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

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Abstract

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

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

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

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

Key messages:

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

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

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

Abbreviations:

CVID: Common variable immunodeficiency

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

HA: Hemagglutinin

HI: Haemagglutination inhibition

HLA: Human leukocyte antigen

Igs: Immunoglobulins

IFN-γ: Interferon gamma

IL: Interleukin

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PID: Primary immunodeficiency disorder

PHA: Phytohemagglutinin

RDE: Receptor-destroying enzymes

WHO: World Health Organization

Introduction

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

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

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

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

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

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

Material and methods

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

Patients

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

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

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

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

Study overview

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

Investigation and definition of vaccination response

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

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

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

Clinical and immunological phenotyping and immunological assays

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

Thirty-eight of the patients had previously been classified based on two main classifications for B-cell subsets including the Paris, Freiburg, EUROclass[25] and the B-cell pattern classification.[26] In 27 of the patients, ionizing radiation sensitivity assays had previously been performed on primary fibroblasts irradiated with different doses and serial dilutions.[27] This is a method used for classification of patients with CVID and defective DNA repair machinery required for variable (V), diversity (D) and joining (J) genes rearrangement and class-switch recombination.[28-31]

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

Screening of positional candidate genetic markers

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

Ethical considerations

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

Based on the Ethical Review of Research Involving Humans[35] the vaccination including blood sampling and follow-up schedule were approved by the regional Ethical Committee (approval number 2009/1646-31-3). The patients were given oral and written information

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

Statistical analyses

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

Results

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

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

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

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

Clinical phenotyping

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

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

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

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

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

Immunological phenotyping and classification

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

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

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

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

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

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

Discussion

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

Cross-reactive A(H1N1) antibodies are present in healthy populations[38] and consequently, antibodies against the pandemic influenza A(H1N1) antigen may be present in Ig preparations.[39] All participants in the study were therefore tested for pre-excising A(H1N1) antibodies before entering the study. One 73-year old male had a HA inhibition titer of 1:10 at the study start but this patient did not respond to the vaccination. All patients continued with the same brand and batch of Ig during the study period.

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

adults and elderly.[40] In the study by Pedersen *et al*. about the immunological response to the antigen X179a in CVID[19], the intention was to give two doses of 3.75 µg to three patients with CVID but the patients were accidentally given a double dose of Pandemrix® at study start. Two of the patients were then given a second dose of 3.75 µg after three weeks whilst the third patient declined being given the second dose. The two patients receiving two doses (7.5 µg + 3.75 µg) responded with a >4-fold humoral response while the patient receiving one dose (7.5 µg) had a HI titer below ≥1:40. In our study, four of the responders developed protective levels of antibodies against the X179a antigen already after one dose of 3.75 µg, while the four others needed two doses to produce protective levels of antibodies (**Figure 1**). The four early responders were not older at diagnosis or study start than the four late responders, which could have explained the result as older patients might have been in contact with cross-reactive antigens earlier. Based on the current study and the study by Pedersen *et al*.[19], it seems reasonable to assume that two doses of 3.75 µg of the antigen X179a would be required to obtain humoral immunity in patients with CVID. Whether these patients should be offered one or two doses of inactivated seasonal influenza vaccine has never been addressed in the four groups of patients with CVID where the outcome of this type of vaccine has been investigated[9, 17, 41, 42] and any answer to this question is therefore not available. However, Eibl & Wolf[16] suggest that the primary immunization of inactivated influenza vaccine should follow the same scheme as for healthy individuals, but that more frequent booster immunizations might be necessary depending on the assessment of vaccination response in PID.

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

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

There are to date no studies regarding the clinical phenotypes of patients who seroconvert or not after influenza vaccination.[46] The finding among the responders of a significantly higher proportion of enteropathies, a phenotype that usually presents due to increased apoptotic bodies in the gastrointestinal crypts[47], may suggest that the immunological phenotype of the responders with normal B cell subsets could be a result of an increased apoptosis of the long-term plasmacells. In line with this notion these patients can produce normal antibodies against specific antigens during a short-term, but these antibody-producing plasmacells subsequently disappear. The increased susceptibility to apoptosis in the gastrointestinal crypts could be a trigger of chronic inflammation. Regarding the severe lower respiratory tract infections and the bronchiectasis among the non-responders it appears that the non-responders are less likely to mount an immune response against bacteria and consequently an effective elimination in the lower respiratory tract, resulting in persistent tissue damage in the lungs.

