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Correction of bleeding in experimental severe hemophilia A by systemic delivery of factor VIII-encoding mRNA

Short title: mRNA-based therapy for hemophilia A

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Jules Russick<sup>1</sup>, Sandrine Delignat<sup>1</sup>, Peter Milanov<sup>2</sup>, Olivier Christophe<sup>3</sup>, Gábor Boros<sup>4</sup>, Cécile V Denis<sup>3</sup>, Peter J Lenting<sup>3</sup>, Srinivas V Kaveri<sup>1</sup>, Sébastien Lacroix-Desmazes<sup>1</sup>

<sup>1</sup>Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, USPC, Université Paris Descartes, Université Paris Diderot, F-75006 Paris, France; <sup>2</sup>DRK-Blutspendedienst, Institut für Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Germany; <sup>3</sup>HITH, UMR\_S1176, INSERM, Univ. Paris-Sud, Université Paris-Saclay, Le Kremlin-Bicêtre, France; <sup>4</sup>BioNTech RNA Pharmaceuticals, An der Goldgrube 12, 55131 Mainz, Germany

Address correspondence to: Sébastien Lacroix-Desmazes, INSERM UMR S 1138, 15 rue de l'Ecole de Médecine, 75006, Paris, France. Email: Sebastien.Lacroix-Desmazes@crc.jussieu.fr

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## **Abstract**

The treatment or prevention of bleeding in patients with hemophilia A rely on replacement therapy with different factor VIII containing products or on the use of by-passing agents, i.e., activated prothrombin complex concentrates or recombinant activated factor VII. Emerging approaches include the use of bispecific anti-factor IXa/factor X antibodies, anti-Tissue Factor Pathway Inhibitor antibodies, interfering RNA to antithrombin, APC-specific serpins or gene therapy. The latter strategies however meet with short term clinical experience and potential adverse effects including the absence of tight temporal and spatial control of coagulation or risk for uncontrolled insertional mutagenesis. The systemic delivery of mRNA allows the endogenous production of the corresponding encoded protein. Thus, injection of lipid nanoparticles-formulated erythropoietin-encoding mRNA resulted in increased erythropoiesis in mice and macaques. Here, we demonstrate that a single injection of *in vitro* transcribed B domain-deleted factor VIII-encoding mRNA to factor VIII-deficient mice allows the endogenous production of pro-coagulant factor VIII. Circulating FVIII:C levels above 5% of normal levels were maintained for up to 72 hours, with an estimated half-life of factor VIII production of 17.9 hours, and corrected the bleeding phenotype in a tail clipping assay. The endogenously produced factor VIII however exhibited low specific activity and induced a potent neutralizing IgG response upon repeated administration of the mRNA. Our results suggest that the administration of mRNA is a plausible strategy for the endogenous production of proteins characterized by poor translational efficacy. At term, the use of alternative mRNA delivery systems and improved factor VIII-encoding mRNA should foster the production of functional molecules and reduce their immunogenicity.

**Keywords:** Hemophilia A, factor VIII, *in vitro* transcribed mRNA, mRNA therapy

## Article Summary

1. We investigated whether the systemic delivery to FVIII-deficient mice of *in vitro* transcribed mRNA encoding FVIII allows the endogenous production of procoagulant FVIII.
2. The systemic delivery of FVIII-encoding mRNA yielded therapeutic levels of circulating FVIII, but induced a brisk neutralizing immune response.

## Introduction

Hemophilia A is a rare X-linked hemorrhagic disorder that results from insufficient plasma levels of pro-coagulant factor VIII (FVIII).<sup>1</sup> Replacement therapy using exogenous FVIII is to date the most efficient strategy to treat or prevent bleeds. It is however extremely expensive owing to the elevated production costs and short half-life of therapeutic FVIII and need for life-long treatment. Several alternative strategies to correct bleeding include the use of FVIII bypassing agents, such as activated prothrombin complex concentrates (aPCC), recombinant factor VIIa (rFVIIa) or monoclonal FVIII-mimicking bispecific antibodies,<sup>2</sup> the injection of anti-Tissue Factor Pathway Inhibitor (TFPI)<sup>3</sup>, of interfering RNA to antithrombin (AT)<sup>4</sup> or of APC-specific serpins,<sup>5</sup> and gene therapy.<sup>6</sup> Each of these promising therapies has however intrinsic challenges that may limit broad application at term.

