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On-demand treatment with the iron chelator deferasirox is ineffective in preventing blood-induced joint damage in haemophilic mice

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Abstract

Introduction: Early intervention in the devastating process of haemophilic arthropathy (HA) is highly desirable, but no disease-modifying therapy is currently available. Considering the pivotal role of iron in the development of HA, iron chelation is considered a promising therapeutic approach. A previous study in haemophilic mice demonstrated that treatment with the iron chelator deferasirox (DFX) 8 weeks before joint bleed induction, attenuated cartilage damage upon blood exposure. However, in haemophilia patients this approach is not opportune given the unpredictable occurrence of hemarthroses.

Aim: To evaluate the effectiveness of on-demand DFX treatment, initiated immediately after joint bleed induction.

Methods: A joint bleed was induced in 66 factor VIII-deficient mice by infra-patellar needle puncture. Mice were randomly assigned to treatment with either placebo (drinking water) or DFX (dissolved in drinking water) throughout the study. Five weeks after joint bleed induction, inflammation and cartilage damage were assessed histologically. Joints of ten bleed naive haemophilic mice served as controls.

Results: A joint bleed resulted in significant inflammation and cartilage damage in the blood-exposed joint compared with those of control animals, in both the placebo and DFX group (all $p = <.05$). No differences in tibiofemoral or patellar inflammation ($p = .305$ and $p = .787$, respectively) nor cartilage damage ($p = .265$ and $p = .802$, respectively) were found between the blood-exposed joints of both treatment groups.

Conclusion: On-demand treatment with DFX does not prevent joint damage following blood exposure in haemophilic mice. DFX seems unable to reach the joint in time to exert its effect before the irreversible harmful process is initiated.

KEYWORDS
arthropathies, cartilage, deferasirox, haemophilia, hemarthrosis, iron, synovium

1 | INTRODUCTION

Spontaneous joint bleeding is a characteristic manifestation of the inherited coagulation disorder haemophilia. Even a single bleed can lead to significant joint tissue damage, affecting the synovium, cartilage and bone.[1,2] Prophylactic clotting factor replacement reduces the risk of hemarthrosis, but cannot fully prevent it.[3] Moreover, patients in developing countries do not have access to this expensive treatment. Reduction in treatment efficacy by the development of neutralizing antibodies (inhibitors), suboptimal adherence and subclinical (and thus untreated) bleeding are concerns as well. As a consequence, a significant proportion of haemophilia patients encounters recurrent joint bleeding, ultimately leading to the disabling condition haemophilic arthropathy (HA).

Treatment options in established HA are limited and focus on relieving symptoms and maintaining mobility, but do not intervene in the pathophysiology of HA. Iron is essential in the process of blood-induced joint damage and is as such considered a promising target for therapy.[2]

Iron is involved in several mechanisms resulting in synovial inflammation[4] and cartilage degeneration.[5] Following a joint bleed, blood components including toxic iron (Fe^{2+}) derived from red blood cells are cleared by the synovium and invading macrophages.[6,7] In case of repeated or ongoing hemarthroses, iron accumulates as synovial hemosiderin deposits and induces synovial inflammation, proliferation and angiogenesis.[1,8,9] The triggered synovium affects cartilage by producing cartilage-destructive pro-inflammatory cytokines[9] and matrix-degrading proteinases.[8] In addition, iron contributes to direct cartilage damage induced by oxidative stress.[5,10] Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are produced by activated mononuclear cells and also chondrocytes.[11] In the presence of erythrocyte-derived iron, H_2O_2 reacts according to the Fenton reaction, resulting in the formation of highly toxic hydroxyl radicals, which in the vicinity of chondrocytes cause apoptosis and with that permanent cartilage damage.[10,12]

Based on the above, restricting the role of iron might prevent lasting joint damage upon blood exposure. In pathological conditions such as chronic systemic iron overload, iron chelators are used to reduce iron levels in plasma and several tissues.[13] Deferasirox (DFX) is such an iron chelator with oral availability and a long plasma half-life, approved for treatment of iron overload syndromes.[14]

