RESEARCH PAPER

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Humoral and cellular immune responses after influenza vaccination in patients with postcancer fatigue

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Abbreviations: CBT, cognitive behavior therapy; CIS-fatigue, Checklist Individual Strength fatigue subscale; HI, hemagglutination-inhibition; HSP90α, human heat shock protein 90 alpha; IFN- γ, interferon gamma; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; Radboudumc, Radboud University Medical Center; Treg, regulatory T lymphocytes

The aim of this study was to compare humoral and cellular immune responses to influenza vaccination in cancer survivors with and without severe symptoms of fatigue. Severely fatigued (n = 15) and non-fatigued (n = 12) disease-free cancer survivors were vaccinated against seasonal influenza. Humoral immunity was evaluated at baseline and post-vaccination by a hemagglutination inhibition assay. Cellular immunity was evaluated at baseline and post-vaccination by lymphocyte proliferation and activation assays. Regulatory T cells were measured at baseline by flow cytometry and heat-shock protein 90 alpha levels by ELISA. Comparable humoral immune responses were observed in fatigued and non-fatigued patients, both pre- and post-vaccination. At baseline, fatigued patients showed a significantly diminished cellular proliferation upon virus stimulation with strain H3N2 (1414 ± 1201 counts), and a trend in a similar direction with strain H1N1 (3025 ± 2339 counts), compared to non-fatigued patients (3099 ± 2401 and 5877 ± 4604 counts, respectively). The percentage of regulatory T lymphocytes was significantly increased (4.4 ± 2.1% versus 2.4 ± 0.8%) and significantly lower amounts of interleukin 2 were detected prior to vaccination in fatigued compared to non-fatigued patients (36.3 ± 44.3 pg/ml vs. 94.0 ± 45.4 pg/ml with strain H3N2 and 28.4 ± 44.0 pg/ml versus 74.5 ± 56.1 pg/ml with strain H1N1). Pre-vaccination heat-shock protein 90 alpha concentrations, post-vaccination cellular proliferation, and post-vaccination cytokine concentrations did not differ between both groups. In conclusion, influenza vaccination is favorable for severely fatigued cancer survivors and should be recommended when indicated. However, compared to non-fatigued cancer survivors, fatigued cancer survivors showed several significant differences in immunological reactivity at baseline, which warrants further investigation.

Introduction

Severe fatigue is one of the long-term problems of cancer survivors. Postcancer fatigue is an invalidating problem, which occurs frequently and impairs the quality of life.[1,2] Severe fatigue is defined by a cut-off score of at least 35 points on the fatigue severity subscale of the Checklist Individual Strength (CIS-fatigue).[3,4] Already during cancer treatment 70–96% of the patients suffer from fatigue symptoms.[5,6] Postcancer fatigue has a prevalence ranging from 19 to 39% as observed in longitudinal studies.[7-11] The cancer diagnosis and treatment characteristics are not related to postcancer fatigue.[6,12-16] There is evidence, however, that patients who receive only surgery have a decreased risk for postcancer fatigue[11] and patients who receive both surgery and adjuvant treatment have an increased risk for postcancer fatigue.[7,17] Cognitive behavior therapy (CBT), particularly

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composed for postcancer fatigue, is proven to be efficacious in treating severely fatigued cancer survivors.[14,18] However, the nature of the underlying physiology of postcancer fatigue remains unclear.

Possible explanations for the pathophysiological mechanism of cancer-related fatigue are dysregulation of brain serotonin, dysregulation of the hypothalamic-pituitary-adrenal axis responsiveness, disruption of the circadian rhythm, alterations in muscle and ATP metabolism, activation of the vagal afferent nerve, and dysregulation of cytokines (for a review, see Ryan *et al* 2007[19], and for a meta-analysis, see Schubert *et al* 2007[20]).

The presence of an immunological imbalance was also put forward as an explanation for postcancer fatigue. As a response to the presence of the tumor or its treatment, the immune system is activated, which results in the release of cytokines and other immune factors, including receptor antagonists, soluble receptors, and products of cellular activation.[21] Cytokines steer both the innate and adaptive immune response, but also mediate neural symptoms like fatigue.[22] Alterations in pro-inflammatory cytokines have been observed in fatigue-related disorders like chronic fatigue syndrome and depression.[23,24] Most of these changes in immune parameters resolve following completion of cancer treatment, but an imbalance in the immune system persists, which could explain the fatigue.