The *in vivo* production of proteins following the administration of mRNA was demonstrated in the early 90's in the case of luciferase and beta-galactosidase,<sup>7</sup> leading to the first clinical trial with mRNA a decade later.<sup>8</sup> Concomitantly, both double- and single-stranded RNA were found to trigger innate immunity upon ligation of TLR3, 7 and 8, and RIG-1.<sup>9-12</sup> The replacement of uridines by 1-methylpseudouridines and the removal of double-stranded RNA by HPLC was demonstrated to abrogate the activation of innate immune cells,<sup>13, 14</sup> and allowed the *in vivo* production of different proteins, including erythropoietin (EPO), factor IX and anti-HIV antibodies without the induction of overt neutralizing immune responses.<sup>15-19</sup> Conversely, the administration of synthetic mRNA was also used in vaccination strategies either by direct injection<sup>20</sup> or upon adoptive transfer of *ex vivo* transfected dendritic cells.<sup>21, 22</sup>

Because of its monogenic nature and the requirements for low amounts of FVIII to correct the bleeding phenotype of the patients, hemophilia A is a particularly suitable disease for treatment with mRNA. Furthermore, transfection with mRNA is by essence not integrative, and thus avoids risks of uncontrolled insertional mutagenesis that may occur in DNA-based gene therapy

approaches. In addition, mRNA is translated only transiently and is degraded by physiological pathways, thus ensuring its safety and facilitating the control of the bio-availability of the encoded protein. Here, we investigated whether the intravenous administration of FVIII-encoding mRNA allows the production of therapeutic levels of pro-coagulant FVIII in FVIII-deficient mice.

## **Methods**

### **Cloning of FVIII**

The cDNA encoding human B domain-deleted (BDD) FVIII (FVIII<sup>HSQ</sup>), containing the 14-amino acid segment SFSQNPPVLKRHQR in place of the B domain, cloned in the ReNeo mammalian expression plasmid with a geneticin resistance, has been described previously.<sup>23</sup> Codon optimization of the DNA sequence encoding human BDD-FVIII was adapted to the bias of Homo Sapiens using an in-house proprietary software (GeneOptimizer) from GeneArt (Thermo Fisher, Darmstadt, Germany). The GeneOptimizer software also calculates removal of cis-acting sequence motifs, including internal TATA-boxes, chi-sites and ribosomal entry sites, AT- or GC-rich sequence stretches, AU-rich elements, inhibitory and cis-acting repressor sequence elements, repeat sequences, RNA secondary structures, and all cryptic splice sites. The codon optimized BDD-FVIII-encoding cDNA was also cloned in the ReNeo vector.

### ***In vitro* transcription of mRNA**

mRNAs were transcribed as previously described<sup>15</sup> using the linearized plasmids encoding BDD-FVIII (FVIII<sup>HSQ</sup>), the codon-optimized BDD-FVIII (CoFVIII<sup>HSQ</sup>) and firefly luciferase (Luciferase). The Megascript T7 RNA polymerase kit (Thermo Fisher) was used for transcription, and UTP was replaced with 1-methylpseudouridine triphosphate (m1ΨTP; TriLink, San Diego, CA) to generate m1Ψ-containing mRNA. All mRNAs were transcribed to

contain 100-nt long poly(A) tails. To obtain cap1, RNA was incubated with guanylyltransferase and 2'-O-methyltransferase (Vaccinia capping system; New England Biolabs, Frankfurt). All mRNAs were purified and stored at -20°C.

### ***In vitro* transfection**

For transient *in vitro* production of FVIII, baby hamster kidney (BHK) cells (0.5 million cells in 48-well plates) were transfected with FVIII<sup>HSQ</sup> or CoFVIII<sup>HSQ</sup> cloned in the ReNeo vector (0.1 µg) using lipofectamine (Invitrogen, Carlsbad, CA, USA). For *in vitro* transfection using mRNA, mRNA (0.4 µg) was mixed with TransIT®-mRNA reagent (0.45 µl, Mirus Bio, Madison, WI, USA) and Boost reagent (0.29 µl) in a final volume of 50 µl of Dulbecco's Modified Eagle's Medium for 2 min at room temperature. HEK293 cells (50,000 cells/130 µL) were incubated with the formulated mRNA overnight in DMEM-F12 (DMEM-F12, Thermo Fisher). FVIII was measured in the supernatant after 24 hours. Supernatant was kept frozen at -80°C until use.