The capability to respond to certain vaccines *in vivo*, strongly suggest that some patients with CVID can produce class-switched isotypes, as has been shown by cytokine stimulation *in vitro.*[48] Although $CD27^{+}$ IgD^{-}isotype-switched memory B cells are generally reduced in CVID patients[49], our findings confirm that responders had a higher level of plasmablasts and lower counts of $CD21^{low}$ B cells (compatible with a Euroclass $SmB^{-}Tr^{norm}21^{norm}$ and post-germinal center B-cell pattern). In contrast, we found that the non-responders at diagnosis presented with lower mean serum level of IgG1 and a higher mean serum level of IgM, highlighting a severe defective class switching recombination in this group of patients.

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

Stimulating peripheral blood mononuclear cells with PHA enhanced the production of IL-12 at significantly higher mean level in non-responders as compared to responders, suggesting a significant immune regulation in this group toward activation of cellular immunity. Up-regulation of the IL-12 as an initial factor in a subgroup of patients with CVID skews the immune response away from Ig production towards a polarized Th1-type chronic inflammation.[50, 51] Increased expression of the IL-12β1 receptor has also been reported in these patients.[52] Although the IFN-γ level, another marker of Th1-response, did not differ between the two groups, Ig replacement may alter the serum pattern of this cytokine but not the IL-12 level.[53] Failure of an immune deviation from a systemic Th1 response to a Th2 immune response may explain the mechanism responsible for the numerically lower proportion of IgE mediated atopic diseases observed in the non-responders as compared to responders.[54]

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

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

The presence of the familiar form of CVID only in non-responders, taken together with the finding of predisposing HLA and non-HLA factors, suggest a strong association of CVID pathogenetic in this group of patients.[57] Moreover, the progressive primary antibody deficiency was exclusively found in the non-responders, a phenomenon that has previously been linked to genetic susceptibility markers in patients with CVID, in particular HLA A1, B8, DR3 and DQ2.[58, 59] Investigation of non-genetic etiologies among responders should be prioritized, including epigenome and microbiome assays. The essential role of specific HLA haplotype in response to vaccine has been investigated also in healthy individuals; particularly CVID HLA susceptibility markers such as DQ2-DR3 phenotypes have been linked with non-responsiveness to hepatitis B antigen in vaccine in the normal population.[60, 61] Specific antibody response to vaccines has also been investigated in patients with *TNFRSF13B* mutated alleles and transmembrane activator and CAML interactor (*TACI*) knockout mice due to the role of TACI in signaling for induction of Ig class switching. Most of the animal studies have showed consistent defective responses to vaccination with T independent antigens.[62] However, humans with heterozygous *TNFRSF13B* mutations show a wide range of ability to respond to vaccine from absent to some antibody production[63, 64] even within the same family with same mutation[65], suggesting a presence of other genetic modifier rather than TACI.

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

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

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

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Author contributions

(1) The conception and design of the study

(2) Acquisition of data,

(3) Analysis and interpretation of data,

(4) Drafting the article

(5) Revising it critically for important intellectual content,

(6) Final approval of the version to be submitted

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

Parameters	Total group	Responders	Non-responders	*p*-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±18.0	40.5±15.4	44.9±18.5	0.26
Mean age at study start (years)	57.7±14.9	54.3±16.2	58.3±17.4	0.60
Mean years from onset of infections to diagnosis (years)	12.9±10.6	9.4±8.2	13.6±9.7	0.44
Mean follow-up after diagnosis (years)	13.4±8.8	13.8±8.0	13.4±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	*p*-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***