Recently, a proof-of-concept study was conducted in haemophilic mice to study the effect of DFX on blood-induced joint damage.[15] Mice were treated prophylactically with DFX, systemically administered 8 weeks prior to induction of a joint bleed. Treatment was continued for an additional 5 weeks post-hemarthrosis. Prophylactic treatment with DFX attenuated blood-induced cartilage damage upon blood exposure, confirming the role of iron in the pathophysiology. However, translating this approach to clinical use is hampered by the unpredictability of the occurrence of joint bleeds. As a consequence, haemophilic patients without systemic iron overload should use DFX chronically, exposing them to undesirable side effects such as renal insufficiency.[14] Therefore, the present study evaluated the effectiveness of on-demand DFX treatment initiated immediately after joint bleed induction in haemophilic mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Factor VIII (FVIII)-deficient mice (B6;129S4-F8tm1Kaz/J) were bred and housed as previously described.[15] Sample size calculation using Cohen's effect size (effect size: 0.8, alpha: 0.05, power: 0.8, based on previous data)[15] resulted in a group size of 26 animals to demonstrate a relevant difference in cartilage damage between the treatment regimens (G-power version 3.1.9.2). Taking into account an expected 25% loss,[16] a total of 66 animals (30 males and 36 females) were included in the study. In addition, both knee joints of joint bleed naive haemophilic mice (10 animals; 20 joints) were included as external controls, since the use of an internal control for this model is under debate due to the observation of contralateral joint damage upon blood exposure.[17] All animals were between three and 4 months of age. This study was performed according to the European Convention on Animal Care and was approved by the institutional and national animal ethical committee (project number AVD115002016451).

2.2 | Joint bleed induction

Mice were anaesthetized with isofluran/O2 and hair over both knees was removed by an electric shaver. A single joint bleed was induced in the right knee joint (day 0) by insertion of a 30-gauge needle through the subpatellar ligament, as described previously.[18] The left knee of each animal served as an unaffected internal control. The 20 knee joints of the 10 control animals were left untouched. The extent of the induced bleed was quantified by the joint diameter (JD; mm) and visual bleeding score (VBS; 0-3)[19] at baseline (before induction of the joint bleed), and 2, 14 and 35 days after induction of the bleed. The diameter of each knee joint was based on the mean of three measurements using a micrometre calliper.[18] An increase in JD less than 0.5 mm in combination with a VBS lower or equal to 1 at day 2 was considered as an unsuccessful bleeding induction. These animals were removed from further analysis.

2.3 | Treatment regimen

Immediately after the joint bleed induction, treatment was randomly assigned per cage and initiated by changing the drinking water for either placebo (regular drinking water) or DFX (dissolved in drinking water). DFX was kindly and unrestricted provided by Novartis Pharma AG (Basel, Switzerland). The powder was dissolved in drinking water by stirring thoroughly overnight at a concentration of 0.2 mg/ml, within the range of attainable

concentrations reported in literature.[20] Adjusted for the average weight of a mouse [30 g] and daily water intake [15 ml/100 g], this corresponded to a calculated estimate intake of DFX of 30 mg/kg per day, which is considered an effective and safe dose in mice.[21] Treatment was continued during the 5 weeks of the experiment. To prevent precipitation, a new solution was prepared and provided three times a week.

2.4 | Blood analysis

Blood was obtained by puncture of the submandibular vein just before euthanasia and anticoagulated by adding citrate. Haemoglobin (Hb) levels were measured in whole blood by the Cell-Dyn Emerald 18 hematology Analyzer (Abbott diagnostics).

2.5 | Histopathological evaluation

At day 35, all animals were euthanized by cervical dislocation. The hind legs were removed, knee joints isolated and prepared for histological staining.[22] So far, the focus of histological evaluation has been mainly on the tibiofemoral compartment, while blood-induced patellar cartilage[17] and bone damage[18,23–26] are noticed in rodent models as well. This is supported by the finding that blood spreads throughout the joint cavity and ascends to the patella upon hemarthrosis in haemophilic mice.[27] Therefore, evaluation of the patellar compartment is included in this study.

Perls Prussian blue staining was performed to evaluate the presence of synovial iron deposits. Synovial inflammation in the tibiofemoral compartment was scored on haematoxylin-eosin (H&E)–stained sections according to the Valentino score.[28] To evaluate peri-patellar inflammation, an adapted version of the score originally published by Koizumi was used on Safranin-O Fast-Green (Saf-O)-stained sections, based on the amount of pannus formation (0: none, 1: slight, 2: moderate, 3: marked).[29] Tibiofemoral and patellar cartilage damage were evaluated using the modified Osteoarthritis Research Society International (OARSI) score on Saf-O-stained sections.[15] The tibiofemoral score for each joint was the average of the individually scored femoral condyle and tibial plateau. All histopathological scores were performed by two independent observers blinded for the experimental conditions. In case of more than two points, difference consensus was sought (Valentino score: 11 cases, modified OARSI score for tibiofemoral cartilage: 4 cases). For further calculations, the mean of two observers' scores was used.