### **Treatment of mice**

Mice were 8 to 12-week-old FVIII exon 16 knock-out C57BL/6 mice (a kind gift from Prof H.H. Kazazian, Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA). Mice were injected intravenously with recombinant BDD-FVIII (rFVIII, Refacto®, Pfizer, 150 IU/kg), or with mRNA (1 to 5 µg) formulated in TransIT® (100 to 350 µL final volume). Blood was collected from retro-orbital sinus 6, 24, 48, 72 or 120 hours following the injection of mRNA. Plasma was kept frozen at -80°C until use. Animals were handled in agreement with local ethical authorities (Approval by ethics committee Charles Darwin, ethical authorization #3335 2015121718044892). FVIII:Ag, FVIII:C, anti-FVIII IgG and FVIII inhibitors were measured as described in Supplemental Methods online.

## Results

### **Codon-optimization of cDNA encoding FVIII<sup>HSQ</sup> improves *in vitro* FVIII production**

We first investigated whether codon-optimization of FVIII<sup>HSQ</sup> improves the production of FVIII by BHK transfected cells. To this end, the codon-optimized cDNA encoding B domain-deleted FVIII (CoFVIII<sup>HSQ</sup>) was synthesized and inserted in the Reneo vector. BHK cells were transiently transfected with 0.1 µg FVIII<sup>HSQ</sup> or CoFVIII<sup>HSQ</sup>-encoding cDNA (Figure 1A). FVIII:Ag and FVIII:C were measured in the supernatant 24 hours later by ELISA and chromogenic assay. Transfection with CoFVIII<sup>HSQ</sup>-encoding cDNA produced 4.2-fold more FVIII:Ag (mean±SEM: 0.11±0.01 IU/mL vs 0.03±0.00 IU/mL, respectively, P<0.01) and 4.8-fold more FVIII:C (0.14±0.02 IU/mL vs 0.03±0.01 IU/mL, respectively, P<0.01), than transfection with the non-optimized FVIII<sup>HSQ</sup>-encoding cDNA. Our data confirm previous findings obtained upon gene therapy in preclinical models of hemophilia A,<sup>24, 25</sup> on the capacity of codon optimization to increase the yields of FVIII production.

### **Transfection with FVIII-encoding mRNA leads to FVIII production *in vitro***

We then validated the capacity of mRNA transcribed *in vitro* using the FVIII<sup>HSQ</sup> and CoFVIII<sup>HSQ</sup>-encoding cDNA as templates, to promote FVIII production. As the codon-optimization is for Homo Sapiens, mRNA encoding FVIII<sup>HSQ</sup> or CoFVIII<sup>HSQ</sup> formulated in TransIT® were used to transfect human HEK293 cells (Figure 1B). As a negative control, HEK293 cells were transfected with luciferase-encoding mRNA. Transfection with the two FVIII-encoding mRNA lead to the *in vitro* production of FVIII to similar amounts (FVIII:Ag - 0.28±0.03 IU/mL vs 0.36±0.04 IU/mL for FVIII<sup>HSQ</sup> and CoFVIII<sup>HSQ</sup>, respectively) and similar activities (FVIII:C - 0.20±0.01 IU/mL vs 0.24±0.02 IU/mL, respectively). Accordingly, the specific activity of FVIII produced using both mRNA did not differ (0.72±0.06 and 0.68±0.08,



respectively), suggesting that codon optimization has no beneficial effect on the production of FVIII when cells are transfected with mRNA, in our experimental system.

### **Systemic delivery of FVIII-encoding mRNA to FVIII-deficient mice leads to the endogenous production of FVIII**

FVIII-deficient mice were injected intravenously with 1  $\mu\text{g}$  FVIII<sup>HSQ</sup> or CoFVIII<sup>HSQ</sup>-encoding mRNA formulated in TransIT® (Figure 2A). Plasma levels of FVIII:Ag and FVIII:C, measured after 24 hours, were  $1.00\pm 0.15$  IU/mL and  $0.34\pm 0.07$  IU/mL in the case of FVIII<sup>HSQ</sup>-encoding mRNA, and  $1.30\pm 0.19$  IU/mL and  $0.36\pm 0.06$  IU/mL in the case of CoFVIII<sup>HSQ</sup>-encoding mRNA. Likewise, the specific activities of FVIII<sup>HSQ</sup> and CoFVIII<sup>HSQ</sup> (defined as the ratios of FVIII:C over FVIII:Ag) did not differ significantly:  $0.31\pm 0.06$  and  $0.25\pm 0.02$ , respectively, but were more than 2-fold lower than specific activities measured *in vitro* (Figure 1B). To investigate for the presence of non-functional FVIII molecules, we measured plasma levels of FVIII light chain using a dedicated ELISA. Levels of FVIII:Ag and light chain were perfectly correlated (Figure 2B: slope= $1.15\pm 0.10$ ,  $P < 0.0001$ ). However, 14 of the 20 tested samples showed >10% more FVIII light chain than total FVIII, indicating that some FVIII in the plasma samples was missing its A2 domain (A2 domain dissociation is a feature of FVIII inactivation<sup>26</sup>).