An immunological imbalance in postcancer fatigue patients may be reflected in an altered response to vaccination. Hence, the aim of this study was to compare humoral and cellular immune responses upon vaccination, using seasonal influenza vaccination as a representative vaccination, in severely fatigued and non-fatigued disease-free cancer survivors.

Results

Baseline characteristics of the participants

Patient characteristics at baseline are presented in **Table 1**. The group suffering from severe postcancer fatigue did not significantly deviate from the non-fatigued group in terms of sex, age, average time since cancer treatment, and history of influenza vaccination.

Humoral immune responses in postcancer fatigue upon influenza vaccination

Participants were vaccinated against seasonal influenza. Pre- and post-vaccination, humoral immune responses were assessed by the hemagglutination-inhibition (HI) antibody test. Comparable antibody titres were observed for the vaccination viral strains in fatigued and non-fatigued patients, both pre- and post-vaccination (**Fig. 1**). Influenza vaccination caused a near identical increment in antibody titres for strains H3N2 and H1N1 in

Table 1. Baseline characteristics

	Postcancer fatigue (n=15)	No postcancer fatigue (n=12)	*p*-value
Sex			
Male	7 (47)	5 (42)	0.795
Female	8 (53)	7 (58)	
Age (years)			
Mean	49.6 ±11 .9	48.8 ±9 .1	0.856
Cancer diagnosis			
Breast cancer	7 (47)	7 (58)	
Testicular cancer	1 (7)	3 (25)	
Melanoma	2 (13)	1 (8)	
(Non-)Hodgkin	2 (13)	1 (8)	
Other	3 (20)	0	
Cancer treatment			
Surgery only	3 (20)	1 (8)	
Surgery and chemotherapy	2 (13)	3 (25)	
Surgery and radiotherapy	2 (13)	2 (17)	
Surgery, chemotherapy, and radiotherapy	2 (13)	3 (25)	
Surgery, chemotherapy, radiotherapy, and hormonal therapy	3 (20)	2 (17)	
Chemotherapy only	1 (7)	0	
Chemotherapy and radiotherapy	1 (7)	1 (8)	
Chemotherapy and radiotherapy Chemotherapy and immunotherapy	1 (7)	0	
Time since cancer treatment (months)	66.8 ±84.9	64.4 ±32.2	0.928
Influenza vaccination prior to 2010			
Yes	5 (33)	2 (17)	0.326
No	10 (67)	10 (83)	

Data are presented as mean ± standard deviation or as frequencies with percentages in brackets.

patients suffering from postcancer fatigue as well as in non-fatigued patients (**Fig. 1A, B**, respectively). Fatigued and non-fatigued patients showed comparable seroprotection rates (pre-vaccination and post-vaccination, **Table 2**) and similar seroresponse rates against the 3 influenza strains (**Table 2**). The number of severely fatigued and non-fatigued patients without a humoral response to influenza vaccination was not significantly different (**Table 2**).

