RESEARCH LETTER

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Investigating potential mechanisms underlying FVIII inhibition in acquired hemophilia A associated with mRNA COVID-19 vaccines

Acquired hemophilia A (AHA) is a rare bleeding disorder caused by functional insufficiency of coagulation factor VIII (FVIII). Autoantibodies targeting FVIII may neutralize its procoagulant effect, thereby causing severe bleeding. Such inhibitory autoantibodies have been detected in autoimmune diseases, pregnancy, infections, or malignant diseases. Older age and certain drugs are known co-risk factors.[1] To our knowledge, only two reported cases document AHA diagnosed 8 and 20 days after influenza vaccination.[2,3]

Vaccines have been rarely associated with autoimmune disease occurrence or disease flares. Recently, vaccine-induced immune thrombocytopenia and thrombosis (VITT) has been characterized as a new entity.[4] Immunological studies established a pathogenetic role of platelet-activating autoantibodies targeting platelet factor 4 (PF4) in VITT. VITT-associated anti-PF4-IgG were not cross-reactive with the SARS-CoV2 spike antigen, suggesting that the vaccine-specific antibody response is not directly causing VITT.[5] A recent study linked the occurrence of VITT to the interaction of the adenoviral vector with the coxsackie and adenovirus receptor and PF4, thus instigating memory B cell differentiation and the release of anti-PF4 auto-antibodies.[6]

Our group recently reported three cases of AHA occurring in temporal association with mRNA COVID-19 vaccine immunizations.[7] Statistically, we found no strong evidence that the AHA incidence during the COVID vaccination campaign in Switzerland was substantially higher than the background AHA incidence. In our previous report, we did not address the possibility of FVIII cross-reactivity of the vaccine-induced anti-spike IgG (anti-S-IgG). Excluding cross-reactivity of anti-foreign IgG with a self-antigen is critical to refute 'molecular mimicry' in the immunopathogenesis of an autoimmune disease.

Here, we studied the binding, function, and cross-reactivity of the vaccine-induced anti-S-IgG in our previously reported three cases of AHA diagnosed in temporal association with COVID vaccination.[7] The main goal was to address whether the vaccine-induced

antibody response against the SARS-CoV2 spike protein may exhibit FVIII inhibitory functions.

The sequence alignment of the FVIII (UniProtKB accession number P00451) and the SARS-CoV2 spike protein (UniProtKB accession number P0DTC2) revealed minimal sequence similarity. We identified one region (amino acid position 540–570 within the A2 domain of FVIII) with 13/35(37%) amino acid sequence similarity using the NCBI blast sequence alignment tool. *In silico* antigenic peptide prediction (http://imed.med.ucm.es/Tools/antigenic.pl) revealed 95 and 63 antigenic determinants in the FVIII and spike protein, respectively. Of those, a single overlapping potential epitope was present in both proteins, locating to the region with the sequence similarity (Figure 1A; SDPRCLTRYYS-S in the FVIII sequence [FVIII 543–554]; underlined amino acids indicate homology to the SARS-CoV2 spike protein). Since only a few amino acids are shared between the FVIII and spike protein in this region, the likelihood of a cross-reactive B cell epitope is, however, low.

Next, we addressed this experimentally. The presence of vaccine-specific antibodies is a pre-requisite for a potential cross-reactivity to FVIII. Serological analyses proved considerable anti-Spike IgG (anti-S-IgG) levels in the serum of all three vaccinated patients (Figure 1B). Anti-S-IgG is the only antigen-specificity induced by the mRNA COVID vaccines. To explore the FVIII inhibitory potential of the anti-S-IgG fraction, we performed a bead-based antibody pull-down to deplete and enrich for anti-S-IgG (Supplementary Data). The anti-S-IgG enrichment and -depletion was confirmed in a Luminex assay using spike protein-coated beads (Figure 1C) and in western blot loaded with recombinant spike protein (Figure 1D). Despite efficient depletion and enrichment, the 'anti-S-IgG enriched' fraction contained residual non-anti-S-IgG based on total IgG measurements (mean total-IgG in the anti-S-enriched fraction 0.49g/l. Moreover, we found traces of other serum proteins as assessed by gel electrophoresis (Figure S1). To determine which of the serum fractions contained the FVIII inhibitory factor, we first performed a mixing FVIII assay (Supplementary Methods). The non-manipulated serum and anti-S-IgG-depleted fractions showed similar FVIII inhibition of 75%. In