The endogenous production of FVIII was then followed over 72 hours after the injection of 1, 3 or 5  $\mu\text{g}$  of CoFVIII<sup>HSQ</sup>-encoding mRNA. At 24 hours (Figure 3A, left panel), the levels of FVIII:C reached in the circulation ranged from  $0.27\pm 0.11$  to  $0.42\pm 0.14$  IU/mL. The effect of the dose of injected mRNA on the level of endogenously produced FVIII was statistically significant at 72 hours (Figure 3A, right panel). Interestingly, the mean residual FVIII activity 72 hours after the injection of 3 and 5  $\mu\text{g}$  of mRNA, was above 5% of the theoretical FVIII:C in normal plasma ( $0.06\pm 0.02$  IU/mL and  $0.11\pm 0.03$  IU/mL, respectively). We then estimated

the total amount of FVIII:C produced over 72 hours following injection of FVIII-encoding mRNA. To this end, mice were injected intravenously either with 3 IU of human rFVIII or with 3  $\mu$ g of CoFVIII<sup>HSQ</sup>-encoding mRNA formulated in TransIT® (Figure 3B). The areas under the curves depicting the changes in FVIII:C plasma levels over time were 2.9 and 17.8 IU/mL x hours, respectively, showing that the injection of 3  $\mu$ g of mRNA allows the endogenous production over a period of 72 hours of amounts of FVIII:C 6-fold greater than the amount of rFVIII injected at once. The half-life of rFVIII in the circulation was fitted using a two-phase decay equation: short and long half-lives of 0.9 and 4.7 hours were calculated. An apparent half-life for the production of FVIII of 17.9 hours was calculated by fitting the FVIII:C levels measured at 24, 48 and 72 hours with a one-phase decay equation.

### **Correction of the bleeding phenotype of FVIII-deficient mice by the endogenously produced FVIII**

We then investigate the effect of a single injection of FVIII-encoding mRNA on the bleeding phenotype of FVIII-deficient mice. FVIII-deficient mice were injected with PBS, with FVIII-encoding mRNA or with 1.2 or 3 IU rFVIII (Figure 4). The tip of the mouse tails was clipped after 30 minutes in the case of rFVIII or after 24 hours in the case of PBS or mRNA, which correspond to the activity peak of each individual treatment. Blood loss was followed over 20 min. While the PBS-injected mice lost  $274.3 \pm 53.4$   $\mu$ L of blood, injection of rFVIII protected the mice from major bleeding with blood loss of  $86.0 \pm 44.9$   $\mu$ L and  $12.2 \pm 0.9$   $\mu$ L for 1.2 IU ( $P < 0.05$ ) and 3 IU ( $P < 0.001$ ) rFVIII, respectively. Interestingly, mice injected with FVIII-encoding mRNA lost  $45.8 \pm 24.6$   $\mu$ L of blood ( $P < 0.001$  as compared to PBS-treated mice), showing correction of the bleeding phenotype.

### **Multiple injections of FVIII-encoding mRNA trigger an anti-FVIII immune response**

The repeated administration of rFVIII is known to trigger the production of inhibitory anti-FVIII IgG in mice after 3-5 injections.<sup>27</sup> We thus assessed the effect of multiple injections of FVIII-encoding mRNA on the onset of an anti-FVIII immune response. We treated FVIII-deficient mice with 1  $\mu$ g FVIII-encoding mRNA once a week for 7 weeks. As a control, FVIII-deficient mice were injected once a week with 5 IU rFVIII. First, we measured residual plasma FVIII levels 24 hours after rFVIII or mRNA injection (Figures 5A and B). Consistent with the short half-life of FVIII in mice, very low FVIII:Ag and FVIII:C were measured in plasma 24 hours following injection of rFVIII during the first 3 weeks of treatment. In the case of mRNA-treated mice, a sharp decrease in both FVIII:Ag and FVIII:C was observed between the first ( $1.13 \pm 0.23$  IU/mL and  $0.37 \pm 0.11$  IU/mL, respectively) and second ( $0.26 \pm 0.06$  IU/mL and  $0.05 \pm 0.05$  IU/mL, respectively) injections. From the third week of treatment onwards, FVIII:C levels were below detection levels.

