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Letter to the Editors-in-Chief

Preexisting anti-PF4 antibodies are not further triggered upon vaccination with SARS-CoV-2 vector vaccines in a cohort of 400 health care workers

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Vaccine-induced immune thrombotic thrombocytopenia (VITT) was reported after adenovector-based COVID-19 vaccination with ChAdOx1 and Ad26.COV2·S as a rare, but very severe complication [1,2]. Thrombosis/hypercoagulation has been reported also as a common complication of (severe) COVID-19 and is probably multifactorial due to endothelial dysfunction caused either by direct viral infection or by hyperinflammation, which also leads to activation of platelets and the clotting cascade as well as due to alteration of the blood flow, as explained by the Virchow's triad [3]. In VITT, the hypothesis states that platelet activation, aggregation, thrombocytopenia and thrombotic events could occur either due to boosting of preexisting anti-platelet factor 4 (PF4) antibodies or due to de-novo induction of these antibodies by the vaccine since thrombotic events and PF4 antibodies have been detected during severe SARS-CoV-2 infection [4,5]. It is assumed that auto-antibodies against platelet factor 4 (PF4) may lead to VITT similar as in heparin-induced thrombocytopenia that shares clinical and immunological similarities with VITT [1]. However, despite one study reporting binding of anti-PF4 antibodies to the receptor binding domain (RBD) of the spike protein with possible formation of complexes, it has been demonstrated that anti-PF4 antibodies do not cross-react with SARS-CoV-2 spike protein [6]. Moreover, revaccination of VITT patients did not lead to VITT for a second time and PF4 levels decreased within months, thus arguing for VITT being an antigen-unrelated event [7].

In contrast to the full clinical feature of VITT, oligosymptomatic courses have been described [8], which responded quickly to treatment without development of thromboembolic events. It might even be the case that anti-PF4 antibodies occur without the typical clinical feature of VITT. Data on pre- and post-vaccination anti-PF4 antibodies in a large cohort vaccinated with ChAdOx1 are not yet available.

In this retrospective analysis, we investigated in a cohort of 400 health care workers (HCW) the prevalence of anti-PF4 antibodies prior to the first dose of ChAdOx1and three to four weeks after vaccination.

Blood samples were obtained for anti-SARS-CoV-2 antibody testing upon personal choice from HCW immediately prior to and 3 to 4 weeks after the first dose of ChAdOx1 (Vaxzevria, Astra Zeneca) during the University's vaccination campaign between March and June 2021 (n = 1255). From these vaccinees, a random sample of 400 participants was drawn, in whom pre- and post-vaccination samples intended for SARS-CoV-2 antibody testing were available, and tested for anti-PF4 antibodies. Age at vaccination and sex were recorded. The study was approved by the Ethic Committee of the Medical University of Vienna (EK: 2274/2021).

Anti-PF4/heparin IgG was tested by using the Zymutest HIA IgG immunoassay (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instructions. Cut-off for positivity was an optical density (OD) value above 0.5. Intra-assay and inter-assay reproducibility are reported with 3.07 % and 7.11 % respectively by the manufacturer.

SARS-CoV-2-specific IgG antibodies against the subunit 1 (S1) of the spike protein were measured by ELISA (Quantivac®, Euroimmun) in diluted sera (1:101) according to the manufacturer's instructions. Results are expressed in binding antibody units/ml (BAU/ml) with values above 35.2 BAU/ml considered positive.

Only descriptive, graphical methods with scatterplots and case-profile line plots were applied.

Sample size was determined on the following assumptions: The study should have an 80 % power to detect an odds-ratio of at least 2 for anti-PF4 positivity after vaccination to before vaccination at a (two-sided) significance level of 5 % assuming 18 % discordant pairs.

Among the 400 participants, (61.7 % females) the median age was 37.0 years (± 9.9 standard deviation (SD)). No thrombosis, thrombocytopenia or clinical VITT were reported from the participants until the second dose of ChAdOx1.