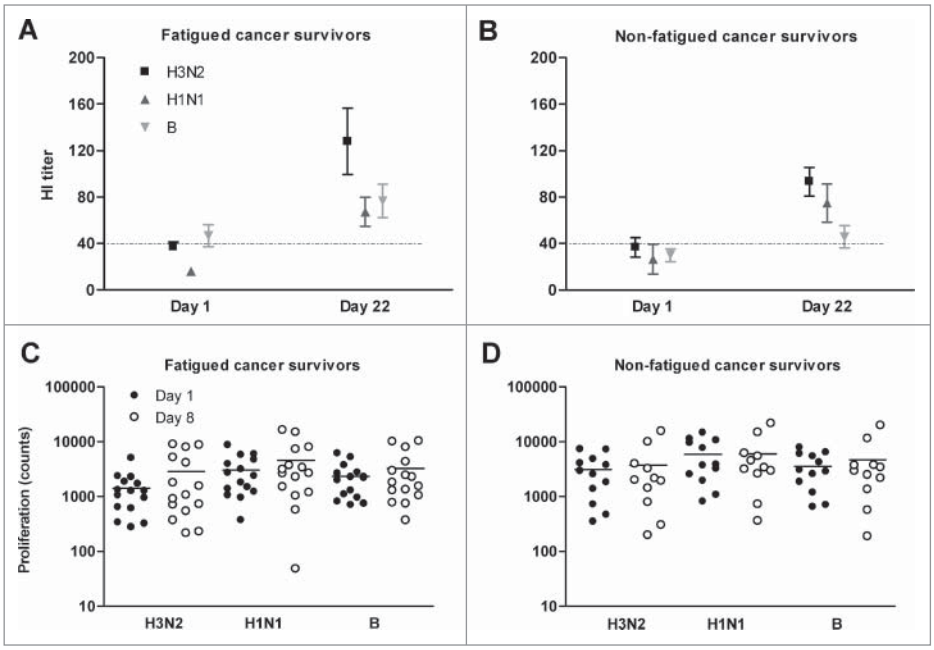

Figure 1. Hemagglutination inhibition antibody responses before (day 1) and after (day 22) influenza vaccination, and cellular proliferation before (day 1) and after (day 8) influenza vaccination. Both immune responses are presented for the 3 influenza strains of the vaccine (H3N2, H1N1, and B), in fatigued (**A and C**) and non-fatigued (**B and D**) cancer survivors. Antibody titres are presented as absolute numbers and mean on a linear scale, the dotted line indicates the protective titer cut-off value. Proliferation counts are presented as absolute numbers and mean (horizontal lines) on a logarithmic scale.

Cellular immune responses in postcancer fatigue upon influenza vaccination

Pre-vaccination

T cell proliferation upon stimulation with phytohemagglutinin (PHA) and influenza virus in both patient groups, indicate the presence of functional T cells (**Table 2**, **Fig. 1C, D**). At baseline, postcancer fatigue patients showed a diminished cellular proliferation upon stimulation with virus-strain H3N2 and with virus-strain H1N1, compared to non-fatigued controls (**Table 2**).

To investigate the cytokine secretion of peripheral blood mononuclear cells (PBMC) at baseline, interleukin 2 (IL-2) and interleukin 10 (IL-10) concentrations were measured. We detected significantly less IL-2 in patients suffering from postcancer fatigue compared to non-fatigued patients upon stimulation with virus-strains H3N2 and H1N1, whereas detected IL-10 concentrations were identical between the 2 groups (**Table 2**).

Regulatory T lymphocytes (Treg), which are known to negatively regulate T cell responses, were measured at baseline. Compared to non-fatigued controls, the percentage of Treg was significantly increased at baseline in severely fatigued patients (**Table 2**).

To test our hypothesis that impaired human heat shock protein 90 alpha (HSP90α) functioning resulted in Treg accumulation, HSP90α concentrations were measured in serum at baseline. Human HPS90α concentrations were not significantly different between fatigued and non-fatigued patients (**Table 2**).

In postcancer fatigue patients, the high percentage of Treg indeed correlated significantly with lower cellular proliferation upon stimulation with virus-strain H3N2 ($p = 0.015$), H1N1 ($p = 0.031$), and B ($p = 0.005$). Also, the percentage of Treg correlated significantly with IL-2 concentrations upon stimulation with virus-strain H3N2 ($p = 0.017$) and H1N1 ($p = 0.001$) in fatigued patients, but not upon stimulation with virus-strain B ($p > 0.05$). Non-fatigued patients did not demonstrate significant correlations between the percentage of Treg and cellular proliferation or between Treg and IL-2 concentrations ($p > 0.05$).

Post-vaccination

The cellular immune responses to the influenza vaccine were measured at day 8 by cytokine secretion of PBMC and T lymphocyte proliferation. Post-vaccination cellular proliferation was not significantly different between fatigued and non-fatigued cancer survivors (**Table 2**). Interferon gamma (IFN-γ), interleukin 4 (IL-4), and interleukin 5 (IL-5) production were not significantly different between both patient groups upon stimulation with the 3 individual influenza strains (**Table 2**).

There is no significant correlation ($p > 0.05$) between pre-existing strain-specific antibody titres or cellular proliferation and post-vaccination strain-specific antibody titres or cellular proliferation, neither in fatigued, nor in non-fatigued patients (data not shown).

Discussion

We believe that we are the first to explore both the humoral and cellular immune responses upon influenza vaccination in patients suffering from severe postcancer fatigue. The hypothesized immunological imbalance in postcancer fatigue was confirmed by our data, as patients suffering from postcancer fatigue show several significant differences in immunological reactivity at baseline, compared to non-fatigued patients.