2.6 | Statistical analysis

Differences in joint diameter and histology scores between paired samples (contralateral and experimental joint of the same animal) were analysed using the paired t-test or the Wilcoxon signed rank test. Differences in Hb value and histology scores across treatment groups were analysed using the Mann-Whitney test. Results were considered significant if $p < .05$. Graphic presentation and statistical analyses were performed using GraphPad Prism (Version 8.0.1; GraphPad Software Inc, San Diego, CA, USA).

3 | RESULTS

3.1 | Needle puncture results in gross joint bleeding

Inducing a joint bleed did not result in a difference in survival rate between both treatment groups and survival rates were within anticipated ranges (placebo: 26 out of 34 animals (76%), DFX: 28 out of 32 animals (88%), $p = .246$). A clear increase in the diameter of the experimental joint was seen 2 days after joint bleed induction as compared to the baseline value (Figure 1A,B; both groups $p = <.001$). The VBS also increased after the joint bleed (Figure 1C,D; both groups $p = <.001$). No differences in JD or VBS of the experimental joint were found between the treatment groups at day 2 ($p = .364$ and $p = .322$, respectively). The joint bleed was considered unsuccessful in three animals of the control group and none of the DFX group, and these animals were excluded from further analysis.

Hb levels were decreased in both the placebo and the DFX group compared with control animals (Figure 2; controls ($n = 9$, one missing due to clotting): median 8.50 mmol/L, interquartile range (IQR) 8.15–8.80, placebo ($n = 23$): 8.07 mmol/L, 7.33–8.40, DFX ($n = 28$): 8.34 mmol/L, 7.84–8.56, $p = .018$ and $p = .057$, respectively). The decrease was not statistically significantly different between both treatment groups ($p = .208$).

3.2 | On-demand treatment with DFX does not attenuate inflammation upon joint bleed induction

Joint bleed induction led to an increase in tibiofemoral inflammation according to the Valentino score in the experimental compared with the contralateral joint in the placebo group (Table 1 and Figure 3A; median score +3, $p = <.001$), as well in the DFX group (median score +2.3, $p = <.001$) (Figure S1 for representative images). The contralateral joint of the placebo and DFX group showed comparable tibiofemoral inflammation ($p = .842$), but this was significantly increased compared with the bleeding naive control animals ($p = .005$ and $p = .018$, respectively). The Valentino score in the experimental joint did not differ between the placebo and DFX group ($p = .305$), although a slightly lower median score was seen in the DFX group (5.5 vs 4.8). In addition, the change (experimental minus contralateral joint) in Valentino score was similar between both treatment groups (Figure 3B; $p = .506$). No differences in hemosiderin depositions, based on the Perls Prussian blue staining (Figure 4) and a subcategory of the Valentino score, were observed between the placebo and DFX group.

To evaluate peri-patellar inflammation, the adapted Koizumi score was applied to Saf-O stained sections. In line with tibiofemoral

FIGURE 1 Joint diameter and visual bleeding score increased 2 days post-hemarthrosis. Change in joint diameter (JD) in the blood-exposed joint over time, in the placebo (panel A) and deferasirox (DFX; panel B) treated group, as well as visual bleeding score (VBS) in the placebo (panel C) and DFX group (panel D). A and B: JD was measured using a micrometre calliper on day 0, 2, 14 and 35. Grey lines represent the diameter of the blood-exposed joints in individual animals, the mean of all animals is indicated by the black line. The dotted line indicates the mean of the contralateral joints. C and D: VBS was assessed at day 0, 2, 14 and 35 depicted as mean ± SD. *indicates $p = <.001$ for comparison with baseline (day 0), Wilcoxon signed rank test
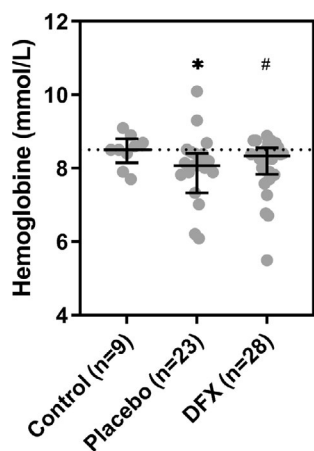
FIGURE 2 Haemoglobin values slightly decreased at day 35. Haemoglobin (Hb) values are assessed in whole blood at the day of euthanasia (day 35). Two data points in the placebo group: Hb 2.0 and 3.5 mmol/L were outside the axis limits, but included in the analysis. Dotted line represents the median of the control group. Data depicted as median with interquartile range, *$p = .018$, #$p = .057$, one-tailed Mann-Whitney test. The levels of both treatment groups were not statistically significantly different ($p = .208$)

