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Does haemophilia slow down the development of liver fibrosis?

Dear Editor,

Haemophilia is an X-linked recessive bleeding disorder, resulting from deficient or dysfunctional coagulation factor VIII (haemophilia A) or IX (haemophilia B). Depending on the severity of their factor deficiency, patients present with a range of bleeding diathesis from traumatic injury or surgically induced bleeding to spontaneous haemorrhage in the soft tissues, joints or muscles.

Before the 1960s, the prognosis of severe haemophilia A patients was just 11 years or less,[1] then the introduction of fresh-frozen plasma, cryoprecipitate, and finally blood-pooled clotting factor concentrates considerably improved the survival and quality of life of patients with haemophilia or with other bleeding disorders.[2] However, the epidemic of HCV and human immunodeficiency virus (HIV) then erupted with severe consequences. Until the 1989 discovery of HCV,[3] almost all haemophilia patients treated with clotting factor products were infected with HCV.[4] The clinical presentation of HCV is characterized by chronic liver disease that can progress to fibrosis with development of (de)compensated cirrhosis and hepatocellular carcinoma.

An unexpected new finding was reported by Assy et al[5] who found that liver disease following HCV infection appeared more benign in haemophilia subjects than in others. This was unexpected considering that HCV infection was one of the major causes of mortality in patients with haemophilia before the introduction of direct acting antivirals. The authors investigated the histological severity of liver fibrosis in haemophilia HCV-infected patients compared with HCV-infected patients with no bleeding disorder, demonstrating significantly lower histologic inflammatory activity and fibrosis scores in patients with haemophilia. However, the small scale of this study and high heterogeneity of included patients render it difficult to establish a clear link between haemophilia and reduced liver damage following HCV infection.

Haemophilia A murine models that mimic patients with severe haemophilia A are currently available. These well-characterized models have been extensively employed to evaluate new treatments.[6] In this report, we have studied the progression of chemically induced liver fibrosis by means of an haemophilia A mouse model.

The study was carried out using 12 control mice (=CTL mice) with normal factor VIII levels (8 weeks old, C57BL/6, sourced from The Jackson Laboratory, Bar Harbor, Maine, USA) and 12 haemophilia mice (=HEMO mice) with factor VIII levels <1% (7 weeks old; B6; 129S-F8^{tm1Kaz}/J, sourced from The Jackson Laboratory). Each group was divided into subgroups receiving either thioacetamide (TAA) in their drinking water (200 mg TAA/L; CTL-TAA, and HEMO-TAA) or only water without TAA (CTL-H2O and HEMO-H2O). TAA (purchased as white crystalline powder from Sigma-Aldrich; St Louis, MO, USA) is an organosulfur compound that induces liver fibrosis while being water soluble. The mice were housed in a temperature-controlled environment (12-hour light cycle) with food and water ad libitum. In the HEMO groups, mice were housed individually to avoid injuries. The amount of water the mice consumed and their weight were monitored weekly to determine the exact dose of TAA administered. After 25 weeks, the mice were sacrificed and their livers harvested. Three haemophilia mice died unexpectedly before Week 25 due to external haemorrhages (two in the HEMO-H2O group; one in the HEMO-TAA group) and were excluded from analysis.

At the time of harvest, liver sections were fixed in formalin and embedded in paraffin. Slices were stained with PicroSirius Red then