Table 2. Humoral and cellular immune responses

	Postcancer fatigue (n=15)	No postcancer fatigue (n=12)	*p*-value
Seroprotection rate baseline (day 1)			
H3N2	11 (55)	6 (50)	0.212
H1N1	2 (13)	4 (33)	0.214
B	10 (67)	5 (42)	0.194
H3N2, H1N1, and B	1 (7)	2 (17)	0.411
Seroprotection rate post-vaccination (day 22)			
H3N2	12 (80)	12 (100)	0.100
H1N1	12 (80)	10 (83)	0.825
B	13 (87)	8 (67)	0.214
H3N2, H1N1, and B	11 (55)	7 (58)	0.411
Seroresponse rate post-vaccination (day 1 to 22)			
H3N2	8 (53)	6 (50)	0.863
H1N1	10 (67)	8 (67)	1.000
B	3 (20)	3 (25)	0.756
H3N2, H1N1, and B	2 (13)	0 (0)	0.189
Proliferation upon stimulation PHA (counts)			
Baseline (day 1)	24736 ±22830	22007 ±9415	0.702
Post-vaccination (day 8)	22286 ±15944	28733 ±18939	0.356
Proliferation upon stimulation H3N2 (counts)			
Baseline (day 1)	1414 ±1201	3099 ±2401	0.042
Post-vaccination (day 8)	2852 ±3241	3755 ±4742	0.569
Proliferation upon stimulation H1N1 (counts)			
Baseline (day 1)	3025 ±2339	5877 ±4604	0.069
Post-vaccination (day 8)	4560 ±5004	5944 ±6458	0.561
Proliferation upon B (counts)			
Baseline (day 1)	2311 ±1622	3551 ±2389	0.121
Post-vaccination (day 8)	3253 ±3336	4685 ±5809	0.434
IL-2 production at baseline (day 1, pg/ml)			
H3N2	36.3 ±44.3	94.0 ±45.4	0.003
H1N1	28.4 ±44.0	74.5 ±56.1	0.030
B	37.0 ±50.9	53.1 ±55.3	0.439
IL-10 production at baseline (day 1, pg/ml)			
H3N2	2.7 ±5.0	6.3 ±10.6	0.286
H1N1	50.0 ±43.2	55.2 ±35.5	0.736
B	34.6 ±80.3	20.6 ±14.0	0.560
Treg at baseline (day 1, %)			
Unstimulated	4.4 ±2.1	2.4 ±0.8	0.033
IFN-γ production post-vaccination (day 8, pg/ml)	167.9 ±216 .8	142.6 ±253 .0	0.787
H1N1	288.7 ±343.6	256.6 ±308.4	0.808
B	326.4 ±450.4	142.4 ±252.1	0.199
IL-4 production post-vaccination (day 8, pg/ml)			
H3N2	< detection limit	3.0 ±9.9	0.341
H1N1	2.2 ±8.5	< detection limit	0.403
B	2.8 ±10.8	< detection limit	0.403
IL-5 production post-vaccination (day 8, pg/ml)			
H3N2	5.5 ±9.9	3.9 ±8.7	0.672
H1N1	4.9 ±8.7	1.0 ±2.5	0.121
B	0.7 ±2.6	< detection limit	0.384
HSP90α expression at baseline (day 1, ng/ml)	37 ±17	41 ±12	0.530

Data are presented as absolute numbers with percentages in brackets or as mean ± standard error of the mean.

Severe fatigued cancer survivors are able to develop sufficient antibody levels and adequate cellular immune reactions after a single shot of seasonal influenza vaccine, which is similar to non-fatigued cancer survivors. Both the humoral and cellular immune responses to influenza vaccination were also comparable in one of our previous studies comparing chronic fatigue syndrome patients and healthy controls.[25] However, interestingly, compared to non-fatigued patients, patients suffering from postcancer fatigue showed significant differences in pre-vaccination cellular immune responses.

