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Citation: Gardulf, A., Abolhassani, H., Gustafson, R., Eriksson, L. E. ORCID: 0000-0001-5121-5325 and Hammarström, L. (2018). Predictive markers for humoral influenza vaccine response in patients with common variable immunodeficiency (CVID). *Journal of Allergy and Clinical Immunology*, doi: 10.1016/j.jaci.2018.02.052

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1 **Predictive markers for humoral influenza vaccine response in**
2 **patients with common variable immunodeficiency (CVID)**

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26

27 **Running title:** Humoral response to A(H1N1) pandemic influenza vaccine in COVID

28

29 **Statement of financial sources:** The study was funded by the Jeffrey Modell Foundation,
30 New York; the Swedish Research Council, Stockholm, Sweden; and research funds from
31 Karolinska Institutet, Stockholm, Sweden.

32

33 **Conflict of interests:** The authors declare that there was no financial or personal relationship
34 with third parties whose interests could be positively or negatively influence the content.

35 **Abstract**

36 **Background:** A subgroup of patients with common variable immunodeficiencies (CVID)
37 responds to vaccination. The aim of the study was to try to identify predictive markers for
38 those who developed a humoral immune response after influenza vaccination.

39 **Methods:** 48 patients with CVID (29 females, 19 males, mean age 59.4 years) were
40 vaccinated with the A(H1N1) influenza vaccine Pandemrix[®] and boosted after one month.
41 Blood samples were collected prior to each vaccination and two months later. Patients with a
42 4-fold titer increase of the hemagglutinin inhibition test ($\geq 1:40$) were considered responders
43 and compared to non-responders for clinical, immunological and genetic markers.

44 **Results:** Eight (16.7%) patients responded to the vaccination. A significantly higher
45 proportion of the responders, who showed a Euroclass SmB^{norm}Tr^{norm}21^{norm} profile ($p=0.03$) with
46 a post-germinal center B cell pattern ($p=0.04$) in blood, suffered from enteropathies ($p=0.04$)
47 as compared to non-responders. Bronchiectasis on the other hand, was exclusively found
48 among non-responders ($n=7$), as was autoimmune cytopenia ($n=5$). Non-responders with a
49 Euroclass SmB^{low}Tr^{norm}21^{norm} profile ($p=0.02$), had a significantly higher prevalence of
50 progressive antibody deficiency ($p=0.048$) and, at diagnosis, a higher mean serum IgM level
51 ($p=0.03$), a lower mean serum IgG1 level ($p=0.007$), an expansion of absolute counts of
52 cytotoxic CD8⁺ T-cells ($p=0.033$) and an increased proportion of memory CD8⁺ T-cells
53 ($p=0.044$) in blood. CVID associated HLA markers were not detected in non-responders
54 ($p=0.03$).

55 **Conclusion:** About one-fifth of the CVID patients achieved protective antibody levels after
56 A(H1N1) vaccination and selected clinical and immunological markers were identified that
57 may predict a positive outcome of influenza vaccination.

58 **Key messages:**

- 59 • A subgroup of patients with CVID develops a humoral immunological response from
60 influenza vaccination.
- 61 • Selected clinical and immunological markers may help to identify a possible positive
62 influenza vaccination outcome and are enteropathy, Euroclass SmB⁺Tr^{norm}21^{norm} and
63 post-germinal center B-cell pattern.
- 64 • HLA CVID genetic predisposing markers, pronounced IL-12 production and Th1
65 polarity were observed only in non-responders.
- 66 • Despite the fact that not all patients with CVID will develop protective antibody levels
67 after influenza vaccination it is concluded that they should be offered this prophylactic
68 measure due to the potential severity of influenza and risk for bacterial complications.

69

70 **Capsule Summary:** One-fifth of the patients with CVID vaccinated against pandemic
71 influenza A(H1N1) converted to a $\geq 1:40$ titer of specific antibodies against the antigen and
72 selected clinical and immunological predictive markers were identified in this subgroup.

73

74 **Keywords:** Common variable immunodeficiency, CVID, specific antibody deficiency,
75 vaccination, influenza, pandemic influenza, immune response, A(H1N1), Pandemrix

76 **Abbreviations:**

77 **CVID:** Common variable immunodeficiency

78 **DNA:** Deoxyribonucleic acid

79 **ELISA:** Enzyme-linked immunosorbent assay

80 **HA:** Hemagglutinin

81 **HI:** Haemagglutination inhibition

82 **HLA:** Human leukocyte antigen

Unmarked

- 83 **Igs:** Immunoglobulins
- 84 **IFN- γ :** Interferon gamma
- 85 **IL:** Interleukin
- 86 **PBS:** Phosphate-buffered saline
- 87 **PCR:** Polymerase chain reaction
- 88 **PID:** Primary immunodeficiency disorder
- 89 **PHA:** Phytohemagglutinin
- 90 **RDE:** Receptor-destroying enzymes
- 91 **WHO:** World Health Organization
- 92

93 Introduction

94 Common variable immunodeficiency (CVID) is a heterogeneous primary
95 immunodeficiency disorder (PID) characterized by low serum concentrations of
96 immunoglobulins (Igs) and impaired specific antibody production.¹⁻³ Specific antibody
97 deficiency is a diagnostic criterion in most of the standard clinical guidelines and constitutes
98 the absence of natural Igs (e.g. isohemagglutinins) or poor response to novel protein or
99 polysaccharide antigens (e.g. in vaccines).^{2, 4, 5}

100 Approximately 10-20% of clinically diagnosed patients with CVID have a residual
101 response to vaccination against protein antigens and, to a lesser extent, against polysaccharide
102 antigens.⁶⁻¹² The heterogeneity in specific antibody defects in CVID patients may be due to
103 the association of this complex disease with several different genetic defects.^{13, 14} Although it
104 is uncertain whether vaccination with killed/inactivated vaccines will protect an individual
105 with CVID, the use of these vaccines is nevertheless recommended for diagnostic purposes.^{10,}
106 ¹⁵ Moreover, the use of killed/inactivated vaccines to patients with PID is indeed
107 recommended to follow the same schemes as in general populations.¹⁶

108 Current evidence suggests that a subgroup of patients with CVID is not only capable of
109 re-stimulation *in vitro* for production of class-switched Igs but may show residual antibody
110 production *in vivo*.¹⁷⁻¹⁹ Characterization of this subgroup of patients regarding their clinical,
111 immunological and genetic profile could potentially help developing targeted therapy for
112 these patients^{18, 20} and provide evidence-based advises regarding administering influenza
113 vaccine to patients with CVID.

114 To our knowledge, the immunological response to the same antigen as used in the current
115 study - A(H1N1) pandemic influenza vaccine (antigen X179a 2009, Pandemrix[®]) - has so far
116 only been investigated in three patients with CVID and one patient with X-linked
117 agammaglobulinemia in a 3-months long follow-up study.¹⁹ Two of the patients with CVID
118 responded to the vaccination by a >4-fold rise in haemagglutination inhibition (HI) antibodies

119 and all three showed a Th1-cell response. It was concluded that some patients with CVID
120 might produce an influenza-specific humoral immune response, but that this should be
121 confirmed in larger studies.

122 The aim of the study was to try to identify clinical, immunological and genetic predictive
123 markers for those patients with CVID who developed a humoral immune response after
124 influenza vaccination. This was performed by investigating to what extent a group of patients
125 with CVID responded to vaccination by producing protective antibodies against the
126 glycopeptide pandemic influenza A(H1N1) antigen.

127 Material and methods

128 The present study had a prospective design, monitoring patients with CVID before and up to
129 one year after vaccination with the pandemic influenza A(H1N1) vaccine (Pandemrix[®],
130 GlaxoSmithKline, Belgium).