inflammation, an increase in peri-patellar inflammation was observed in the experimental joint compared with the contralateral joint in the placebo and DFX group (Table 1 and Figure 3C; both groups median score +3, $p = <.001$) (Figure S1). Comparison between the treatment groups demonstrated no differences between the contralateral nor the experimental joints ($p = .214$ and $p = .787$, respectively). Also, the change in adapted Koizumi score did not differ between both groups (Figure 3D; $p = .794$).
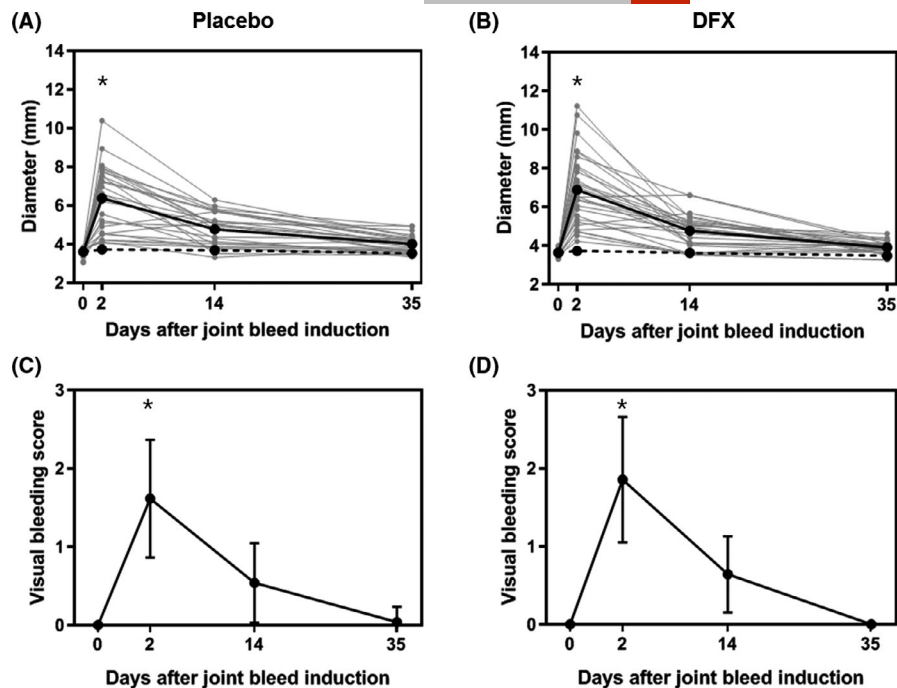
3.3 | Cartilage degeneration is not limited by DFX on demand

A significant increase in cartilage damage in the tibiofemoral compartment of the experimental joint of the placebo and DFX group was noted as compared with the joints of the bleeding naive control animals (Table 1; both $p = .003$) (Figure S1). Also, the contralateral joints of the placebo and DFX group demonstrated a significant increase in tibiofemoral cartilage degeneration compared with the control animals (Table 1; $p = <.001$ and $p = .002$, respectively). Because of this contralateral damage, no statistically significant increase between experimental blood-exposed joints and contralateral joints was found in the tibiofemoral compartment (Table 1 and Figure 5A). Neither was a difference observed in tibiofemoral cartilage damage of the contralateral or experimental joints between the placebo and DFX group ($p = .265$ and .802, respectively). The change in cartilage damage (blood-exposed minus contralateral joint) was marginal in both groups and comparison between the treatment groups did not indicate any protective effect of DFX on tibiofemoral cartilage damage (Figure 5B; $p = .143$).