We then investigated the presence of inhibitory anti-FVIII IgG 5 days after each administration of mRNA or rFVIII to the mice (Figures 5C and 5D). As described previously, the intravenous administration of rFVIII induced a progressive increase in levels of anti-FVIII IgG that reached a plateau after 5 injections ( $10168 \pm 4501$  AU after 7 injections, Figure 5C). This was mirrored by a gradual increase in levels of FVIII inhibitors, that crossed 10 BU/mL 5 days after the fourth injection to plateau at  $111 \pm 79$  BU/mL (Figure 5D). In the case of FVIII-encoding mRNA, levels of anti-FVIII IgG as high as those obtained after 5 injections of rFVIII were detected as early as 5 days following the second treatment. The plateau after 7 weeks of treatment ( $98060 \pm 8251$  AU) was 10-fold higher in the case of mRNA than in that of rFVIII. Likewise, while no FVIII inhibitory activity was detected 5 days after the first injection of mRNA, levels of  $383 \pm 196$  BU/ml were generated 5 days after the second injection, which gradually increased to reach a plateau at  $2258 \pm 669$  BU/mL after 7 weeks of treatment. The plateau of FVIII inhibitors was thus 20-fold greater in mice treated with mRNA than in mice treated with rFVIII.

Of note, the incubation FVIII-encoding mRNA, TransIT or TransIT-formulated mRNA with immature human MODCs failed to induce the production of TNF-alpha and IL-6 (Figures 5E and 5F), indicating that mRNA and TransIT® do not activate innate immune cells and are not responsible for the triggering of the anti-FVIII immune response. In additional experiments, we investigated the site of production of mRNA-encoded proteins following formulation in TransIT®. To this end, Balb/c mice were injected with luciferase-encoding mRNA formulated in TransIT. The luminescence measured 24 hours later was at least 2 folds greater in the spleen as compared to the liver (supplemental Figure S1), suggesting that the liver and hepatocytes are not the main target for TransIT-formulated mRNA. Attempts to detect FVIII in FVIII-encoding mRNA-treated FVIII-deficient mice lead to occasional FVIII signal in the marginal zone of the spleen and constant absence of FVIII detection in the liver (data not shown).

## Discussion

The present work documents the sustained endogenous production of pro-coagulant FVIII following the injection of FVIII-encoding mRNA into FVIII-deficient mice, and is of potential relevance for the improvement of treatments for hemophilia A patients. FVIII levels achieved 24 hours after the injection of mRNA were about 40% of the levels in normal plasma. The expression levels of FVIII are notoriously low owing to poor transcriptional and translation efficacies<sup>27-29</sup> as well as retention of the protein in the ER.<sup>30, 31</sup> Attempts to improve the levels of expression of FVIII include the partial removal of the B domain with conservation of essential N-glycosylation sites,<sup>24, 31</sup> mutation of a BiP binding site<sup>32</sup> to ensure improved transfer from ER to Golgi,<sup>33</sup> and codon optimization. Here, mRNA was generated using wild-type<sup>23</sup> or codon-optimized cDNA encoding human BDD-FVIII. While codon optimization improved FVIII production following transfection of eukaryotic cells with plasmid DNA *in vitro*, it did not increase the levels of FVIII:C or FVIII:Ag following *in vitro* mRNA transfection of cells or *in vivo* transfection of FVIII-deficient mice. Codon optimization aims at improving translation rates by using codons for which the cognate tRNA levels are not limiting. Thus, codon optimization of FVIII-encoding cDNA cloned in lentiviral vectors or adenoviral-associated vectors lead to more than 10-fold increased FVIII levels after *in vitro* transfection of cell lines and after injection to wild-type or FVIII-deficient mice.<sup>24, 25</sup> The lack of improvement in protein production associated with the administration of codon-optimized mRNA encoding FVIII suggests that codon optimization of FVIII preferentially targets transcriptional rather than translational events, as previously shown.<sup>34, 35</sup> Alternatively, levels of mRNA introduced in each cell upon *in vivo* transfection with TransIT® may be much lower than that transcribed endogenously following transfection using DNA, and insufficient to exhaust non-abundant tRNA. Increasing the amount of mRNA injected *in vivo* had however no drastic effect on the FVIII levels reached in the circulation.