131

132 Patients

133 Patients with CVID cared for and receiving Ig replacement therapy at the
134 Immunodeficiency Unit at the Karolinska University Hospital in Stockholm, Sweden, and
135 who had not already been vaccinated against the A(H1N1) pandemic virus were invited to
136 participate in the study carried out during 2010-2011. None of the patients used any steroids
137 or any other immuno-suppressive therapy during the study period and 3 months prior to study
138 start. All patients had been diagnosed as having CVID based on the diagnostic criteria
139 established by the European Society for Immunodeficiencies
140 (<http://esid.org/WorkingParties/Registry/Diagnosis-criteria>) and the American Academy of
141 Allergy, Asthma and Immunology practice parameter for the diagnosis and management of
142 PID.^{4, 21} Before study start, all patients were re-evaluated for fulfilling the diagnostic criteria
143 of CVID and secondary causes of dysgammaglobulinemia were ruled out.²² None of the
144 invited patients presented with protein losing enteropathy, nor with pre-study protective
145 A(H1N1) pandemic virus antibody levels ($\geq 1:40$ titer).

146 Fifty-seven patients gave their informed consent and volunteered to participate in the
147 study by receiving the first dose of the vaccine. Nine patients (five women and four men,
148 mean age 48.8 \pm 20.3) dropped out of the study; seven at the first follow-up visit and two
149 patients at the second follow-up visit. Totally 48 patients (60.4% females) participated in all
150 appointments and were included in the final vaccine response analyses. Forty-seven of the
151 patients received regular weekly subcutaneous (minimum 100 mg/kg/week) and one patient
152 received monthly intravenous (400 mg/kg/month) Ig replacement therapy before and during

153 the entire study period. Different brands were used but each patient received the same brand
154 and batch of Ig at least three months before and at least until the last blood sampling.

155 An evaluation document was used to summarize the demographic information of each
156 patient including age at diagnosis, family history and history of previous vaccinations shown
157 as specific antibody responses, and clinical, immunological and genetic data.

158

159 ***Study overview***

160 The A(H1N1) pandemic influenza vaccine Pandemrix[®], a monovalent X179a 2009
161 vaccine, was injected intramuscularly at study start and boosted once after one month. In each
162 vaccination dose, 3.75 µg of hemagglutinin (HA) of the X179a antigen was administered. At
163 study start, concentrations of total IgG, IgG subclasses, IgA and IgM were determined in fresh
164 serum samples taken immediately prior to the next Ig substitution. Specific antibody titers for
165 antibodies against the pandemic influenza A(H1N1) antigen were measured in samples
166 collected prior to the first and the second vaccination and also two months after the second
167 dose. Follow-up visits due to the vaccine study were scheduled for the patients at regular
168 intervals until one year after study start for the recording of any influenza-like symptoms
169 and/or incidental side effects of the vaccination.

170

171 ***Investigation and definition of vaccination response***

172 One volume of serum was treated with four volumes of neuraminidase receptor
173 destroying enzyme (RDE, Seiken, Japan) in phosphate-buffered saline (PBS) at 37°C
174 overnight before inactivation at 56°C for 30 minutes to prevent non-specific inhibition. Serial
175 two-fold dilutions of RDE treated sera were then incubated with eight haemagglutinating
176 units of the X179a pandemic influenza A(H1N1) antigens for one hour, followed by the
177 addition of 0.7% ([volume of solute/volume of solution] ×100%) turkey erythrocytes. After
178 30 minutes of incubation, the HA inhibition titers were read as the reciprocal of the highest

179 dilution at which 50% HA was inhibited. Each serum sample was run in duplicate in two
180 independent experiments and the titers are presented as a mean of these two experiments for
181 each patient.

182 A positive response to the pandemic influenza A(H1N1) antigen was defined as a 4-fold
183 titer increase of the HA inhibition test ($\geq 1:40$).²³ Patients with such increase are hereinafter
184 referred to as responders.

185

186 ***Clinical and immunological phenotyping and immunological assays***

187 The clinical phenotyping of the 48 patients was based on the suggested division into
188 distinct clinical phenotypes by Chapel *et al.*²⁴ and included patients with infections but no
189 other disease-related complications or patients with infections and autoimmunity, infections
190 and enteropathy and/or infections and polyclonal lymphocytic infiltration. Patients with ≥ 3
191 clinical findings were classified as overlapping phenotypes. Clinical data were collected from
192 the medical and nursing records and covered the time from diagnosis to the study start.

193 Thirty-eight of the patients had previously been classified based on two main
194 classifications for B-cell subsets including the Paris, Freiburg, EUROclass²⁵ and the B-cell
195 pattern classification.²⁶ In 27 of the patients, ionizing radiation sensitivity assays had
196 previously been performed on primary fibroblasts irradiated with different doses and serial
197 dilutions.²⁷ This is a method used for classification of patients with CVID and defective DNA
198 repair machinery required for variable (V), diversity (D) and joining (J) genes rearrangement
199 and class-switch recombination.²⁸⁻³¹

200 Peripheral blood mononuclear cells from all 48 patients were stimulated for 48 hours with
201 phytohemagglutinin (PHA) and cytokine production of interferon gamma (IFN- γ), interleukin
202 2 (IL-2), IL-5, IL-10 and IL-12 were measured by enzyme-linked immunosorbent assay
203 (ELISA) using a method described previously.³²

204

205 ***Screening of positional candidate genetic markers***

206 HLA-A, -B, -DQ and -DR alleles were determined using low-resolution DNA-based
207 typing (polymerase chain reaction [PCR]/sequence specific oligonucleotide probe).³³ The
208 PCR amplification of tumour necrosis factor receptor superfamily member 13 B and C
209 (*TNFRSF13B* and *TNFRSF13C*) and their Sanger sequencing were performed using primers
210 and conditions as previously described.³⁴ Of note, selected monogenic forms of PID were
211 excluded in studied patients according to the CVID diagnostic criteria using a targeted gene
212 panel sequencing (covering 260 known monogenic diseases) including *BTK*, *BLNK*, *CD79A*,
213 *CD79B*, *IGHM*, *IGLL1*, *TCF3*, *CD19*, *CD20*, *CD21*, *CD81*, *AICDA*, *UNG*, *INO80*, *MSH6*,
214 *CARD11*, *NFKB1*, *NFKB2*, *PI3KCD*, *PIK3R1*, *PTEN*, *DOCK2*, *IKAROS*, *IRF2BP2*, *MOGS*,
215 *TWEAK*, *IL21*, *IL21R*, *LRBA* and *CTLA4* genes. Moreover, whole exome sequencing in 17
216 patients were also performed, but neither non-responders nor responders had a confirmed
217 candidate monogenic disease.

218

219 ***Ethical considerations***

220 The World Health Organization declared in June 2009 that the outbreak of A(H1N1)
221 influenza fulfilled the criteria of a pandemic situation. As a consequence, the National Board
222 of Health and Welfare in Sweden recommended the general population to be vaccinated and
223 certain risk groups, including individuals with an impaired immune system, to be prioritized.
224 The pandemic influenza A(H1N1) vaccine had not previously been used in a mass vaccination
225 situation, but the potential severity of the disease motivated patients with CVID to be offered
226 the new vaccine.

227 Based on the Ethical Review of Research Involving Humans³⁵ the vaccination including
228 blood sampling and follow-up schedule were approved by the regional Ethical Committee
229 (approval number 2009/1646-31-3). The patients were given oral and written information

230 about the study. The principle of volunteering was emphasized and informed written consent
231 to participate including a one-year follow-up was obtained from all patients.