In the patellar compartment, an evident increase in cartilage damage was found in the experimental joint as compared to the contralateral joint in both the placebo (Table 1 and Figure 5C; median score +4.5, $p = <.001$) and DFX group (median score +5.5, $p = <.001$) (Figure S1). No differences in the contralateral ($p = .802$) or the experimental joint ($p = .882$) were noted when the placebo and DFX treated group were compared. In accordance with the tibiofemoral compartment, the modified OARSI scores applied to the patella of the contralateral joints of both treatment groups were significantly increased compared with the bleeding naive control animals ($p = .001$ and $p = <.001$, respectively). The change

TABLE 1 Histological joint damage. Histological joint damage was scored at day 35 by two observers blinded for the intervention in bleeding naive control, placebo and deferasirox (DFX)-treated animals

		Placebo group			DFX group		
	Control group	JB−	JB+	*p*-value	JB−	JB+	*p*-value
Tibiofemoral inflammation (Valentino score)	2.0 (0.0–2.0)	2.5* (2.0–3.0)	5.5* (4.0–6.5)	<.001	2.5* (2.0–3.0)	4.8* (3.0–6.0)	<.001
Patellar inflammation (adapted Koizumi score)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	3.0* (1.0–3.0)	<.001	0.0 (0.0–0.0)	3.0* (0.0–3.0)	<.001
Tibiofemoral cartilage damage (modified OARSI)	0.0 (0.0–0.6)	1.3* (0.5–3.5)	0.5* (0.3–1.3)	.012	0.5* (0.3–3.5)	0.5* (0.1–2.5)	.449
Patellar cartilage damage (modified OARSI)	0.0 (0.0–0.5)	0.5* (0.0–2.5)	5.0* (3.0–6.0)	<.001	0.5* (0.4–2.3)	6.0* (2.0–6.0)	<.001

Note: Synovial (in the tibiofemoral compartment) and patellar inflammation were assessed according to the Valentino score (0–10) and adapted Koizumi score (0–3), respectively. Tibiofemoral and patellar cartilage damage were evaluated using the modified Osteoarthritis Research Society International (OARSI) score (0–6). Data are expressed as median (interquartile range) and *p*-values (Wilcoxon signed rank test) for comparison between the contralateral (joint bleed (JB)−) and experimental (JB+) are given.

*Indicates a significant difference (*p* = <.05) compared with control (bleeding naive) animals (Mann-Whitney test).
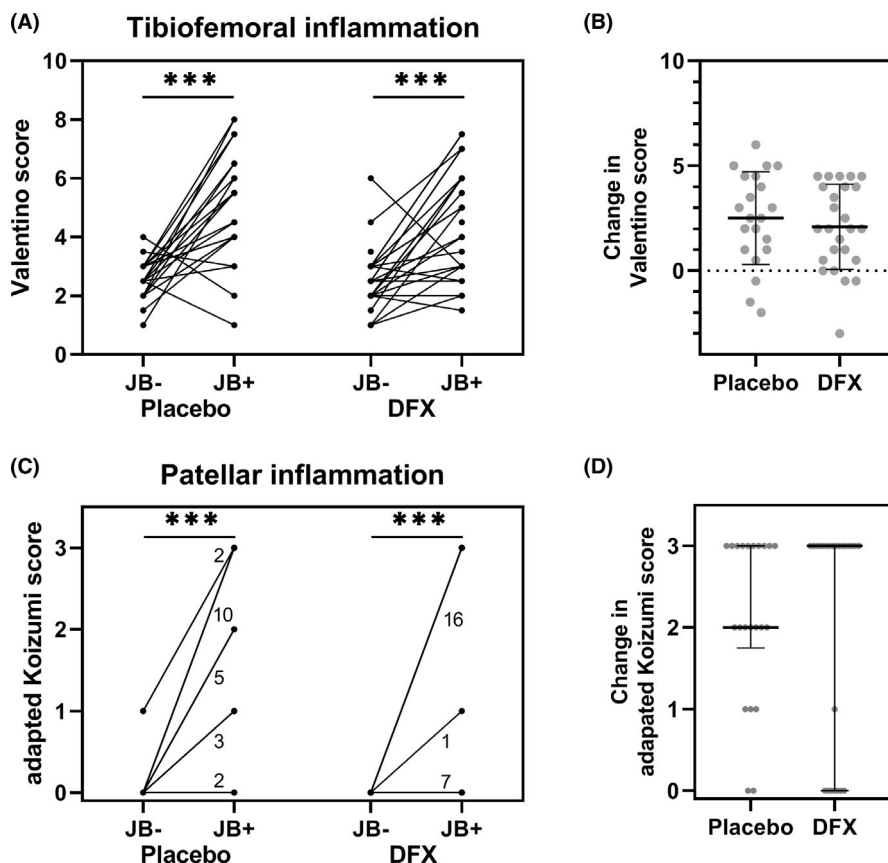