We observed that, in comparison to non-fatigued patients, the T cell capacity to proliferate was significantly diminished at baseline in fatigued patients upon stimulation with virus-strain H3N2 and for strain H1N1. To study this finding in more detail, we investigated 2 immunological mechanisms generally known to suppress T cell immunity. First, the baseline

production of the immunosuppressive cytokine IL-10, which has the capacity to inhibit T cell proliferation,[26] was measured. However, baseline IL-10 production was comparable in fatigued and non-fatigued patients. Secondly, since Treg are known to negatively modify T cell responses, we examined pre-vaccination Treg percentages.[27] Indeed, Treg percentages were significantly increased at baseline in postcancer fatigue patients, compared to non-fatigued controls, and could not be explained by a difference in the number of lymphocytes between both patient groups (data not shown). This finding could explain the diminished cellular proliferation in fatigued patients compared to non-fatigued patients before vaccination.

Consistent with the increase in Treg, the baseline production of IL-2, which drives T cell growth,[28] is decreased in fatigued compared to non-fatigued patients. The reduction in IL-2 levels is likely the result of IL-2 consumption by Treg,[29] thereby driving their expansion.

We hypothesized that impaired HSP90α functioning in patients suffering from postcancer fatigue resulted in Treg accumulation. Treg accumulation might lead to the development of functional somatic syndromes, like chronic fatigue syndrome.[30] HSP90 is highly up regulated during cell stress,[31] which occurs as a result of the tumor or its treatment, and HSP90 is involved in controlling the functions of FoxP3+ Treg.[32] The difference in Treg was not caused by HSP90 because fatigued and non-fatigued patients did not differ in baseline HSP90α concentrations in serum.

The influenza virus slightly changes its antigenic makeup every season, but still viral proteins are identical between the different viral strains (shared epitopes). At baseline the reactivity against these shared antigens is measured since the novel epitopes on the vaccination strains are not yet exposed to the immune system. Apparently, the Tregs were able to inhibit the response to these shared antigens. Both patient groups showed a normal level of proliferation when stimulated with the new viral strains. This indicates that naïve T cells were equally responsive toward those novel viral antigens. This was confirmed by the IL-2 production, which did not significantly differ between fatigued and non-fatigued patients (data not shown). We conclude that patients suffering from postcancer fatigue expand the percentage of Treg after vaccination. Seemingly, the naïve T cell response is normal between the 2 groups but the memory response is hampered in the severe fatigue patient group. This would imply that patients suffering from postcancer fatigue have to invest more energy in handling immunity against pathogens that are encountered earlier in live. This leads to less energy available for other tasks of the organism, explaining their fatigue. In contrast, cancer survivors who are able to handle the immune response to recurrent immunological challenges more efficiently may suffer less from fatigue.

In conclusion, assumed abnormalities in immune responses in postcancer fatigue patients were demonstrated for a memory immunological response. We show comparable humoral and cellular immune reactions to influenza vaccination in fatigued and non-fatigued cancer survivors indicating a normal naïve T cell response in both groups. Therefore, influenza vaccination is favorable for patients suffering from postcancer fatigue and should be recommended when indicated.

Patients and Methods

Participants

Fifteen severely fatigued and 12 non-fatigued cancer survivors were included in this study. From a previous influenza study in cancer patients with similar sample size and using identical measurement techniques, we know that our group sizes are sufficient to detect significant differences in immunological parameters.[34] Fatigue severity was determined according to the CIS-fatigue.[3,4] The CIS-fatigue was shown to be sensitive to detect changes and was used in previous research to assess fatigue in cancer patients.[11,18,35,36] A score of $\geq$35 on the CIS-fatigue is an indicator for severe fatigue. Cancer survivors suffering from severe fatigue who were referred for CBT to the Expert Center for Chronic Fatigue of the Radboud University Medical Center (Radboudumc) were asked for participation. Non-fatigued patients (score of <27 on the CIS-fatigue) were recruited from the outpatient clinics of Medical Oncology and Radiation Oncology of the Radboudumc. All participants were between 18 and 60 y old, used no corticosteroids during the previous 2 weeks, did not have an immune disorder or allergy to chicken eggs, and had no symptoms of influenza or an influenza-like illness on the day of the vaccination. For each patient, the oncological treatment history was retrieved from the medical chart. The study was approved by the local ethics committee of the Radboudumc and all participants provided a written informed consent.