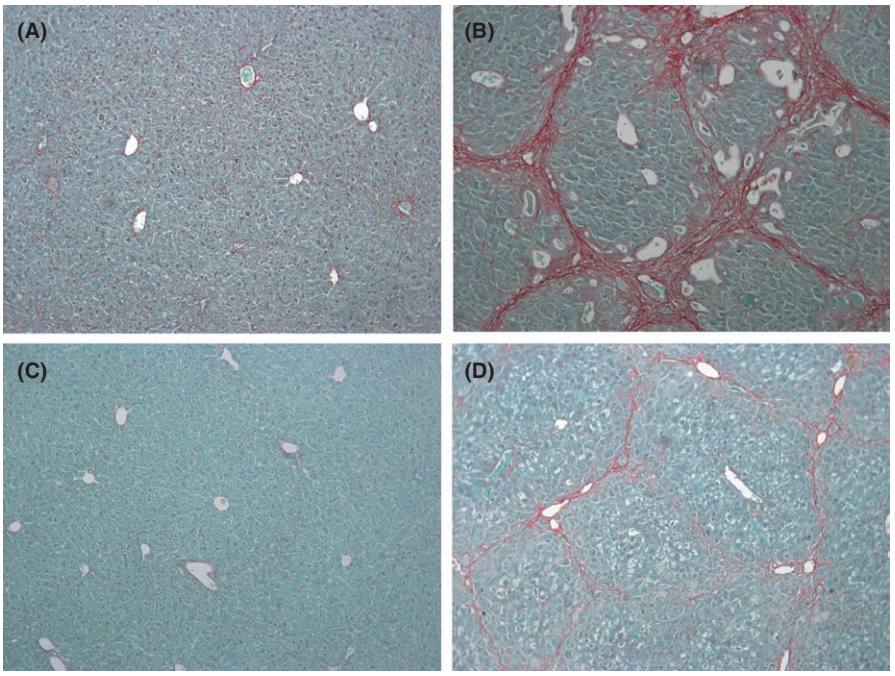

FIGURE 1 Collagen staining of liver sections. Collagen was stained with PicroSirius Red in control mice receiving water (CTL-H2O) (A), control mice receiving thioacetamide (CTL-TAA) (B), haemophilia mice receiving water (HEMO-H2O) (C) and haemophilia mice receiving thioacetamide (HEMO-TAA) (D)

scanned using a Leica scanner for collagen deposition quantification. Morphometric analysis was performed with Leica Tissue IA2.0 software by analysing a minimum of five fields per slide, as described by Van Hul et al.[7] Percentage of liver fibrosis was expressed as the ratio of PicroSirius-Red-stained area to the total area (leaving out the white areas).

Total RNA was isolated from frozen liver tissue using TriPure isolation reagent (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Hepatic mRNA expression levels of murine Type I collagen-alpha-1 were quantified by real-time polymerase chain reaction (PCR). Primer pairs for transcripts of interest were designed using the express design primer software v2.0 (Applied Biosystems, Foster City, California, USA). The ABI Prism 5700 PCR platform and SYBR green master mix (Applied Biosystems) were used to detect the amplification product. The relative mRNA amount was calculated by reference to a calibration curve and normalized to the expression level of RPL19 mRNA, an invariant control.

Statistical analysis (Mann-Whitney *U* test) was carried out using Analyse-It Software, Ltd (Leeds, UK).

The mean TAA intake during the whole period was 37.9 mg/kg/d (95% confidence interval [CI]: ±4.4 mg/kg/d) and 24 mg/kg/d (95% CI: ±1.1 mg/kg/d) for HEMO-TAA and CTL-TAA mice, respectively. Haemophilia mice (HEMO-TAA) were exposed to a significantly higher ($P < 0.001$) dose per body weight than the treated control mice (CTL-TAA).

Significant bridging fibrosis developed in all TAA-treated mice. In CTL-TAA mice, thick bundles of collagen intersected the liver, while much thinner and incomplete bands of fibrotic scarring were observed in HEMO-TAA livers (Figure 1). Morphometrical analysis confirmed significant fibrosis in the CTL-TAA mice, with an average of 18.7% (95% CI: ±3.2%) compared to 0.1% (95% CI: ±0.03%) in the control group (CTL-H2O; $P < 0.01$). HEMO-TAA mice also developed significant fibrosis (2.9%; 95% CI: ±0.5%) compared to the controls (HEMO-H2O; 0.1%; 95 CI: ±0.02%; $P < 0.05$). Haemophilia mice treated with TAA (HEMO-TAA) developed a significantly lower level of fibrosis than the CTL-TAA group ($P < 0.01$, Figure 2). An increase in Type I collagen mRNA concentration was described as an early event in the development of liver fibrosis, caused by activation of hepatic stellate cells.[8] The amount of Type I collagen mRNA significantly differed ($P < 0.05$) between the HEMO-TAA and CTL-TAA groups. To the best of our knowledge, this is the first work that shows, while using an animal model, that a genetic coagulation defect, namely