In total, six of 400 participants (1.5 %) tested positive for anti-PF4 antibodies before the first dose (Fig. 1A). Five of the six samples were considered low to moderately reactive with OD < 1. Only one sample was highly reactive with an OD of 1.5. The median age of the anti-PF4 antibody positive participants was 47.3 (± 12.1 SD) years that is non-significantly higher than in the anti-PF4 antibody negatives (37.0 years ±12.0 SD). Four of the six positive individuals were females (Table 1). Comparison of positive anti-PF4 levels before and after vaccination revealed no change (mean change in OD was 0.997-fold) and levels remained stable (Fig. 1A). In none of the participants de novo positivity occurred. None of the six participants, positive for anti-PF4 antibodies displayed serological evidence of past SARS-CoV-2 infection since all had negative SARS-CoV-2 spike-specific IgG at baseline (Fig. 1B). In addition, the 12 individuals in this study identified as

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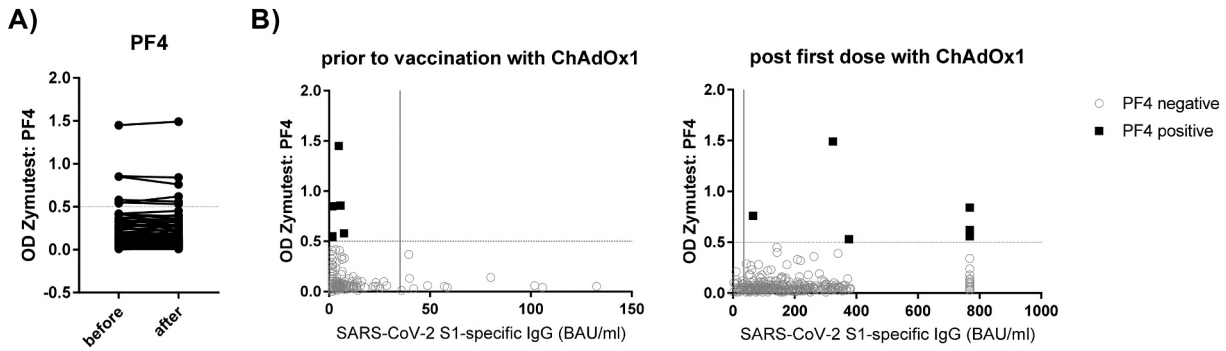

Fig. 1. Anti-PF4 antibody values before and after the first dose of ChAdOx1 and the correlation with SARS-CoV-2 S1-specific IgG values. (A) The anti-PF4 IgG values measured before (day 0) and 21 days after the first dose of ChAdOx1 are expressed in OD. The dotted line represents cut-off for positive anti-PF4 IgG values at OD 0.5. (B) Anti-PF4 antibody and SARS-CoV-2 antibody values were correlated upon measuring of sera taken from the vaccinees either immediately before vaccination with ChAdOx1 (A) and 21 days after the first dose of ChAdOx1 (B). The dotted lines represent the cut-off for positive anti-PF4 antibody values at OD 0.5 and the black lines represent the cut-off for positive SARS-CoV-2 S1-specific IgG at 35.2 BAU/ml.

Table 1
Demographic characteristics of anti-PF4 positive individuals.

Subject	Sex	Age	Anti-PF4 antibody OD before/ after	Comorbidities
1	m	25	0.58/0.56	Information not available
2	f	34	1.45/1.49	None
3	f	39	0.55/0.53	Information not available
4	m	55	0.85/0.84	Seasonal allergy
5	f	56	0.54/0.62	Seasonal allergy
6	f	56	0.86/0.84	Type 1 diabetes

recovered from COVID-19 by SARS-CoV-2 IgG seropositivity prior to vaccination did not show anti-PF4 antibodies before (mean 0.084 ± 0.098) or after (mean 0.078 ± 0.079) the first dose of ChAdOx1. Furthermore, there was no correlation of the anti-PF4-antibody levels and the immunological responses to the first dose of ChAdOx1 in our study cohort measured by anti-SARS-CoV2 IgG antibody levels before and after the first dose for titers neither below nor above the cut-off of 35.2 BAU/ml (Fig. 1B).