Vaccination and blood collection

All participants visited the outpatient clinic of Medical Oncology of the Radboudumc between September 2010 and January 2011 and were intramuscularly vaccinated with a single dose of the inactivated trivalent split influenza vaccine (Vaxigrip^{R}, Sanofi Pasteur MSD, Hoofddorp, the Netherlands), which contained inactivated, split virion of the 3 influenza strains (A/H3N2/Perth/16/2009, A/H1N1/California/7/2009, and B/Brisbane/60/2008).

PBMC were harvested at baseline (day 1) and 7 d post-vaccination (day 8). PBMC were collected from heparinized venous blood by density-gradient centrifugation on lymphoprepTM (Axis-Shield PoC AS, Oslo, Norway) and cells were frozen using a cryo 1°C freezing container (Nalgene, Rochester, New York, USA) in freezing medium (49% X-VIVO15 (Lonza, Verviers, Belgium), 1% human serum (Sanquin blood bank, Nijmegen, the Netherlands), 40% human serum albumin (Albuman; Sanquin blood bank), and 10% DMSO (Cryo Sure; Wak Chemie Medical GMBH, Steinbach, Germany)). After 24h in a freezing container at −80°C, PBMC were stored in liquid nitrogen until analysis.

Serum was collected at day 1 and 21 d post-vaccination (day 22).[34] Serum samples were stored at −80°C until analysis.

Humoral immune response

Humoral immune reactions to the influenza vaccine were studied in serum by the HI antibody test as described previously.[37] Virus antibody responses were assessed at day 1 and day 22 for the 3 individual virus-strains of the vaccine (A/H3N2/Perth/16/2009, A/H1N1/California/01/2010, and B/Florida/004/2006). Seroprotection represents an antibody titer of 1:40 or higher.[36,37] Participants were considered fully protected if the titres to all 3 virus-strains reached the criteria for seroprotection, partially protected when having protective titres to only one or 2 virus-strains, and non-protected when titres to all 3 virus-strains were below 1:40. Post-vaccination seroresponse represents an at least fold 4- increase in antibody titer.[37]

Cellular immune response

Cellular immune reactions were studied by T lymphocyte proliferation and cytokine secretion of PBMC collected at day 1 and 8, the presence of Treg at day 1, and the concentration of HSP90α at baseline.

Cells were simultaneously thawed. For the analysis of lymphocyte proliferation and cytokine secretion, 1.5×10^5 PBMC were added per well of a 96-wells plate in culture medium (RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 4% human serum albumin). In the proliferation assay, PBMC were incubated with 1μg/ml PHA and a 1:10 dilution of the individual virus-strains (A/H3N2/Perth/25/11/2008, A/H1N1/California/01/2010, and B/Florida/05/11/2008). Supernatant was collected to measure cytokine production after 48 hours of culture. The Th1/Th2 11plex kit (eBioscience, San Diego, CA) was used according to the manufacturer's protocol to measure levels of IL-10 at day 1 and IFN-γ, IL-4, and IL-5 at day 8. After four days of culture, 1 μCi ^{3}[H]-thymidine (MP Biomedicals, Santa Ana, California, USA) was added to each well for 8 hours of incubation to measure T lymphocyte proliferation.

Multi-color flow cytometric analyses were performed on unstimulated PBMC harvested at day 1 according to the manufacturer's protocol. Cells were stained for surface antigens anti-CD4/FITC (Beckman Coulter, Woerden, the Netherlands), anti-CD25/PE-Cy7 (BD Biosciences, Breda, the Netherlands), and anti-CD127/PE (BD Bioscience), fixed and permeabilized prior to staining with anti-FoxP3/APC (clone PCH101, eBioscience). Treg were defined as CD4+CD25+CD127-FoxP3+ cells and calculated as a percentage of all CD4+ cells. Data were analyzed using FlowJo software (Treestar).

HSP90α concentrations were detected in serum collected at baseline, using a colorimetric, immunometric enzyme immunoassay kit according to the manufacturer's protocol (Enzo Life Sciences, Antwerpen, Belgium).

Statistics

Statistical analyses were performed using PASW for Windows®, version 18.0.2 (Armonk, New York, USA) and Prism, version 4.0 (San Diego, California, USA). Independent samples *t* tests were performed to assess differences in numerical variables at day 1, 8, and 22. Paired *t* tests were used to assess changes in numerical variables from pre- to post-vaccination. Chi-square tests were performed to compare groups on categorical variables. Differences were considered statistically significant at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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