FIGURE 3 No effect of DFX on tibiofemoral and patellar inflammation. Clear inflammation was demonstrated in the tibiofemoral compartment (A/B; Valentino score) and patella (C/D; adapted Koizumi score, numbers indicate how many animals are represented by a black line) of the blood-exposed joint 35 days after a single joint bleed. The change in inflammation (score of blood-exposed minus contralateral joint) between the placebo and deferasirox (DFX) group is displayed for the tibiofemoral (B; depicted as mean ± SD) and patellar (D; depicted median with interquartile range) compartment. ***p* = <.001 for the difference between blood-exposed and contralateral joint (Wilcoxon signed rank test). No statistical differences between both treatment groups were found. JB, joint bleed

in patellar cartilage degeneration is comparable between the treatment groups (Figure 5D; *p* = .536).

4 | DISCUSSION

This study was designed based on the results reported by Nieuwenhuizen, indicating that prophylactic treatment with the iron chelator DFX attenuates cartilage damage upon blood exposure in haemophilic mice.[15] Since this approach is not opportune in clinical practice due to the unpredictable occurrence of a joint bleed, the effect of on-demand treatment with DFX was investigated in the present study. Equal methods in terms of mouse strain, model, dose of DFX and histopathological evaluation were applied to enable direct comparison. In line with the prophylactically treated mice,[15] on-demand treatment with DFX initiated at the time of the joint bleed
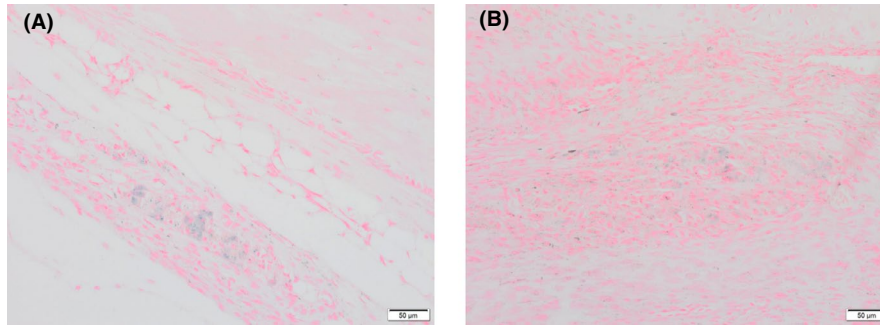
FIGURE 4 On-demand treatment with DFX does not affect synovial iron staining. Representative photomicrographs of the synovial Perls Prussian blue staining of experimental knees, demonstrating similar presence of iron (indicated by the blue colour) in the control (A) compared with the deferasirox (DFX) group following a joint bleed. Magnification: 10× [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 No effect of DFX on tibiofemoral and patellar cartilage degeneration. The modified OARSI score was applied to the tibiofemoral (A/B) and patellar (C/D) compartment 35 days following hemarthrosis. Comparison of the change in modified OARSI (score of blood-exposed minus contralateral joint, depicted as median with interquartile range) is presented for the tibiofemoral (B) and patellar (D) compartment. ***p = <.001 for the difference between the blood-exposed and contralateral joint. No statistical differences between both treatment groups were found. JB, joint bleed
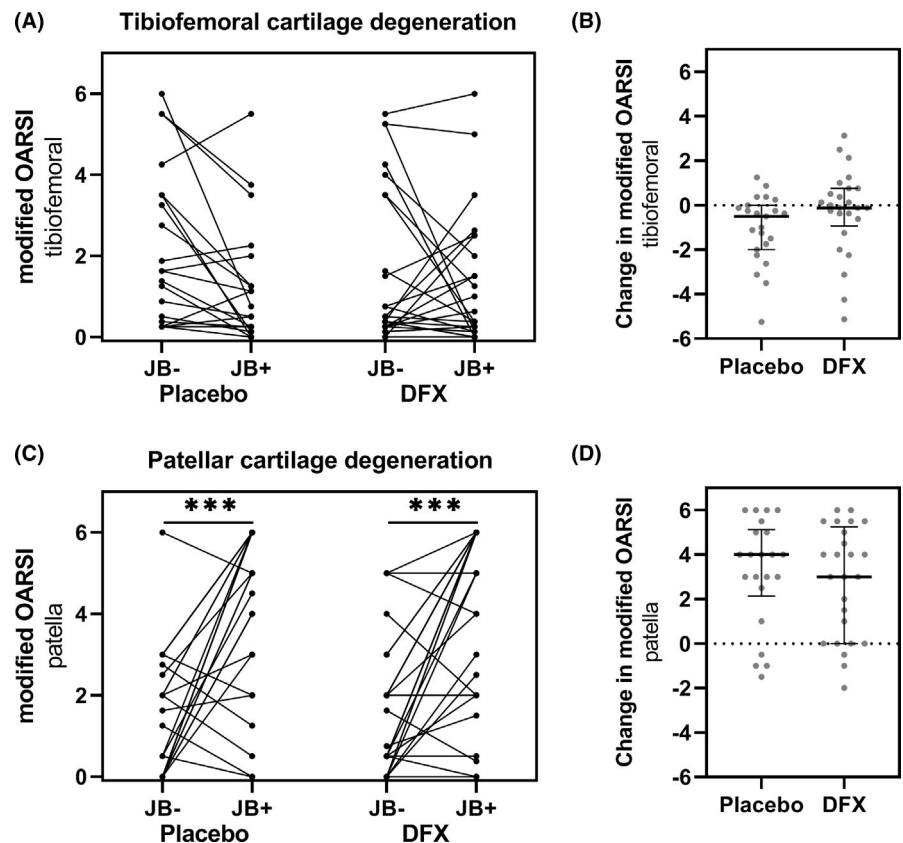
had no protective effect on tibiofemoral or patellar inflammation. In contrast to prophylactic treatment with DFX, on-demand treatment did not prevent cartilage damage in haemophilic mice.