Frequent spontaneous joint and muscle bleeds in patients with severe hemophilia A eventually lead to the development of arthropathy and functional joint impairment. An association between joint bleeds and baseline FVIII activity levels was demonstrated,<sup>36</sup> with FVIII levels above 5% of the normal values drastically reducing the occurrence of joint bleeds. Because the half-life of human FVIII in patients with hemophilia A is comprised between 12 and 18 hours, prophylactic replacement therapy is the gold standard to maintain healthy joint functions.<sup>37</sup> In FVIII-deficient mice, the half-life of human FVIII is comprised between 4 and 6 hours. Interestingly, delivery of FVIII-encoding mRNA yielded FVIII levels that corrected acute bleeding in a tail clipping assay, and were maintained above 5% for up to 72 hours. The longer residence time of FVIII produced after mRNA delivery as compared to that of recombinant FVIII results from the cumulative lifespans of the transfected mRNA in the cells and of the FVIII released in the circulation. The mRNA-based therapy can be improved by engineering the encoded protein to extend its half-life.<sup>38</sup> For instance, a FVIII molecule with an increased half-life has been developed upon fusion of FVIII to the Fc fragment of human IgG1.<sup>2</sup> We anticipate that the use of mRNA encoding long-lasting FVIII glycoproteins will allow a further increase in the duration of FVIII detection *in vivo*. Because peak expression levels of FVIII were reached in the mice between 6 and 24 hours after the administration of mRNA, mRNA-based therapy may represent a surrogate for prophylactic treatment rather than for the on-demand treatment of sudden acute hemorrhagic events.

The specific activity of the recombinant FVIII produced by eukaryotic cells *in vitro* was about 1.2 following transfection using cDNA and about 0.7 following transfection with mRNA. In contrast, the specific activity of the FVIII produced endogenously following injection of mRNA to the mice was about 0.3, suggesting the absence of pro-coagulant activity for a substantial proportion of the endogenously produced FVIII molecules. In parallel, we compared the levels of intact FVIII measured using a standard ELISA, wherein FVIII is captured with an anti-light

chain antibody and detected with anti-heavy chain antibody, with that of FVIII light chain measured using a sandwich light chain-specific ELISA. A perfect correlation was obtained although with an overall tendency for 2-fold more light chains than intact molecules. Importantly, the levels of light chain may be under-estimated owing to the short *in vivo* half-life of the light chain alone. Future experiments will indicate whether the use of mRNA encoding FVIII lacking the furin cleavage site<sup>39,40</sup> between the heavy and light chains, or FVIII mutants with increased FVIII A2 subunit stability<sup>41</sup> yields FVIII with improved specific activity. An alternative and non-exclusive explanation for the poor specific activity of the endogenously produced FVIII is the fact that the TransIT® used to formulate the mRNA does not target particular cell types. In fact, we show here that luciferase is produced by the spleen, rather than by the liver, following injection of mice with luciferase-encoding mRNA formulated with TransIT®. Accordingly, immunofluorescence experiments on mice treated with FVIII-encoding mRNA occasionally detected faint amounts of FVIII in the marginal zone of the spleen but never in the liver (not shown). Hence, TransIT® delivers FVIII mRNA to cells that are not dedicated to the production of FVIII,<sup>42, 43</sup> thus potentially generating FVIII molecules that are improperly folded or have undergone incorrect post-translational modifications.

In patients with severe hemophilia A, inhibitory anti-FVIII IgG (FVIII inhibitors) generally develop within the first 20 cumulated days of exposure to therapeutic FVIII.<sup>44</sup> In the present work, and in agreement with previous studies in FVIII-deficient mice,<sup>45</sup> FVIII-binding IgG and FVIII inhibitors were detected after 2 and 3 intravenous injections of recombinant FVIII, respectively. The levels of anti-FVIII IgG increased to reach a plateau at 10 to 100-fold higher levels after 2 to 3 additional injections of recombinant FVIII. In the case of mRNA administration however, close to maximal levels of FVIII-binding IgG and inhibitory antibodies were reached after only two injections of FVIII-encoding mRNA. Accordingly, endogenous FVIII:C was undetectable as early as after the second administration of mRNA. Different

explanations for such a brisk and intense immune response to the endogenously produced FVIII may be envisaged. Because the mRNA used in our study was engineered to contain 1-methylpseudouridines and was HPLC-purified to remove dsRNA, thus abrogating mRNA recognition by TLR3, TLR7, TLR8 and RIG-I,<sup>9-12</sup> it is not probable that the very mRNA plays an adjuvant role in the onset the anti-FVIII immune response. Indeed, FVIII-encoding mRNA, alone or formulated in TransIT®, failed to activate human immature MODCs *in vitro*. Interestingly, previous work on *in vivo* mRNA transfection without apparent induction of neutralizing immune responses have used TransIT®<sup>15</sup> or other types of nanoparticles.<sup>16, 18</sup> Most studies were however performed in immuno-compromised animals<sup>46</sup> or in animal expressing the corresponding endogenous protein (e.g., the absence of induction of an anti-EPO immune response in animals receiving EPO-encoding mRNA<sup>15, 17</sup> probably relates to the presence of the endogenous EPO molecule and associated ongoing active immune tolerance).