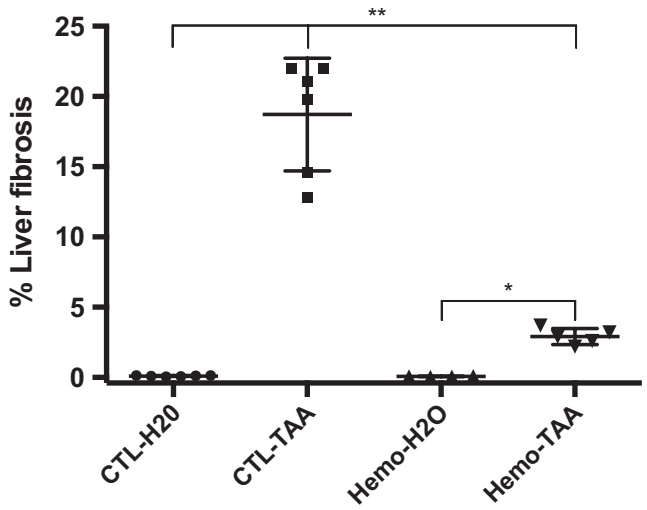

FIGURE 2 Liver fibrosis. The results were expressed as percentage of liver fibrosis per group (**P-value < 0.01; *P-value < 0.05) in control mice receiving water (CTL-H2O), control mice receiving thioacetamide (CTL-TAA), haemophilia mice receiving water (HEMO-H2O) and haemophilia mice receiving thioacetamide (HEMO-TAA)

haemophilia A, may protect the liver against chemically induced fibrosis progression.

In 2008, the impact of the coagulation process on the development and progression of liver fibrosis was studied by Anstee et al[9] in mice with prothrombotic phenotype. Using carbon tetrachloride, more significant liver fibrosis was induced in mice with factor V Leiden mutation compared to normal mice. In addition, warfarin, a potent anticoagulant drug, decelerated the hepatic fibrogenesis observed in the hypercoagulable mice. Similarly, Li et al[10] observed that administering low molecular weight heparin, namely enoxaparin, preserved liver structure and function in a rat model of liver fibrosis.

Thrombin is the final enzyme of the coagulation cascade, enabling the transformation of fibrinogen into fibrin, which stabilizes platelet clotting during the primary haemostasis. Due to haemophiliacs' factor VIII deficiency, the amount of thrombin they generate is 10 times lower compared to a normal subject.[11] Besides its significant role in the coagulation process, thrombin is additionally able to activate cells like hepatic stellate cells (HSCs) and platelets via the protease-activated receptor (PAR) pathway. In normal livers, HSCs are quiescent and maintained in a non-proliferative phenotype. Following liver damage, these cells become activated and switch into a myofibroblast phenotype, causing enhanced production of extracellular matrix (ECM) and collagen deposition in the liver, eventually resulting in liver fibrosis. In 1998, Marra et al[12] demonstrated that PAR expression is up-regulated during acute and chronic liver damage. In 2004, Fiorucci et al[13] reported that PARs are expressed on HSCs, and that activating HSC PAR triggers their proliferation, as well as collagen 1 production and release. More recently, Vilaseca et al[14] observed that injecting rivaroxaban, an inhibitor of activated factor X that reduces thrombin production, was able to deactivate HSCs in a rat model of liver fibrosis.