232

233 *Statistical analyses*

234 Statistical analyses were performed using SPSS (version 21.0.0, Statistics software,
235 SPSS, Chicago, Illinois) and R statistical systems (version 3.4.1., R Foundation for Statistical
236 Computing, Vienna, Austria). The one-sample Kolmogorov-Smirnov test was applied to
237 estimate whether the data distribution was normal and based on the findings of this evaluation
238 independent T-test (in normal distributions) and Mann-Whitney U test (in skewed
239 distributions) were used to compare continuous variables between responders and non-
240 responders. Differences in categorical variables between responders and non-responders were
241 examined using χ^2 tests and Fisher's exact tests (the latter when variables had a low
242 frequency). Pearson's correlation coefficient analysis was used to investigate the relationship
243 between specific antibody responses to different antigens. A *p*-value of 0.05 or less was
244 considered statistically significant.

245 **Results**

246 Three months after the first vaccine dose and two months after the second dose, eight
247 (16.7%) patients had reached a $\geq 1:40$ titer of specific antibodies against the pandemic
248 influenza A(H1N1) antigen (responders) whereas the remaining 40 patients (83.3%) were
249 considered as non-responders. The production of specific antibodies against the antigen is
250 depicted in **Figure 1**. Four out of eight responders reached protective levels already after the
251 first vaccine dose. Comparing the four early responders with the four late responders, no
252 significant differences could be found between their current age (57.0 ± 14.7 vs. 54.2 ± 11.7
253 years) or age at CVID diagnosis (41.5 ± 10.0 vs. 39 ± 16.8 years).

254 During the study period, none of the patients showed any clinical symptoms of having
255 been infected with the ongoing pandemic influenza A(H1N1) and during the one-year follow-
256 up period, no influenza-like symptoms and/or negative side-effects other than local reactions
257 on the injection sites were reported.

258 **Table 1** summarizes the demographic and essential immunological data of responders
259 and non-responders. Response to the A(H1N1) pandemic vaccine was independent of the
260 gender of the patients. There were also no significant differences in the mean age at diagnosis,
261 mean age at study start, mean years from onset of infections to diagnosis or the mean follow-
262 up time after the CVID diagnosis between responders and non-responders, respectively. Nine
263 patients had a familiar form of CVID, all belonging to the non-responder group. In the total
264 group, 14 individuals (29.1%) had evolved from an IgA deficiency (n=12) and/or IgG
265 subclass deficiency (n=2) to a CVID diagnosis during the course of their disease. All these 14
266 patients were non-responders constituting 35.0% of this group ($p=0.048$, compared to absence
267 of this progressive PID form in responders) (**Table 1**).

268 Regarding the Ig replacement therapy there was no difference in the distribution of
269 different Ig brands used between responders and non-responders (data not shown).

270

271 ***Clinical phenotyping***

272 Before diagnosis, all 48 patients had experienced recurrent upper respiratory tract
273 infections and 44 had also been diagnosed as recurrently suffer from lower respiratory tract
274 infections (bronchitis and/or pneumonia). Clinical phenotyping of the 48 patients revealed
275 that 14 (29.2%) of them presented with infections without any other disease-related
276 complications, 14 (29.2%) by infections and autoimmunity, four (8.3%) by infections and
277 enteropathy, and three (6.2%) by infections and polyclonal lymphocytic infiltration. Thirteen
278 patients (27.1%) also manifested overlapping phenotypes (**Figure 2, Table S2**).

279 When dividing the clinical phenotyping between responders and non-responders the
280 proportion of patients with infections without any other disease-related complications was
281 12.5% among the responders and 32.5% among the non-responders ($p=0.12$) (**Figure 2,**
282 **Table S2**). Lower respiratory tract infections were documented in five responders (62.5%)
283 and in 33 non-responders (82.5%, $p=0.20$). None of the responders showed signs of
284 bronchiectasis, while seven non-responders (17.5%; $p=0.10$) suffered from this condition.

285 The phenotype infections and enteropathy was significantly higher in responders (50%
286 vs. 0%, $p<0.001$). Although infections and autoimmunity was present in about the same
287 proportion in both groups (25% in responders and 30% in non-responders, n.s.) (**Figure 2,**
288 **Table S2**), autoimmune cytopenia was exclusively observed among the non-responders ($n=5$).

289 Malignancies were recorded in 12 patients (30%) in non-responders, mainly due to
290 thymoma and lymphoma and in two patients (25%) among the responders (one breast cancer
291 and one colon cancer) ($p=0.3882$) (**Table S2**).

292 No significant difference was observed regarding the proportion of patients with IgE
293 mediated atopic disorders: four responders (50%) and nine non-responders (22.5%, $p=0.12$)
294 (**Table S2**).

295

296

297 ***Immunological phenotyping and classification***

298 At the time of the COVID diagnosis, a significantly higher mean serum level of IgM
299 (34.8 ± 29.6 vs. 17.0 ± 16.0 mg/dl; $p=0.03$) and a significantly lower mean serum level of IgG1
300 (122.4 ± 96.3 vs. 219.2 ± 198.3 mg/dl; $p=0.007$) were noted in non-responders as compared to
301 responders. Lymphocyte subset analyses showed an expansion of absolute counts of cytotoxic
302 T-cells ($p=0.033$) as well as an increased proportion of memory CD8⁺ T-cells ($p=0.044$) in the
303 non-responders as compared to the responders. Furthermore, there was a tendency of a higher
304 number of NK-cells among the non-responders as compared to the responders ($p=0.06$). There
305 was no significant difference between responders and non-responders regarding absolute B-
306 cell counts, although the non-responders presented a decreased number of plasmablast and an
307 increased CD21^{low} percentage ($p=0.007$ and $p=0.041$, respectively) (**Table 2**).
308 Radiosensitivity was only documented in the group of non-responders (4/20 tested non-
309 responders, 20%, vs. 0/7 tested responders, $p=0.09$).

310 Although a positive correlation was observed in responders with increment in the level of
311 protective antibodies after the vaccination with the pandemic influenza A(H1N1) antigen
312 regarding the production of other specific antibodies against protein and polysaccharide
313 antigens ($r=0.75$, $p=0.08$), no statistically significant differences were found between
314 responders and non-responders. Details of the humoral immune response to other antigens are
315 presented in **Table S1**.

316 Immunological classification of the patients revealed that the most frequent immune
317 profile in the responders was Euroclass SmB⁻Tr^{norm}21^{norm} ($p=0.03$) and post-germinal center
318 B-cell pattern (normal naïve, transitional, marginal and memory B-cell subsets) ($p=0.04$) as
319 compared to the non-responders, whereas the most frequent immune profile of non-
320 responders was Euroclass SmB⁻21^{low}Tr^{norm} ($p=0.02$ compared to the responders) (**Figure 3**).

321 The PHA induced cytokine production in vitro in responders and non-responders are
322 presented in **Figure 4**. A significant difference was found in mean IL-12 levels; in non-
323 responders $1,081.2 \pm 651.3$ pg/ml and for responders 283.3 ± 256.5 pg/ml ($p=0.007$).

324

325 *Genetic markers associated with CVID and pandemic influenza A(H1N1) antibody*
326 *production*

327 Four patients with *TNFRSF13B* (10%) and one patient with *TNFRSF13C* (2.5%) CVID
328 susceptibility variants were found among the non-responders while only WT *TNFRSF13*
329 genes were identified among the responders (12.5% vs 0%, $p=0.38$). HLA markers associated
330 with CVID were detected in 16/40 (40%) of the non-responders; HLA-DR3-DQ2 in six
331 patients, HLA-A1-B8 in six patients and HLA-A2-B44 in four patients. HLA markers
332 associated with CVID were not found in any of the responders (40% vs 0%, $p=0.03$).