Figure legends

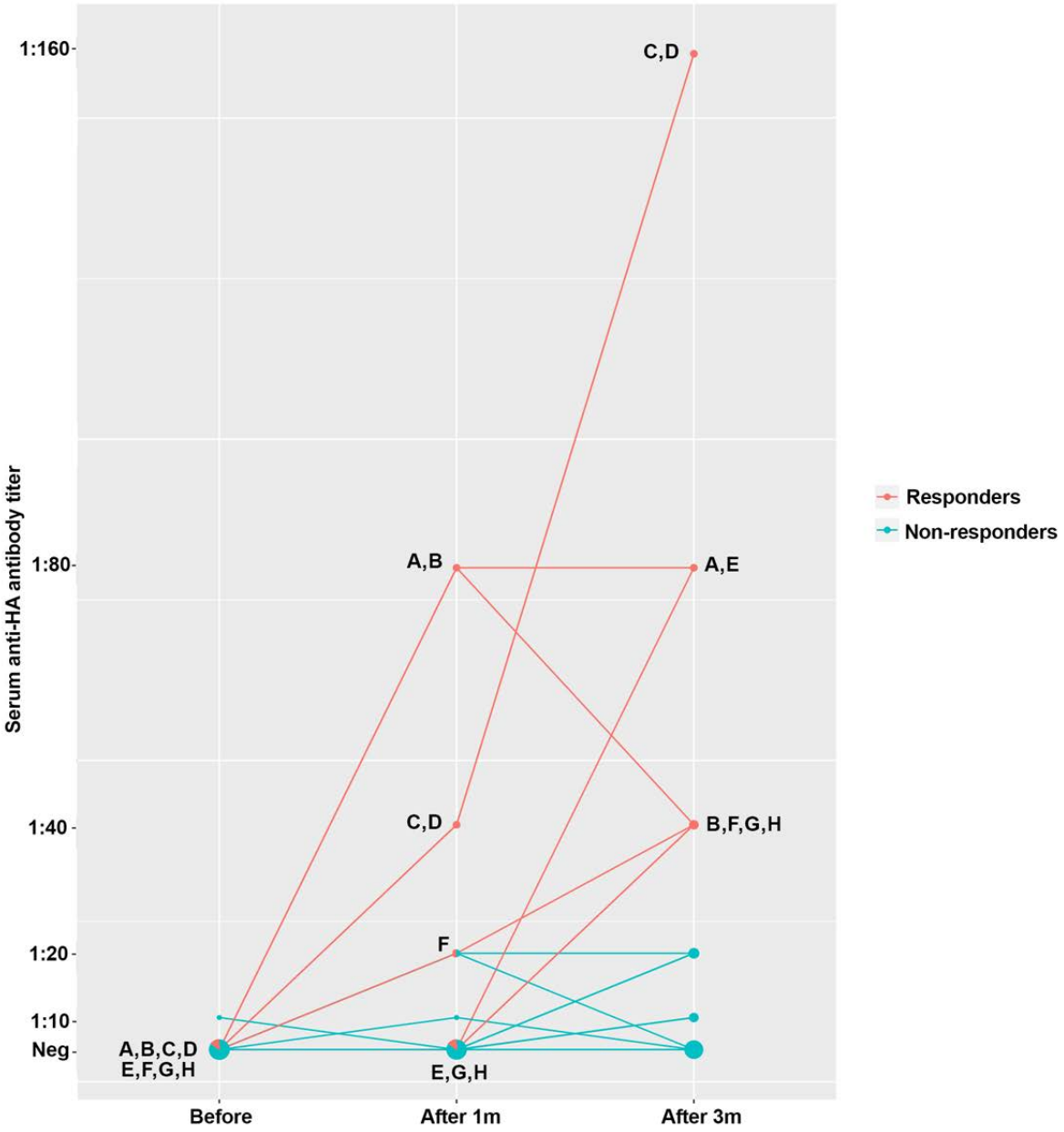
Figure 1- Specific antibody response against A(H1N1) pandemic influenza vaccination after 1 and 3 months in 48 patients with CVID.

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

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

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

Fig 1.


