**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for MGUS.](image)

- \( r = 0.80 \)
- \( P = 0.0005 \)

**Control**

![Graph showing OPA titer vs. IgG ELISA (µg/ml) for Control.](image)

- \( r = 0.85 \)
- \( P = 0.0001 \)