In fact, the very formulation of mRNA in TransIT® may be a reason for induction of a strong anti-FVIII immune response. TransIT® was initially conceived for *in vitro* and not *in vivo* gene transfection. As explained above, protein production following injection of TransIT®-formulated mRNA is not targeted to the liver and not to endothelial cells, that produce FVIII under physiological conditions. This is reminiscent of the early works on gene therapy for hemophilia wherein the use of promoters with poor specificity for hepatocytes was associated with the induction of neutralizing anti-FVIII or anti-factor IX antibodies<sup>47</sup>.

Several lines of evidence suggest that the produced FVIII itself may be responsible for the sharp anti-FVIII immune response. As explained above, FVIII is a particularly immunogenic glycoprotein: e.g., a fusion protein between the light chain of FVIII and the first domain of hemagglutinin 1 (HA1) demonstrated greater anti-HA1 immunogenicity upon intravenous injection to FVIII-deficient mice than the HA1 molecule alone.<sup>48</sup> In contrast, the production of factor IX was induced in mice following administration of mRNA without report of a



neutralizing immune response.<sup>18</sup> Of note, a relationship between the dose of FVIII injected and the kinetics of detection of the anti-FVIII IgG response was reported in mice<sup>45</sup> and in patients.<sup>49</sup> This is particularly relevant in view of the fact that the amount of active FVIII produced over 72 hours after one injection of FVIII-encoding mRNA was equivalent to 6 folds the amount of recombinant FVIII administered in a single injection. Furthermore, based on the poor specific activity of the endogenously produced FVIII, the total amount of FVIII molecules (active and inactive) probably exceeds 18 folds that of injected recombinant FVIII. Further work will indicate whether targeting mRNA delivery to hepatocytes or to endothelial cells using improved lipid nanoparticle-based formulating agents,<sup>50</sup> using mRNA encoding single chain or A2 variant stable FVIII<sup>39, 40, 41</sup> and including miRNA target sequences to prevent off-target expression in hematopoietic cells<sup>51</sup> are plausible strategies to improve the specific activity and reduce the immunogenicity of the endogenously produced molecule.

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### **Authors contributions:**

JR, SVK and SLD designed the research

JR, SD, PM, OC and GB performed experiments;

PJL, CVD contributed essential material;

JR and SLD analyzed results and made the figures;

JR, OC, SVK and SLD wrote the paper

Conflict-of-interest disclosure: The authors declare no competing financial interests.

GB is an employee of BioNTech RNA Pharmaceuticals, a company that develops mRNA-based therapies.

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## Legends to figures

**Figure 1. *In vitro* production of wild-type and codon-optimized FVIII.** DNA in lipofectamin (0.1  $\mu$ g, panels A) or mRNA formulated in TransIT® (0.4  $\mu$ g, panels B) encoding BDD-FVIII (FVIII<sup>HSQ</sup>) and codon-optimized BDD-FVIII (CoFVIII<sup>HSQ</sup>) were used to transfect BHK (panels A) or HEK293 (panels B) cells. mRNA encoding luciferase was used as a control (panels B). FVIII:Ag (left panels) and FVIII:C (middle panels) were measured in cell supernatant 24 hours after transfection. The right panels depict the specific activities as calculated by dividing the FVIII:C values by the FVIII:Ag values. Statistical differences were assessed using a two-tailed t test (ns: non-significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ). Results are depicted as means  $\pm$  SEM for 3 independent experiments.

**Figure 2. Delivery of FVIII-encoding mRNA allows endogenous production of FVIII.** mRNA encoding BDD-FVIII (FVIII<sup>HSQ</sup>) or codon-optimized BDD-FVIII (CoFVIII<sup>HSQ</sup>) was formulated in TransIT®. FVIII-deficient mice were then injected intravenously with 1  $\mu$ g FVIII-encoding mRNA. Panel A. FVIII:Ag and FVIII:C were measured in plasma after 24 hours. Individual symbols on the graphs represent individual mice; horizontal bars represent means  $\pm$  SEM. Statistical differences were assessed using a two-tailed t test (ns: non-significant). Panel B. FVIII:Ag and FVIII light chain were measured in plasma by ELISA. Individual symbols on the graphs represent individual mice. The full line curve represents the linear regression of the experimental data. The dotted line represents the theoretic correlation with a slope of 1.