1:160
1:80
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Neg
Serum anti-HA antibody titer
C,D
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C,D
B,F,G,H
F
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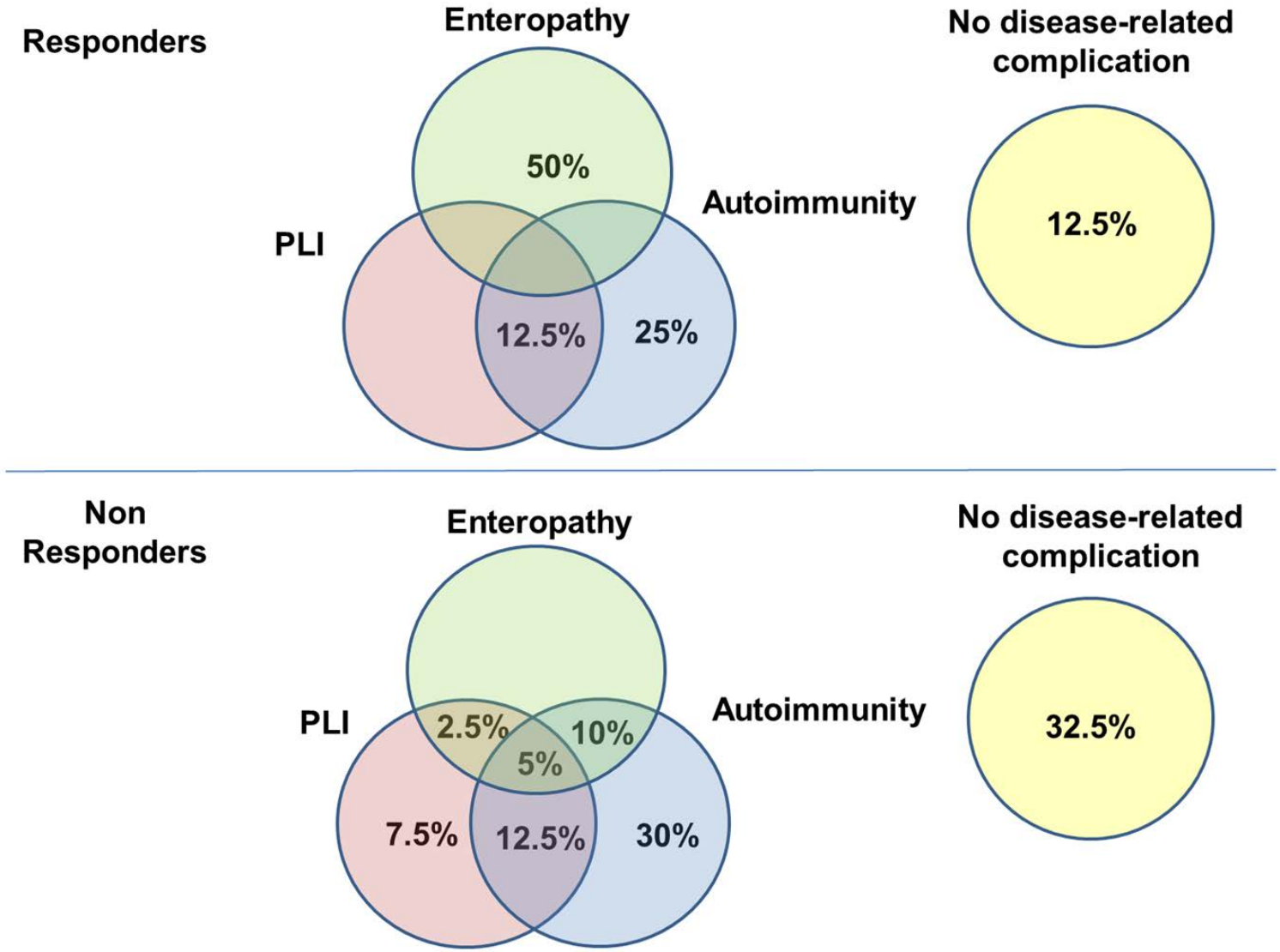
E,F,G,H
E,G,H
Before
After 1m
After 3m
Responders
Non-responders