All these studies support the hypothesis that the protective effect haemophilia offers against liver fibrosis progression may be accounted for by reduced thrombin generation. In addition to its effect on HSCs, thrombin likewise influences liver fibrosis through platelet activation. Activated platelets are not only essential to primary haemostasis, but also provide efficient catalytic surfaces enabling the enzyme complexes of the blood coagulation system to assemble properly. Besides this role, the activation of platelets by thrombin through their PARs also results in the release of up to 300 bioactive proteins from platelet alpha-granules. Among these molecules, the role of PDGF-β and CXCL-4 in the activation of HSCT and the development of liver fibrosis has already been well described.[15] In line with these observations, Li et al[10] demonstrated in a rat model that aspirin, an antiaggregant drug, can alleviate liver fibrosis. Consequently, it is plausible that thrombin platelet activation also influences the progression of liver fibrosis.

We report the following limitations of our study. The different genetic background of the normal mice (C57BL/6) compared to the haemophilia mice (B6; 129S-F8^{tm1Kaz}/J) may have impacted our results. Furthermore, we only looked at Type I collagen production. It would be very interesting to investigate other markers of HSC activation and fibrosis development.

In conclusion, our study suggests that haemophilia A has a protective effect against liver fibrosis development. We believe that this protective role can be attributed to the central role played by thrombin in the activation of HSCs, either directly via PAR activation or indirectly via platelet activation. However, further studies are needed to strengthen this hypothesis.

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DISCLOSURES

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

AUTHOR CONTRIBUTIONS

S. Eeckhoudt, I. Leclercq and Y. Homans were involved in designing and carrying out the study. S. Eeckhoudt, M. Jacquemin, C. Hermans, C. Lambert, and MA van Dievoet contributed to interpreting the study results and preparing the manuscript. The statistical analyses were performed by MA van Dievoet, and all authors had access to the data. All authors reviewed the manuscript and approved the final submitted version.

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The prevalence and burden of hand and wrist bleeds in von Willebrand disease

1 | INTRODUCTION

Von Willebrand disease (VWD), as the most prevalent hereditary bleeding disorder, affects between 0.6% and 1.3% of the population.[1] Typical symptoms of VWD include haematomas, epistaxis, menorrhagia and bleeding from minor wounds. VWD is caused by deficiency of von Willebrand factor (vWF) or by dysfunctional vWF. More severe bleeding occurs with lower vWF and FVIII activity levels depending on the underlying genetic variant and subtype.[1,2]

Large joint bleeds, reported by 23% of moderate and severe VWD patients in the Willebrand in the Netherlands (WiN) study, can cause joint damage (arthropathy) and pain, associated with functional limitations.[2,3] Haemarthrosis in hand and wrist joints has been described in haemophilic patients, some complicated by contractures or neuropathy.[4] It is unknown if joint bleeds also occur in the small joints of hands and wrists in VWD and whether these bleeds result in joint destruction, pain and functional impairment.

The aim of this study was to investigate the prevalence and treatment of hand and wrist bleeds in the three different types of VWD and to explore clinical consequences of those bleeds.

2 | METHODS

The study population consisted of 804 VWD patients (historically lowest VWF activity ≤30 IU/dL) included in the Willebrand in the Netherlands (WiN), a cross-sectional multicentre study, between October 2007 and October 2009. All participants completed an extensive questionnaire.[5] Within this questionnaire, we analysed the occurrence, locations and frequency of hand and wrist bleeds. We compared the proportion of patients reporting hand or wrist bleeds across the different subtypes of VWD and compared VWF and FVIII levels between the WiN participants who did and did not report hand or wrist bleeds.

To analyse the consequences of joint bleeding, we additionally performed a nested case-control study that included 96 participants of the WiN study with a mean age of 46 years (range 18-80) between August 2013 and July 2015. In this study, 48 patients with a medical history of clotting factor treatment for large joint bleeds were compared to a matched control group of 48 patients who did not receive treatment for joint bleeds.[6] During the study visit, all these participants were questioned on the occurrence, frequency