Anti-PF4 antibodies are considered a hallmark of VITT and testing for anti-PF4 antibodies is indicated upon clinically suspected VITT in recently SARS-CoV-2-vaccinated patients with new onset of thrombosis, thrombocytopenia and elevated D-Dimer [9]. Here, we demonstrate within a large cohort of 400 HCW that anti-PF4 antibodies were already prevalent albeit at a low frequency of 1.5 % before vaccination. Furthermore, no new PF4 antibody formation was detected after vaccination. The prevalence of anti-PF4 antibodies in our study appears to be lower than the 6.6 % detected in the pre-pandemic era in a cohort of blood donors representing a preselected healthy study population. However, the discrepancy in prevalence might be explained by the lower cut-off at OD 0.4 used in the former study compared to 0.5 in our analysis. Using this lower cut-off would increase prevalence in our study to 2 %. These data indicate that anti-PF4 antibodies are not solely specific for VITT and can be also detected at low frequency in healthy individuals.

With regard to anti-PF4 antibody levels measured after vaccination with ChAdOx1 a previous study featuring a comparable cohort of HCW showed a prevalence of 1.2 %, all with OD < 1.5 and no onset of VITT [10]. Importantly, we could show that the levels of anti-PF4 antibodies before and after vaccination with ChAdOx1 did not change/increase and remained below OD 1.5 in all individuals. The OD value is important because an OD below 1.5 seem to be unrelated to VITT since patients with confirmed VITT displayed an OD above 2 [2]. Additionally, not all anti-PF4 antibodies seem to be able to activate platelets, which is a prerequisite for the pathogenesis of VITT. We did not test for platelet activation capacity in anti-PF4 positive sera but considering that all participants remained healthy, capacity of platelet activation of the detected antibodies must have been limited.

So far, data are limited on whether anti-PF4 antibodies arise from SARS-CoV-2 vaccination or are already preexisting and expand upon antigen contact. Since anti-PF4 antibody detection is described to be transient [7], comparison of pre- to post-vaccination anti-PF4 levels in close temporal proximity to vaccination seems essential. We did not find de-novo induction of anti-PF4 antibodies upon application of the first dose of the COVID-19 vector vaccine ChAdOx1 within the 400 participants. This is in line with a previous study [11] reporting preexisting low-level anti-PF4 antibodies (OD < 1) at the day of the first dose of ChAdOx1 that remained within the same OD range until two weeks after vaccination in one individual, however, the sample size of this subgroup was very small (n = 7). Taken together these data support the notion that the de-novo induction of high-level anti-PF4 antibodies associated with VITT in the context of the SARS-CoV-2 vector vaccines are rare events [9]. Since vector-based vaccines are a major part of the global COVID-19 vaccine supply, it would be helpful to define markers to predict and to identify vaccinees at risk for developing VITT. However, anti-PF4 antibodies, at least in low concentration, seem not an appropriate prediction marker, since pre-vaccination anti-PF4 antibodies are not associated with development of VITT. Incidence of VITT ranges from 1 case per 26,500 to 127,300 first doses of ChAdOx1 administered according a recent publication [6]. Compared to one anti-PF4 positive case per 67 prior to vaccination, incidence of VITT is at least two orders of magnitude less frequent suggesting additional conditions underlying pathogenesis of VITT.

Since pre-vaccination anti-PF4 antibody levels are rarely known, detection of post-vaccination anti-PF4 antibodies has to be interpreted with caution and testing should only be done in patients with clinical suspicion of VITT. That includes new onset thrombocytopenia, with or without thrombosis five to 30 days after vaccination and elevated D-Dimer [9].

Limitations of this study are the restriction to HCW, mainly younger than 60 years of age that may represent different immunological profiles as compared to the general population of vaccinees and the reliance on clinical symptoms for thrombosis, without performing platelet counts and coagulation assays. However, since VITT mainly occurred in females below age of 60 [9] our study cohort is indeed representative for the population at risk to develop VITT. In addition, we determined anti-PF4 antibodies by ELISA to measure heparin-dependent antibodies of the IgG isotype since the polyanion responsible for VITT has not been identified yet. Furthermore, we are aware that the analyzed anti-PF4 antibodies are only one possible marker involved in the pathomechanism of VITT.

In conclusion, pre-existing anti-PF4 antibodies do not seem to be

relevant for VITT induction, as they did not expand after vaccination and occurrence of new anti-PF4 antibodies were not detected in our cohort.

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Declaration of competing interest

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