333 Discussion

334 The outbreak of the pandemic influenza A(H1N1) and the recommendation to
335 specifically vaccinate individuals with an impaired immune system did not only stress the
336 immediate medical need to offer this new vaccine with a A(H1N1) clade selected by WHO to
337 patients with CVID, but it also opened up a possibility for a scientific evaluation of the
338 vaccination in this group of individuals. The background to the study was the perpetual
339 discussion whether patients with CVID should routinely be offered vaccination against
340 seasonal influenza. Only our study and the study by Pedersen *et al.*¹⁹ have presented data from
341 the use of the specific antigen X179a to individuals with CVID. Our study was designed as an
342 evaluation within the group of patients and therefore no healthy controls were included.
343 However, it has been shown that between 67-98.3% of healthy adults produce protective
344 levels of antibodies against the influenza A(H1N1) vaccine Pandemrix[®] 21 days after a single
345 dose of 3.75 µg of the vaccine.^{36,37}

346 Cross-reactive A(H1N1) antibodies are present in healthy populations³⁸ and
347 consequently, antibodies against the pandemic influenza A(H1N1) antigen may be present in
348 Ig preparations.³⁹ All participants in the study were therefore tested for pre-existing A(H1N1)
349 antibodies before entering the study. One 73-year old male had a HA inhibition titer of 1:10 at
350 the study start but this patient did not respond to the vaccination. All patients continued with
351 the same brand and batch of Ig during the study period.

352 The WHO declaration in 2009 of a pandemic situation in 74 countries and territories
353 rapidly led to a demand for the vaccine that was greater than the supply. On a European level
354 it was considered important to offer the vaccine rapidly to as many individuals as possible.
355 For this reason it was decided by the authorities to start the vaccination campaign with an
356 antigen dose of 3.75 µg to the entire population but immunodeficient patients were to be
357 offered a second dose after one month. It has later been shown that a single dose of 3.75 µg of
358 Pandemrix[®] with the antigen X179a brings about immunity to protective level in healthy

359 adults and elderly.⁴⁰ In the study by Pedersen *et al.* about the immunological response to the
360 antigen X179a in CVID¹⁹, the intention was to give two doses of 3.75 µg to three patients
361 with CVID but the patients were accidentally given a double dose of Pandemrix[®] at study
362 start. Two of the patients were then given a second dose of 3.75 µg after three weeks whilst
363 the third patient declined being given the second dose. The two patients receiving two doses
364 (7.5 µg + 3.75 µg) responded with a >4-fold humoral response while the patient receiving one
365 dose (7.5 µg) had a HI titer below $\geq 1:40$. In our study, four of the responders developed
366 protective levels of antibodies against the X179a antigen already after one dose of 3.75 µg,
367 while the four others needed two doses to produce protective levels of antibodies (**Figure 1**).
368 The four early responders were not older at diagnosis or study start than the four late
369 responders, which could have explained the result as older patients might have been in contact
370 with cross-reactive antigens earlier. Based on the current study and the study by Pedersen *et*
371 *al.*¹⁹, it seems reasonable to assume that two doses of 3.75 µg of the antigen X179a would be
372 required to obtain humoral immunity in patients with CVID. Whether these patients should be
373 offered one or two doses of inactivated seasonal influenza vaccine has never been addressed
374 in the four groups of patients with CVID where the outcome of this type of vaccine has been
375 investigated^{9, 17, 41, 42} and any answer to this question is therefore not available. However, Eibl
376 & Wolf¹⁶ suggest that the primary immunization of inactivated influenza vaccine should
377 follow the same scheme as for healthy individuals, but that more frequent booster
378 immunizations might be necessary depending on the assessment of vaccination response in
379 PID.

380 In the influenza, polysaccharide or protein vaccine studies that have been conducted in
381 patients with CVID^{7, 9, 19, 41-44} they have shown more frequent and earlier decline in antibody
382 responses against polysaccharides compared to proteins, suggesting a preservation of T cell-
383 dependent specific antibody response in a subgroup of patients.^{7, 19, 43} In a previous study,
384 Zhan *et al.*⁴¹ reported generation of specific IgG-secreting memory B cells post seasonal

385 influenza vaccines in 50% of the studied CVID patients. In our study, we found that about
386 one-fifth (16.7%) of the patients responded by producing protective levels of antibodies
387 against the pandemic influenza A(H1N1) antigen. Consistent with other reports, we also
388 observed a functional classification of CVID patients, according to the level of the response to
389 the vaccine.^{43, 45}

390 There are to date no studies regarding the clinical phenotypes of patients who seroconvert
391 or not after influenza vaccination.⁴⁶ The finding among the responders of a significantly
392 higher proportion of enteropathies, a phenotype that usually presents due to increased
393 apoptotic bodies in the gastrointestinal crypts⁴⁷, may suggest that the immunological
394 phenotype of the responders with normal B cell subsets could be a result of an increased
395 apoptosis of the long-term plasmacells. In line with this notion these patients can produce
396 normal antibodies against specific antigens during a short-term, but these antibody-producing
397 plasmacells subsequently disappear. The increased susceptibility to apoptosis in the
398 gastrointestinal crypts could be a trigger of chronic inflammation. Regarding the severe lower
399 respiratory tract infections and the bronchiectasis among the non-responders it appears that
400 the non-responders are less likely to mount an immune response against bacteria and
401 consequently an effective elimination in the lower respiratory tract, resulting in persistent
402 tissue damage in the lungs.

403 The capability to respond to certain vaccines *in vivo*, strongly suggest that some patients
404 with CVID can produce class-switched isotypes, as has been shown by cytokine stimulation
405 *in vitro*.⁴⁸ Although CD27⁺ IgD⁻ isotype-switched memory B cells are generally reduced in
406 CVID patients⁴⁹, our findings confirm that responders had a higher level of plasmablasts and
407 lower counts of CD21^{low} B cells (compatible with a Euroclass SmB⁺Tr^{norm}21^{norm} and post-
408 germinal center B-cell pattern). In contrast, we found that the non-responders at diagnosis
409 presented with lower mean serum level of IgG1 and a higher mean serum level of IgM,
410 highlighting a severe defective class switching recombination in this group of patients.

411 Moreover, radiosensitivity was only found in non-responders but this test was performed only
412 in 27 patients and the difference was not statically significant. These findings emphasize the
413 role of the class-switching capability for developing a vaccine response. In two studies by
414 Goldacker *et al.*⁷ and Chovancova *et al.*¹³, respectively, they presented B cell subset analysis;
415 both reported that group II of the Freiburg classification or the EUROclass group smB+,
416 which represent patients with CVID with nearly normal numbers of class-switched memory B
417 cells, constitutes patient with measurable antibody responses.