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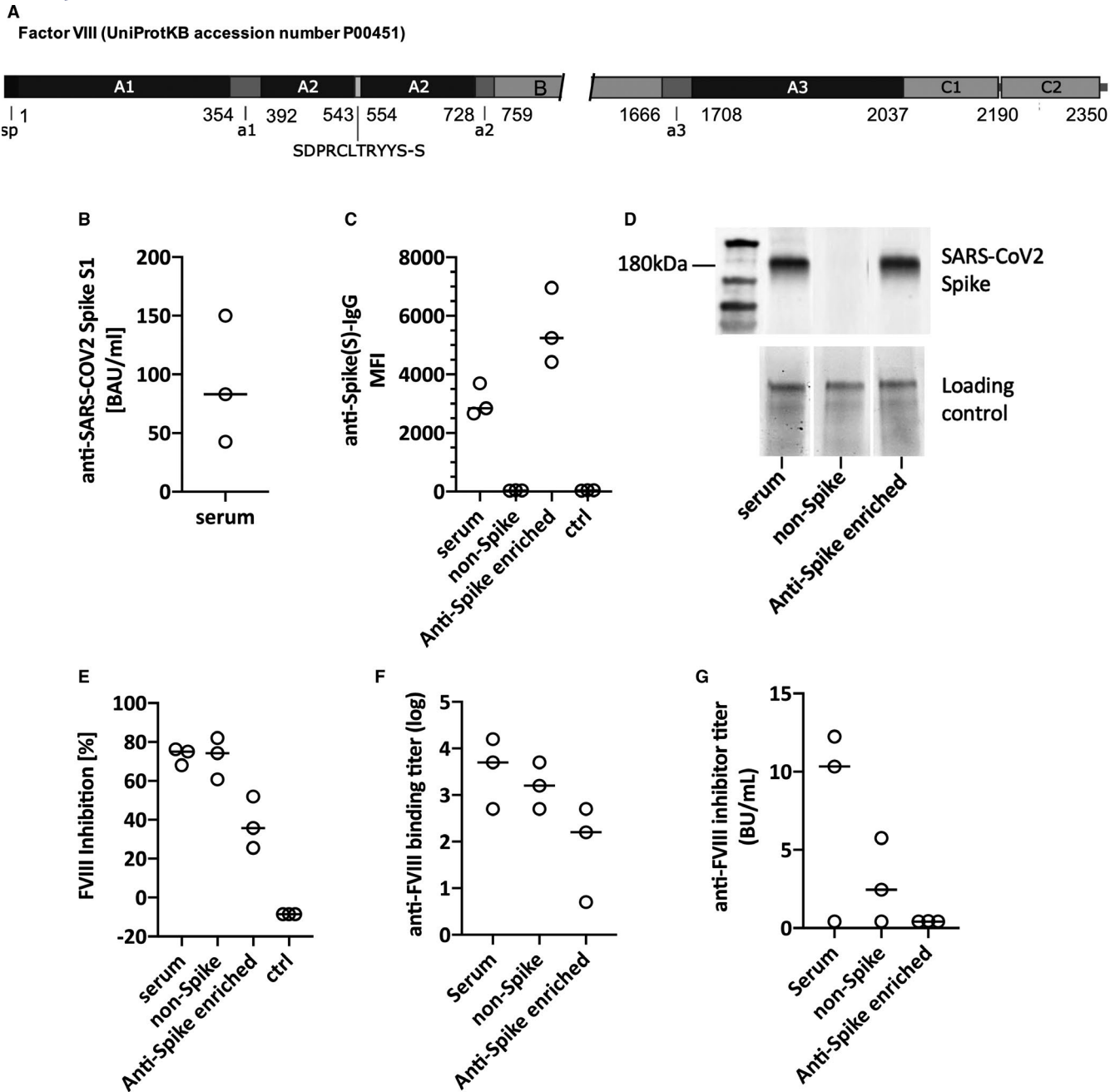**Factor VIII (UniProtKB accession number P00451)**

FIGURE 1 Factor VIII inhibition by anti-SARS-CoV2-spike-IgG and the non-anti-spike-IgG fraction. (A) Localization of the potential epitope with sequence similarity between the Factor VIII and the SARS-CoV2 spike protein. Protein amino acid sequence and gene arrangement were retrieved from UniProtKB, accession number P00451. Amino acid annotations were adapted according to Ref. [14]. (B) Serum anti-Spike-IgG/M (Roche Elecsys® Anti-SARS-CoV-2 assay; BAU = binding antibody units). (C) Anti-spike-IgG antibodies in the serum of the three patients, the anti-spike-IgG-depleted serum, and the anti-Spike-IgG enriched plasma in Luminex. ctrl = serum from three healthy subjects from pre-pandemic timepoints. (D) Western blot confirms the anti-spike-IgG's depletion and enrichment in the respective samples. FVIII mixed serum assay (E), anti-FVIII binding titers (F), and anti-FVIII inhibitor titers (G) from the same samples

contrast, only 35% FVIII inhibition was observed using the anti-S-IgG-enriched fraction, indicating that anti-S-IgG was not the main mediator of FVIII inhibition in this assay (Figure 1E). The application of an enzyme-linked immunosorbent assay for total anti-FVIII IgG yielded detectable levels in serum and anti-S-IgG depleted fractions. However, the anti-S-IgG enriched fractions either showed no detectable ($\log_{10}$ titer <0.7, $n = 1$) or about 1.5 $\log_{10}$