Fig. 2


Responders
Enteropathy
No disease-related complication
50%
Autoimmunity
PLI
12.5%
25%
12.5%
Non Responders
Enteropathy
No disease-related complication
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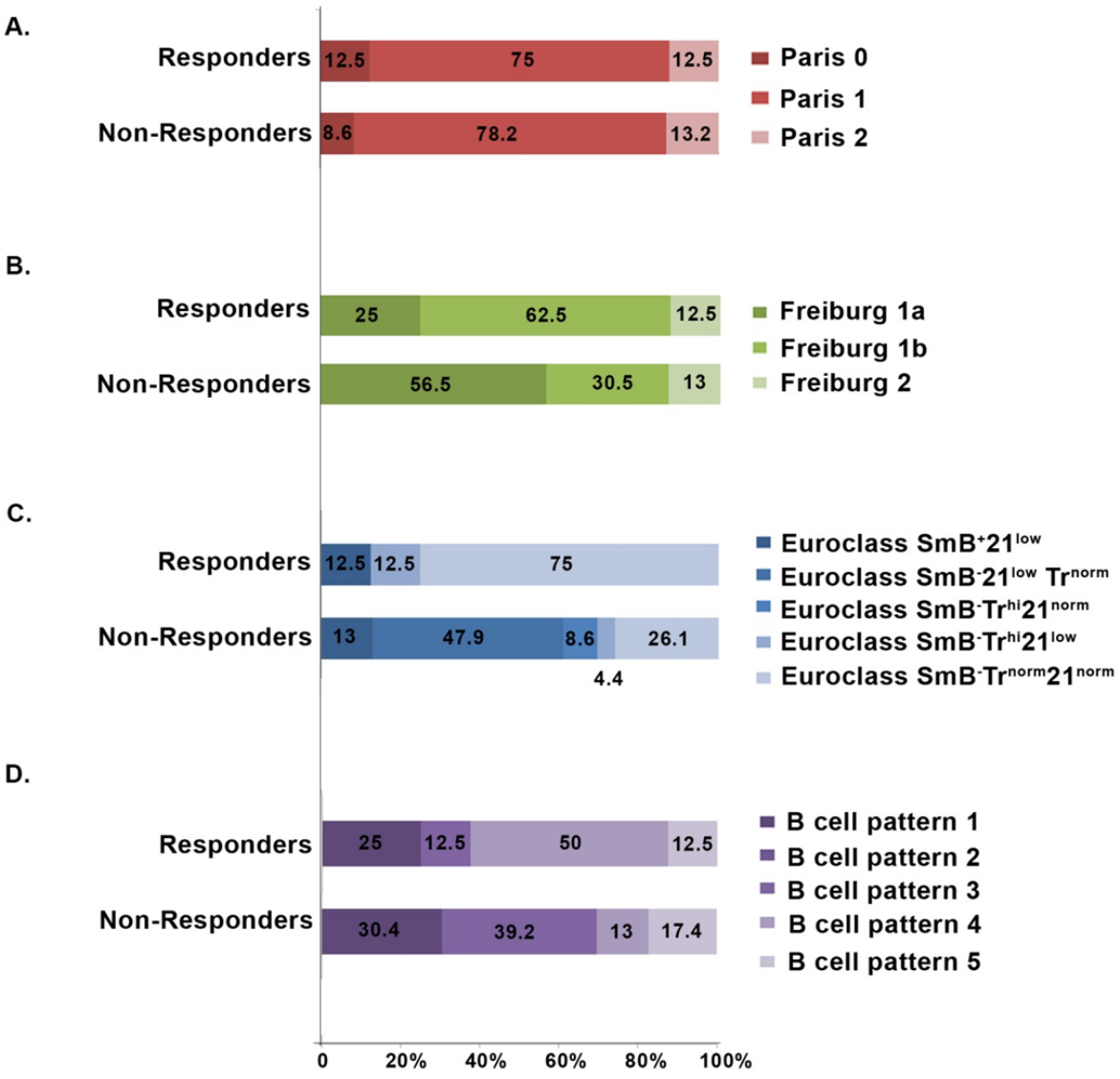
10%
5%
Autoimmunity
32.5%
7.5%
12.5%
30%