418 Stimulating peripheral blood mononuclear cells with PHA enhanced the production of IL-
419 12 at significantly higher mean level in non-responders as compared to responders, suggesting
420 a significant immune regulation in this group toward activation of cellular immunity. Up-
421 regulation of the IL-12 as an initial factor in a subgroup of patients with CVID skews the
422 immune response away from Ig production towards a polarized Th1-type chronic
423 inflammation.^{50, 51} Increased expression of the IL-12 β 1 receptor has also been reported in
424 these patients.⁵² Although the IFN- γ level, another marker of Th1-response, did not differ
425 between the two groups, Ig replacement may alter the serum pattern of this cytokine but not
426 the IL-12 level.⁵³ Failure of an immune deviation from a systemic Th1 response to a Th2
427 immune response may explain the mechanism responsible for the numerically lower
428 proportion of IgE mediated atopic diseases observed in the non-responders as compared to
429 responders.⁵⁴

430 Humoral response against influenza vaccine has been shown to be the major source of
431 protection against infections and individuals with annual injections have a broader antibody
432 recognition profile after pathogenic confrontation.⁵⁵ However, influenza-specific CD8+ T
433 cells can be generated after vaccination targeted to conserved viral proteins (nucleoprotein
434 and the matrix protein) to provide heterosubtypic immunity. Since long-term annual
435 vaccination in the presence of normal humoral immunity may interfere the induction of
436 heterosubtypic immunity, it can be hypothesized that virus-specific CD8+ T cell responses are

437 more pronounced in patients with antibody deficiency.⁵⁶ As the current study only focused on
438 humoral immunity, further evaluation of the cellular immunity in these patients may be of
439 considerable interest.

440 The presence of the familiar form of CVID only in non-responders, taken together with
441 the finding of predisposing HLA and non-HLA factors, suggest a strong association of CVID
442 pathogenetic in this group of patients.⁵⁷ Moreover, the progressive primary antibody
443 deficiency was exclusively found in the non-responders, a phenomenon that has previously
444 been linked to genetic susceptibility markers in patients with CVID, in particular HLA A1,
445 B8, DR3 and DQ2.^{58, 59} Investigation of non-genetic etiologies among responders should be
446 prioritized, including epigenome and microbiome assays. The essential role of specific HLA
447 haplotype in response to vaccine has been investigated also in healthy individuals; particularly
448 CVID HLA susceptibility markers such as DQ2-DR3 phenotypes have been linked with non-
449 responsiveness to hepatitis B antigen in vaccine in the normal population.^{60, 61} Specific
450 antibody response to vaccines has also been investigated in patients with *TNFRSF13B*
451 mutated alleles and transmembrane activator and CAML interactor (*TACI*) knockout mice due
452 to the role of TACI in signaling for induction of Ig class switching. Most of the animal studies
453 have showed consistent defective responses to vaccination with T independent antigens.⁶²
454 However, humans with heterozygous *TNFRSF13B* mutations show a wide range of ability to
455 respond to vaccine from absent to some antibody production^{63, 64} even within the same family
456 with same mutation⁶⁵, suggesting a presence of other genetic modifier rather than TACI.

457 To summarize, we identified protective increment in the level of antibodies against the
458 pandemic influenza A(H1N1) antigen in eight (16.7%) patients of our study population
459 including totally 48 individuals. This is to date the largest study performed investigating
460 influenza vaccine response in patients with CVID. We found that a positive outcome of the
461 influenza vaccination might be expected in patients with certain identified specific B cell
462 patterns. The responders were sporadic cases without genetic susceptibility markers, but with

463 normal class switching recombination and DNA repair machinery. Therefore they could still
464 produce residual specific antibodies against the current antigen, and they presented with low
465 rate of severe lower respiratory tract infections and no infectious complications such as
466 bronchiectasis. Instead, these patients more often presented with enteropathy.

467 Predictive markers for patients with CVID who will respond to influenza vaccine were
468 found to be enteropathy, Euroclass SmB⁻Ti^{norm}21^{norm} and post-germinal center B-cell pattern.
469 Despite the fact that not all patients with CVID developed protective antibody levels after two
470 doses of the vaccine, it is concluded that patients with CVID should be offered vaccination
471 also against seasonal influenza^{9, 16, 17, 42, 46} due to the potential severity of the infection and risk
472 for bacterial complications. Adverse events are not a major issue and inactivated influenza
473 vaccine can safely be given to patients with CVID.

474 **Acknowledgments**

475 We would like to express our sincere gratitude to the participating patients and staff at the
476 Immunodeficiency Unit, Department of Infectious Diseases, Karolinska University Hospital,
477 Huddinge, Stockholm, Sweden.

478 The study was funded by the Jeffrey Modell Foundation, New York; the Swedish
479 Research Council, Stockholm, Sweden; and research funds from Karolinska Institutet,
480 Stockholm, Sweden.

481

482 **Author contributions**

483 (1) The conception and design of the study

484 (2) Acquisition of data,

485 (3) Analysis and interpretation of data,

486 (4) Drafting the article

487 (5) Revising it critically for important intellectual content,

488 (6) Final approval of the version to be submitted

489

490 AG(2,3,4,5,6), HA (3,4,5,6), RG(2,4,5,6), LE(3,5,6) and LH, (1,3,5,6).

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698

699 **Table 1-** Demographic data of 48 patients with CVID including eight responders ($\geq 1:40$ titer) and 40
 700 non-responders to the A(H1N1) pandemic influenza vaccine.

Parameters	Total group	Responders	Non-responders	<i>p</i> -value*
Number	48	8	40	-
Gender (F/M)	29/19	5/3	24/16	0.62
Mean age at diagnosis of CVID (years)	44.2 \pm 18.0	40.5 \pm 15.4	44.9 \pm 18.5	0.26
Mean age at study start (years)	57.7 \pm 14.9	54.3 \pm 16.2	58.3 \pm 17.4	0.60
Mean years from onset of infections to diagnosis (years)	12.9 \pm 10.6	9.4 \pm 8.2	13.6 \pm 9.7	0.44
Mean follow-up after diagnosis (years)	13.4 \pm 8.8	13.8 \pm 8.0	13.4 \pm 9.1	0.72
Familial cases (%)	9(18.7)	0	9(22.5)	0.16
Progressive primary antibody^a deficiency (%)	14(29.1)	0	14(35)	0.048

701

702 ^a Progression of other types of primary antibody deficiency including IgA deficiency and IgG subclass
 703 deficiency to CVID.

704 * *p* values <0.05 were regarded significant and are **bolded**

705 **Table 2-** Immunologic data of 48 patients with CVID at diagnosis including 8 responders and 40 non-
 706 responders to A(H1N1) pandemic vaccine.

Parameters	Normal range	Total CVID	Responders	Non-responders	<i>p</i> -value*
IgM (mg/dl)	50-370	31.7±9.7	17.0±16.0	34.8±29.6	0.03
IgA (mg/dl)	80-380	9.2±8.4	9.5±8.4	9.2±7.0	0.76
IgG (mg/dl)	600-1500	209.2±173.4	243.7±206.1	201.7±167.8	0.20
IgG1 (mg/dl)	280-800	141.3±125.7	219.2±198.3	122.4±96.3	0.007
IgG2 (mg/dl)	115-570	49.3±37.0	48.0±36.1	49.7±37.0	0.74
IgG3 (mg/dl)	24-125	19.9±18.1	19.3±16.5	22.3±21.4	0.30
White Blood cells (cell/ul)	3500-8800	5828.5±2395.2	5971.4±1729.8	5791.4±2565.9	0.49
Lymphocyte (cell/ul)	1130-2720	1364.4±607.2	1237.3±318.0	1397.4±662.6	0.10
T cells (cell/ul)	780-2070	1126.6±489.8	1032.8±264.8	1150.9±534.1	0.056
CD4⁺ T cells (cell/ul)	490-1340	516.4±244.3	504.2±187.6	519.6±260.0	0.46
Naive CD4 (% of CD4⁺ T cells)	11-35	13.0±8.0	16.1±9.5	12.1±7.4	0.24
Memory CD4 (% of CD4⁺ T cells)	31-74	70.4±14.8	70.5±14.5	70.4±15.2	0.8
CD8⁺ T cells (cell/ul)	190-800	625.4±342.3	530.5±230.4	650.0±365.2	0.033
Naive CD8 (% of CD8⁺ T cells)	27-69	35.4±12.1	32.8±14.0	36.1±11.7	0.42
Memory CD8 (% of CD8⁺ T cells)	12-50	35.5±2.0	33.2±9.7	43.1±16.4	0.044
CD4:CD8	1-4	1.1±0.9	1.0±0.4	1.1±1.0	0.28
NK cells (cell/ul)	70-420	94.1±61.6	78.5±23.4	98.1±67.9	0.06
B cell (cell/ul)	90-400	123.7±94.9	114.2±91.2	126.2±94.5	0.56
Naïve B (% of CD19⁺ T cells)	47-84	70.2±21.4	71.6±17.6	69.7±22.8	0.72
Transitional B (% of CD19⁺ T cells)	0-1	4.3±3.6	4.2±4.0	4.4±3.9	0.48
Natural memory B (% of CD19⁺ T cells)	6-29	21.12±17.4	18.9±11.7	22.0±19.0	0.28
Switched memory B (% of CD19⁺ T cells)	9-29	6.4±6.0	8.0±7.8	5.9±5.0	0.41
Plasmablast (% of CD19⁺ T cells)	0-3.2	0.06±0.01	0.1±0.02	0.04±0.03	0.007
CD21^{low} B (% of CD19⁺ T cells)	0.7-10	15.0±10.9	8.5±4.6	17.2±11.6	0.041