(30-fold, $n = 2$) lower titers (Figure 1F) compared to the other fractions. Furthermore, the binding of the anti-S-IgG enriched fraction to different therapeutic FVIII preparations, as assessed by Luminex-based analysis, was negative or very low in all cases (Figures S2 and S3). The FVIII inhibitory potential of the binding antibodies was addressed using the Nijmegen-Bethesda assay (NBA). Using serum rather than plasma in this study, neutralizing

anti-FVIII-activity could not be detected within the original or derived samples of one of the patients. However, using citrated plasma, an inhibitor level of 1.01 BU/ml was previously described in the same patient.[7] For the other two patients, neutralizing anti-FVIII-activity was detected in the original and the anti-S-IgG depleted serum samples, while no inhibition was observed in the anti-S-IgG-enriched fraction (Figure 1G). In patient AHA01, the inhibitory titer was even higher than in the original publication, which was likely due to a combination of another time-point of sample acquisition, different sample material (serum *vs.* plasma), and the use of different FVIII test systems for assay read-out.[7] In order to ensure assay specificity for anti-FVIII activity, we applied control measures with respect to the use of serum instead of plasma samples (Supplementary Data). Anti-phospholipid antibodies may interfere with functional anti-FVIII assays.[8] We therefore screened all samples for anti-phospholipid antibodies (IgG and IgM) that were found to be negative or only at threshold levels (Table S1). Furthermore, to further increase specificity, a chromogenic FVIII assay was applied to determine (remaining) FVIII-activities.[9]

In summary, we found that (i) the likelihood of cross-reactive epitopes between the spike protein and FVIII is low based on *in silico* protein structures; (ii) the anti-S-IgG enriched fraction showed weak FVIII cross-reactivity in binding assays; (iii) weak cross-binding of the anti-S-IgG enriched fraction did not translate into FVIII inhibition. The FVIII binding in the enriched anti-S-IgG fraction may have been due to residuals of anti-FVIII-IgG with low cross-reactivity against the spike protein. The amount of total IgG measured in this fraction indeed indicated a substantial non-anti-S-specific IgG.

We conclude that AHA associated with mRNA COVID vaccination was likely not due to vaccine-induced cross-reactive, FVIII-inhibiting anti-S-IgG. Alternatively, the broad toll-like-receptor stimulation by mRNA vaccines[10] may cause polyclonal B cell activation and thereby trigger autoantibody production in pre-existing self-reactive B cell clones in persons predisposed to AHA. Indeed, several studies indicated that thymic deletion of T cell clones specific for endogenous ("self") FVIII is incomplete and that these cells may expand in persons with AHA following a corresponding immune response.[11] While we are not aware of comparable data on B cell clones, the detection of natural anti-FVIII antibodies in healthy subjects strongly argues for the presence of anti-FVIII specific B cells.[12] Interestingly, the FVIII sequence (epitope) described above (FVIII 543–554), which shows some similarities to SARS-CoV2 Spike, has been identified to overlap with a particularly immunogenic FVIII sequence (FVIII 545–559; patent application WO2009071886), and corresponding peptides are therefore part of a proposed strategy to induce immune tolerance in susceptible HA patients.[13] Thus, due to these sequence similarities, it might be speculated whether the presentation of corresponding SARS-CoV2 Spike peptides by MHC class II led to activation of FVIII-specific pre-existing T cell clones. A detailed analyses of the T cell responses in the patients was, however, limited by sample availability and beyond the scope of this work as extensive T cell assays using peptide arrays would have been needed to experimentally assess this. As another limitation, we studied only three subjects and moreover cannot exclude that ethnical backgrounds or MHC haplotypes may have affected the findings, given that we only studied patients in Switzerland. On a larger scope, the here presented data, combined with our epidemiological analysis,[7] demonstrates that immunological phenotypes occurring related to vaccination may occur unrelated to the vaccine-antigen. Detailed epidemiological and immunological studies, rather than single clinical case reports, are needed to advance the understanding of adverse events following vaccination.

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