Fig 3


A.
Responders
12.5
75
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Non-Responders
8.6
78.2
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Paris 0
Paris 1
Paris 2
B.
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25
62.5
12.5
Non-Responders
56.5
30.5
13
Freiburg 1a
Freiburg 1b
Freiburg 2
C.
Responders
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12.5
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Euroclass SmB+21low
Euroclass SmB-21low Trnorm
Euroclass SmB-Trhi21norm
Euroclass SmB-Trhi21low
Euroclass SmB-Trnorm21norm
D.
Responders
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20%
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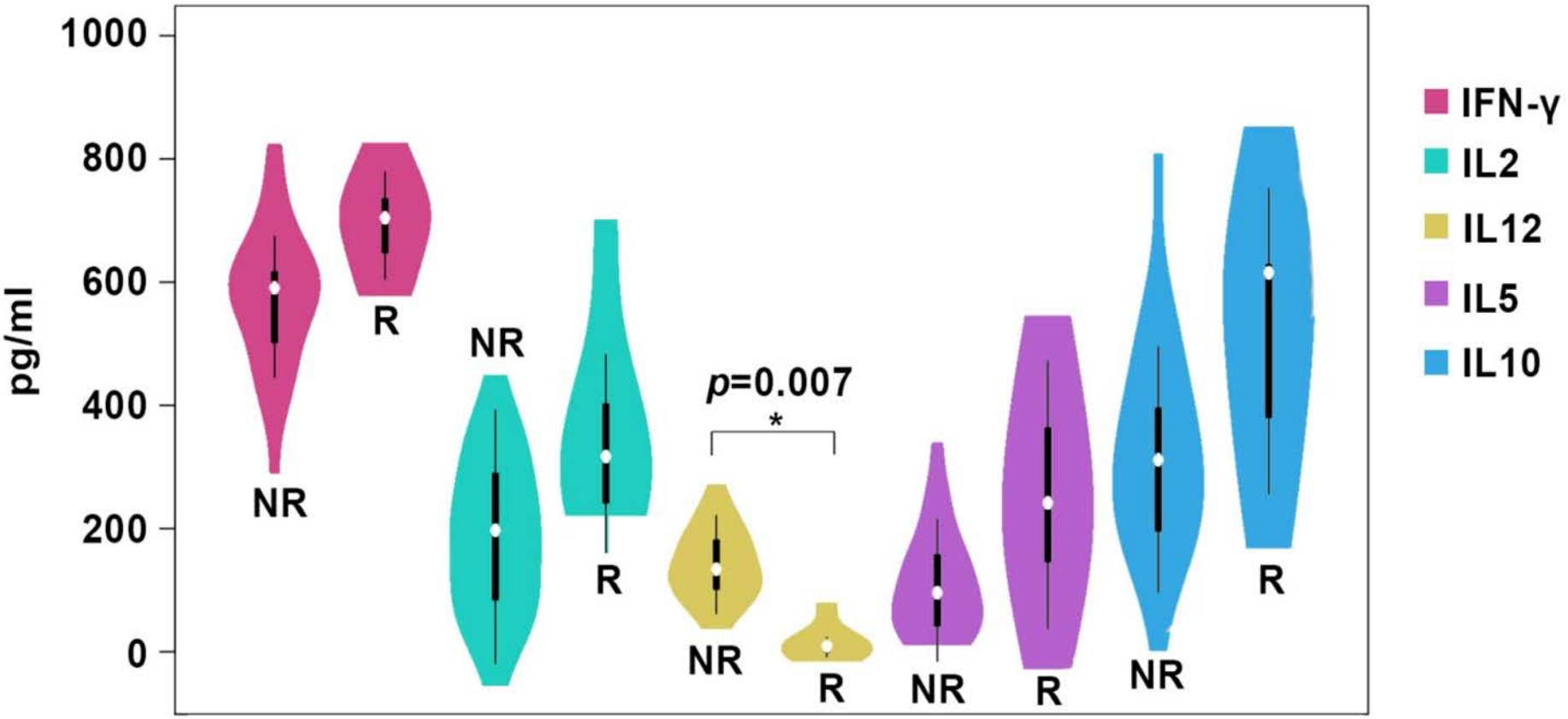
100%

Fig 4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NR
R
IFN-γ
IL2
IL12
IL5
IL10

Supplementary Material

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

Gardulf et al.

Table S1- Specific antibody response to protein and polysaccharides antigens [a]

Parameters	Total CVID Numbers (%)	Responders to Pandemrix® Numbers (%)	Non-responders to Pandemrix® Numbers (%)	*p*-value
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

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

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

Table S2- Medical manifestations and clinical phenotypes of 48 patients with CVID at diagnosis 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 (%)**	***p*-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

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