707 * *p* values <0.05 were regarded significant and are **bolded**

1 **Figure legends**

2 **Figure 1-** Specific antibody response against A(H1N1) pandemic influenza vaccination after 1 and 3
3 months in 48 patients with CVID.

4 **Figure 2-** Clinical phenotyping of 48 patients with CVID including 8 responders and 40 non-
5 responders to A(H1N1) pandemic influenza vaccine. During the course of the disease, all patients
6 presented with the phenotype infections. Thus, the patients had at least the phenotype infections (here
7 presented as “No disease-related complications”) or the phenotype infections together with one or
8 more of the phenotypes in the figure (for detail of clinical phenotyping see **Table S2**). PLI: polyclonal
9 lymphocytic infiltration.

10 **Figure 3-** Immunological phenotyping in percentage of 48 patients with CVID including 8 responders
11 and 40 non-responders to A(H1N1) influenza pandemic vaccine and classification according to the **A.**
12 Paris classification (0: low switch memory[SM] and low total memory [M], 1: low SM and normal M,
13 2: normal SM and normal M), **B.** Freiburg classification (1a: low SM and increased CD21^{low}, 1b: low
14 SM and normal CD21^{low}, 2: normal SM and normal M), **C.** Euroclass classification (according to SM,
15 CD21^{low} and Transitional [Tr] B cells) and **D.** B cell pattern classification (P1:low Tr and low M, P2:
16 low naive and low M, P3: low marginal zone and lowM, P4: lowM and P5: normal).

17 **Figure 4-** Cytokine production of interferon gamma (IFN- γ), interleukin 2 (IL-2), IL-12, IL-5 and IL-
18 10 in 48 patients with CVID, including 8 responders (R) and 40 non-responders (NR) to A(H1N1)
19 pandemic influenza vaccine after phytohemagglutinin stimulation.

Fig 1.