**Figure 3. Time-dependent production of endogenous FVIII after a single injection of FVIII-encoding mRNA.** mRNA encoding codon-optimized BDD-FVIII (CoFVIII<sup>HSQ</sup>) was formulated in TransIT®. Panel A. FVIII-deficient mice were then injected intravenously with

1, 3 or 5 µg FVIII-encoding mRNA. FVIII:C was measured in plasma after 24 and 72 hours. Individual symbols on the graphs represent individual mice; horizontal bars represent means±SEM. The dotted line indicates the critical FVIII level (i.e., 5%) required to drastically reduce joint bleeds. Statistical differences were assessed using a two-tailed t test (ns: non-significant, \*: p<0.05). Panel B. Mice were injected with 3 µg CoFVIII<sup>HSQ</sup>-encoding mRNA formulated in TransIT®, or with 100 µl of 10 nM recombinant BDD-FVIII (3 IU rFVIII). FVIII:C in plasma was measured after 30, 60, 90, 120, 180, 240, 360, 390 and 480 min in the case of rFVIII, and 6, 24, 48 and 72 hours (excluding the 0 and 6-hour time-points) in the case of mRNA. The dotted line depicts the non-linear fit (two-phase exponential decay:  $y=0.62*e^{-0.15x}+0.2211*e^{-0.80x}$ ) of the experimental data obtained with rFVIII. The full circles and full line curve represent means±SEM of 6 mice treated with mRNA (representative of 2 independent experiments; fitted with a one-phase exponential decay for the values obtained at 24, 48 and 72 hours:  $y=0.99*e^{-0.03874x}$ ). Areas under the curves were calculated using Prism GraphPad (Version 6).

**Figure 4. Injection of FVIII-encoding mRNA corrects acute bleeding in FVIII-deficient mice.** FVIII-deficient mice were injected intravenously with 3 µg of FVIII-encoding mRNA (empty squares) formulated in TransIT®, with 1.2 IU (empty circles) or 3 IU (full diamonds) recombinant BDD-FVIII (rFVIII) or with PBS (full triangles). Mice tails were clipped 3 mm from the tip, 24 hours after mRNA and PBS injection, or 30 min after rFVIII injection. Bleeding intensity was measured as the volume of blood lost during 20 min. Horizontal bar depict means and individual dots representing individual mice. Statistical differences were assessed using the double-sided Mann-Whitney test (ns: non-significant; \*: p<0.05; \*\*\*: p<0.001).

**Figure 5. Induction of an anti-FVIII immune response after repeated injections of FVIII-encoding mRNA.** FVIII-deficient mice were injected intravenously with 1  $\mu$ g of coFVIII<sup>HSQ</sup>-encoding mRNA formulated in TransIT® (full circles), or with 5 IU recombinant BDD-FVIII (empty squares), once a week for 7 weeks. FVIII:Ag (panel A) and FVIII:C (panel B) were measured in plasma 24 hours after each injection. Anti-FVIII IgG (panel C) and inhibitory titers (panel D) were measured in plasma 72 hours after each injection. Results are depicted as mean $\pm$ SEM for 4-7 mice per group. The vertical dotted lines represent the days of FVIII/mRNA administration. The horizontal dotted lines represent the background levels (calculated as “mean + 1 x SD”) measured in 6-7 FVIII-deficient mice (panel A, 0.045 $\pm$ 0.013 IU/mL; panel B, 0.001 $\pm$ 0.000 IU/mL; panel C, 0.84 $\pm$ 0.23  $\mu$ g/ml); or the threshold for detection (panel D, 5 BU/mL). Panels E and F. Five-day old immature MODCs were incubated alone (Untreated) or in the presence of Poly:IC, LPS, TransIT® alone, 1  $\mu$ g FVIII-encoding mRNA (mRNA) alone or 1  $\mu$ g FVIII-encoding mRNA formulated in TransIT®. The panels show the levels of TNF-alpha (panel E) and IL-6 (panel F) measured in the culture supernatant 8 (full bars) and 24 (empty bars) hours later. Data depict means $\pm$ SEM from 2 independent experiments. Differences between TransIT, mRNA, TransIT+mRNA and Untreated were not statistically significant (two-sided Mann-Whitney test).