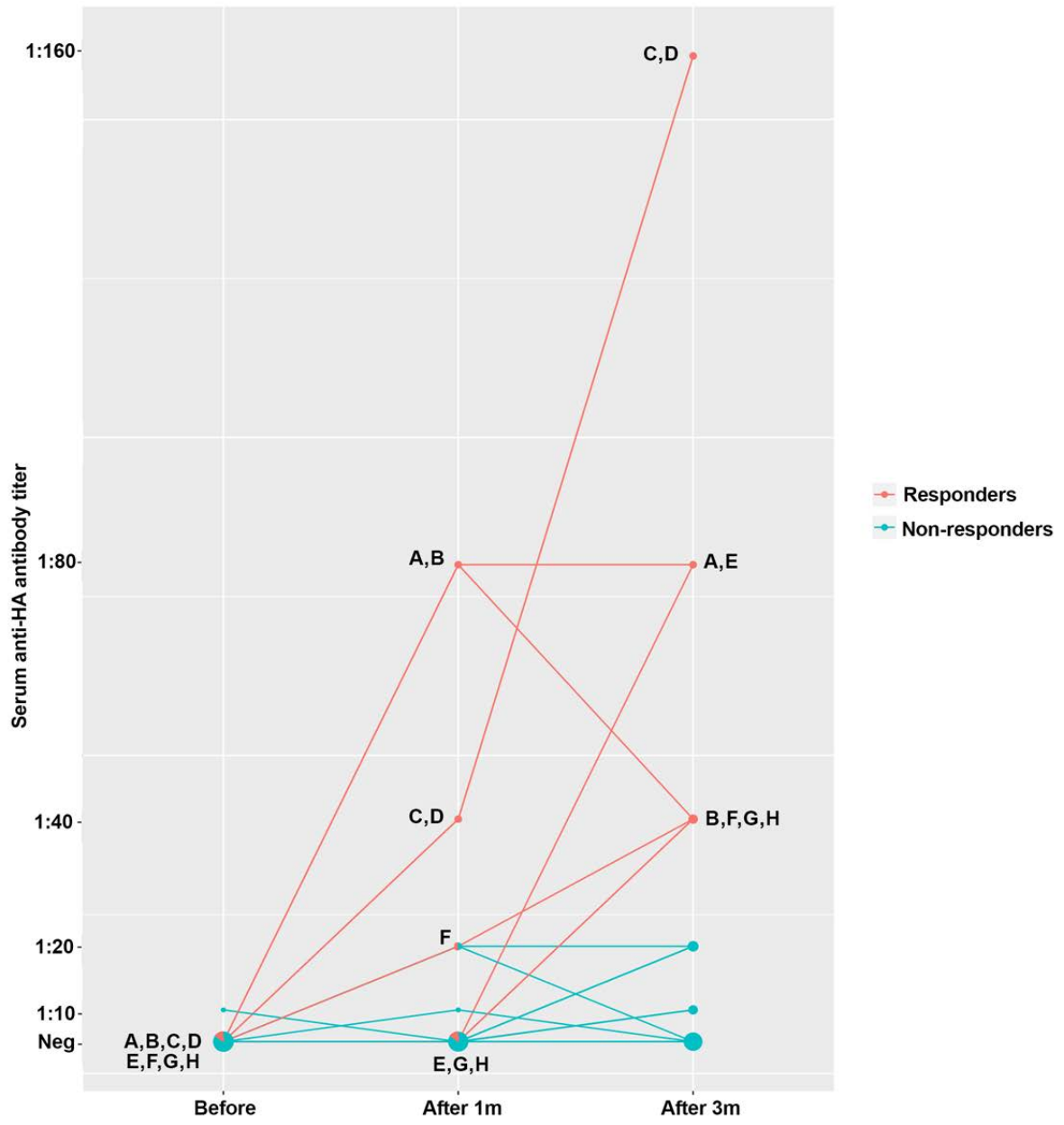


Fig. 2

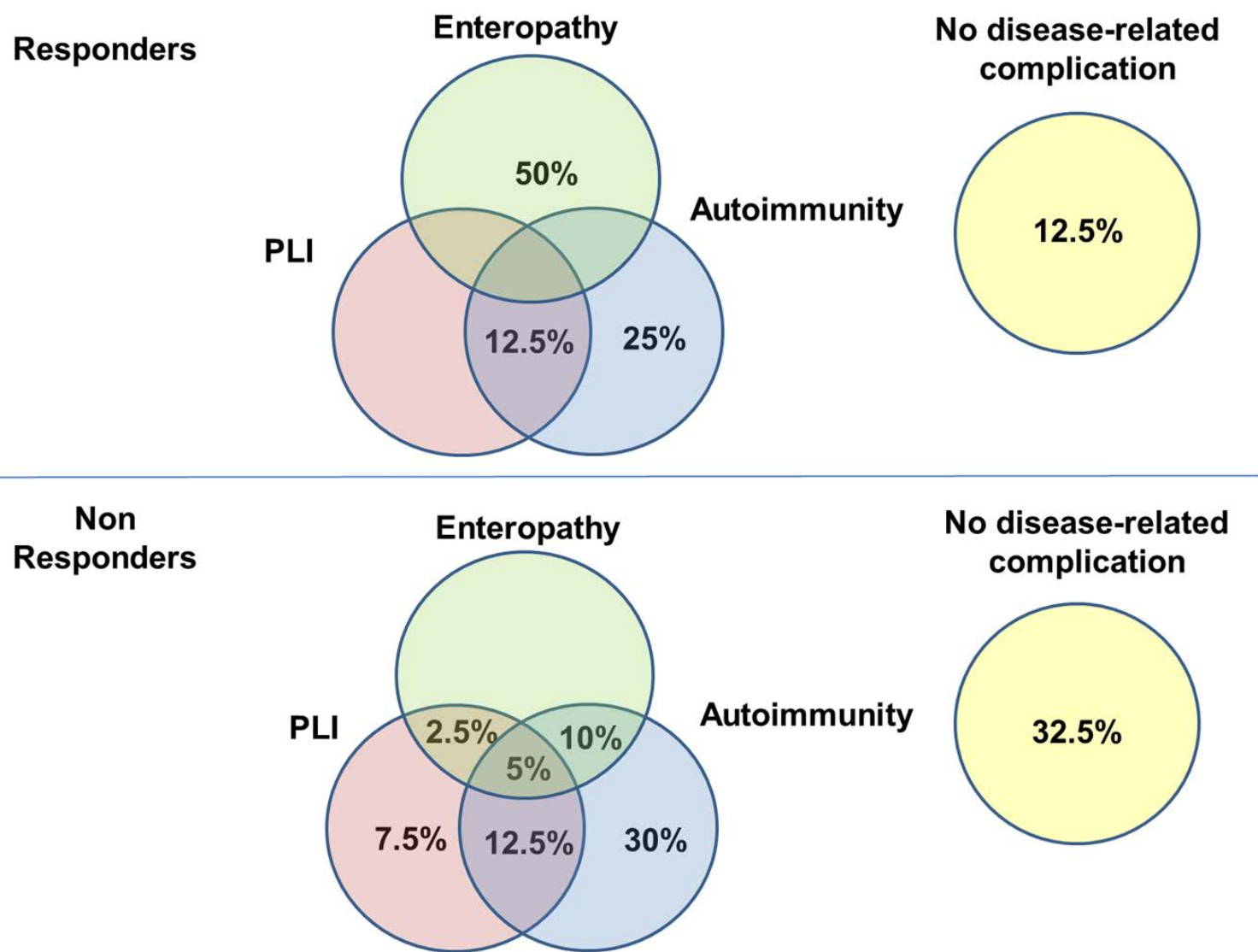


Fig 3

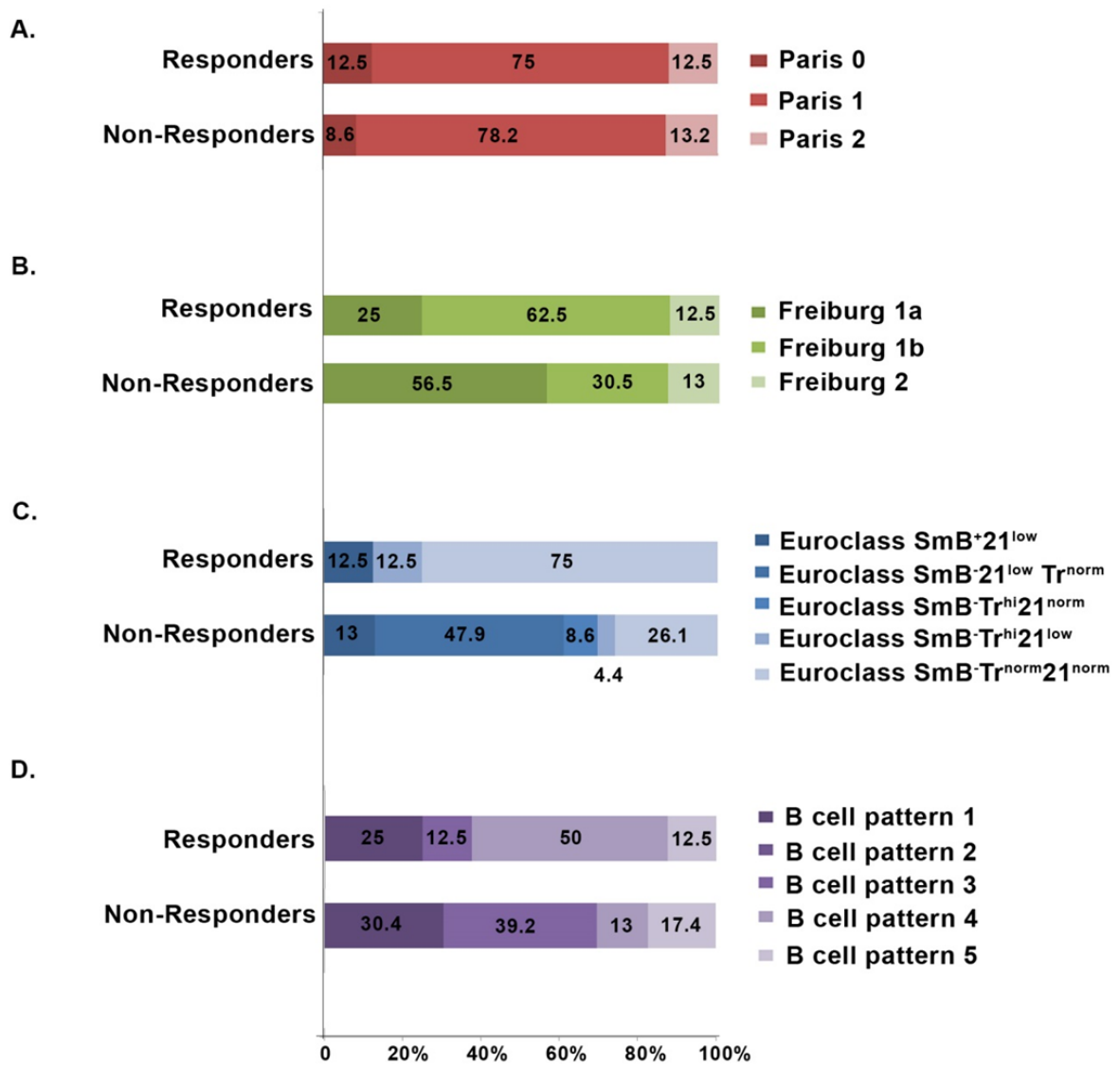
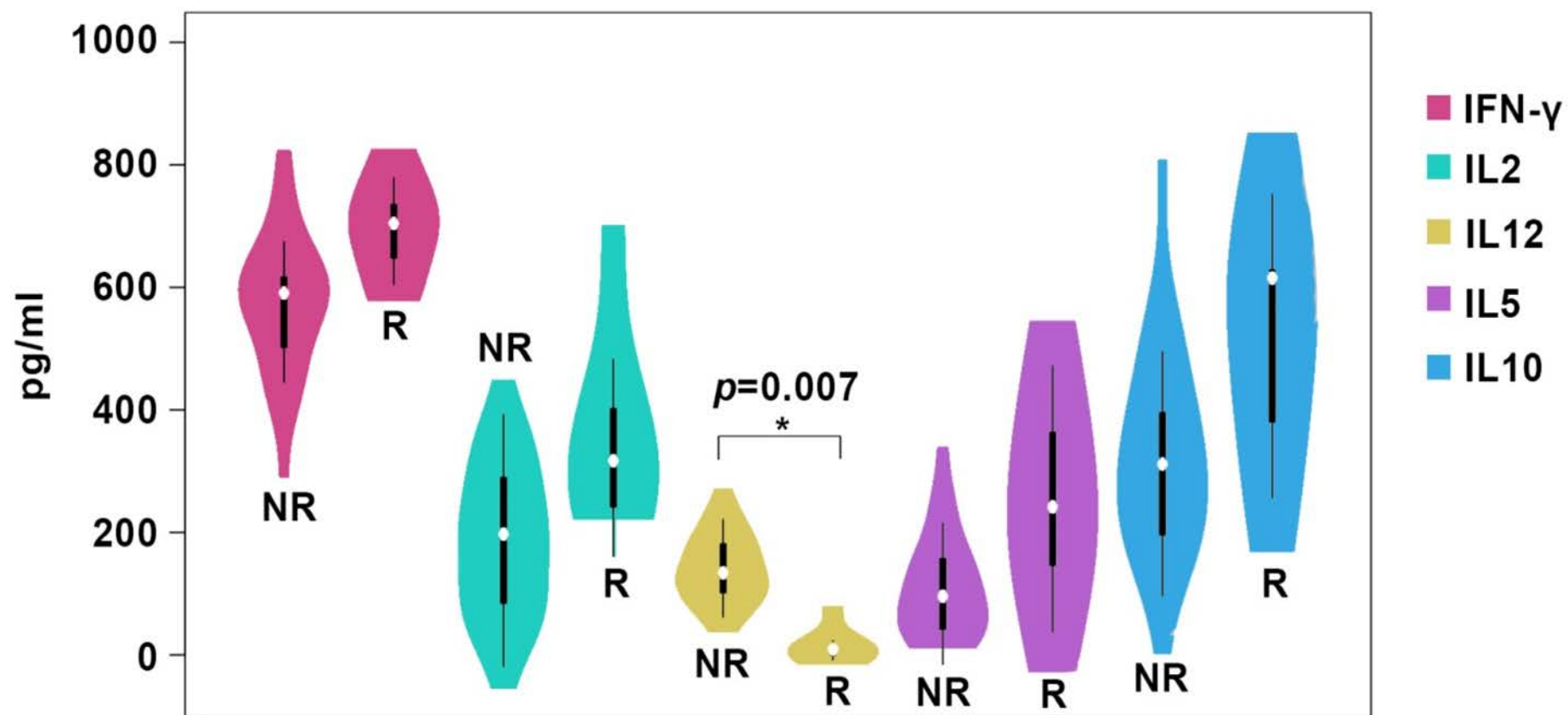


Fig 4



Unmarked

1 **Supplementary Material**

2

3 **Predictive markers for humoral vaccine response in patients with**
4 **common variable immunodeficiency (CVID)**

5

6 Gardulf et al.

7 **Table S1-** Specific antibody response to protein and polysaccharides antigens ^a

Parameters	Total CVID	Responders to Pandemrix [®]	Non-responders to Pandemrix [®]	<i>p</i> -value
	Numbers (%)	Numbers (%)	Numbers (%)	
Diphtheria Ab (normal/total tested)	11/27(40.7)	2/5(40)	9/22(40.9)	1.0
Tetanus Ab (normal/total tested)	13/34(38.2)	3/5(60)	10/29(34.4)	0.34
CMV Ab (normal/total tested)	6/17(35.2)	1/4(25)	5/13(38.4)	1.0
MCV Ab (normal/total tested)	2/10(20)	0/1(0)	2/9(22.2)	1.0
Teichoic acid Ab (normal/total tested)	9/16(56.2)	1/3(33.3)	8/13(61.5)	0.55
Hib Ab (normal/total tested)	6/11(54.5)	1/1(100)	5/10(50)	1.0
Pneumococcus Ab (normal/total tested)	1/9(11.1)	1/1(100)	0/8(0)	0.11
PPS6 Ab (normal/total tested)	7/16(43.7)	1/3(33.3)	6/13(23)	1.0
PPSV23 Ab (normal/total tested)	4 ^b /9(44.4)	1/3(33.3)	3/6(50)	1.0

8 ^a *CMV: Cytomegalovirus, Hib: Haemophilus influenzae type b, MCV: Meningococcal conjugate*
9 *vaccine, PPS6: Phospho-p70 S6 Kinase, PPSV23: nonconjugated polysaccharide, Pneumovax 23-*
10 *valent vaccines.*

11 ^b *These four patients had defective isohemagglutinins tests and therefore, according to the criteria of*
12 *the European Society for Immunodeficiencies (ESID), they have a diagnosis of CVID.*

13 **Table S2-** Medical manifestations and clinical phenotypes of 48 patients with CVID at diagnosis
 14 including 8 responders and 40 non-responders to A(H1N1) pandemic vaccine.

Parameters	Total CVID Numbers (%)	Responders to Pandemrix® Numbers (%)	Non-responders to Pandemrix® Numbers (%)	<i>p</i>-value*
Medical manifestations				
Recurrent upper respiratory tract infections	48(100)	8(100)	40(100)	1
Lower respiratory tract infections	44	5(62.5)	33(82.5)	0.20
Bronchiectasis	7(14.5)	0	7(17.5)	0.10
Autoimmune cytopenia	5(10.4)	0	5(12.5)	0.29
Inflammatory bowel diseases	6(12.5)	1(12.5)	5(12.5)	1
Celiac disease	4(8.3)	1(12.5)	3(7.5)	0.64
Psoriasis	3(6.2)	0	3(7.5)	0.42
Vitiligo	2(4.1)	0	2(5)	0.51
Diabetes mellitus type 1	2(4.1)	0	2(5)	0.51
Systemic lupus erythematosus	1(2.1)	1(12.5)	1(2.5)	0.19
Waldeyer's lymphadenopathy	5(10.4)	1(12.5)	4(10)	0.83
Generalized lymphadenopathy	4(8.3)	0	4(10)	0.35
Splenomegaly	2(4.1)	0	2(5)	0.51
Granulomatous disease	1(2.1)	0	1(2.5)	0.65
Malignancies	14(29.1)	2(25)	12(30)	0.38
Breast cancer	2(4.1)	1(12.5)	1(2.5)	0.19
Colon cancer	2(4.1)	1(12.5)	1(2.5)	0.19
Thymoma/lymphoma	9(12.5)	0	9(22.5)	0.13
Prostate cancer	1(2.1)	0	1(2.5)	0.65
IgE mediated atopic disorders	13(27.1)	4(50)	9(22.5)	0.12
Asthma	4(8.3)	0	4(10)	0.35
Clinical phenotypes				
Infections without any other disease-related complications	14(29.1)	1(12.5)	13(32.5)	0.12
Autoimmunity phenotype	14(29.1)	2(25)	12(30)	0.38
Enteropathy phenotype	4(8.3)	4(50)	0	<0.001
Polyclonal lymphocytic infiltration phenotype	3(6.2)	0	3(7.5)	0.42
Overlapping phenotypes	13(27.0)	1(12.5)	12(30)	0.30

15 * *p* values <0.05 were regarded significant and are **bolded**