Four hypothetical pathophysiological mechanisms regarding the effect of DFX on blood-induced cartilage damage are discussed: 1. decrease in systemic iron load, 2. mobilization of iron overloaded tissue (eg hemosiderin), 3. reducing/scavenging radical formation and 4. inhibiting an upregulated NFκB-pathway. The predominant mechanism whereby DFX removes iron from the body is by binding and eliminating iron systemically.[30] A major difference between the prophylactically and on-demand treated mice is the iron load at the moment of joint bleed induction. Animals in the present study had an unaltered iron status at time of the induced joint bleed, since DFX

treatment was started at the moment the joint bleed was induced. This is in contrast to prophylactically treated mice, in which blood with 30% reduced iron load (represented by plasma ferritin) entered the joint cavity at the moment of the bleed.[15] It can be questioned whether the chondroprotective effect seen in these mice is solely due to the 30% reduction in catalytic iron. *In vitro* data demonstrate that even 10% volume/volume blood exposure already causes prolonged and irreversible cartilage damage.[31] As such, additional effects of prophylactic DFX may also have contributed to its cartilage protective effect.

A second mechanism of action of DFX is its effective and selective mobilization of iron from various iron loaded tissues.[21,32] Upon recurrent hemarthrosis, iron accumulates as hemosiderin in the joint,

causing inflammation and indirect cartilage damage. No differences in hemosiderin depositions could be observed between the placebo and DFX group in the on-demand treated mice, whereas in the prophylactically treated mice reduced hemosiderin depositions were demonstrated.[15] This difference may be caused by the decrease in iron influx during the joint bleed, or the degree of actual iron withdrawal. DFX reaches its peak serum concentration within hours post-administration.[14] However, stress-induced reduced water intake post-injury may have delayed early uptake of DFX in the on-demand-treated mice and with that early iron withdrawal. This time may have been essential, since short blood exposure already causes irreversible cartilage damage.[31]

Previous studies have shown that iron chelators have a chondroprotective effect when applied *in vitro*[33] or locally in non-haemophilic animals.[34] DFX not only has an iron-chelating effect, but also has the capability to reduce oxidative stress caused by ROS and with that inhibiting the NFκB pathway.[12,35] ROS interfere with NFκB signalling pathways,[36] which have been demonstrated to play an important role in blood-induced inflammatory and cartilage degenerative processes in haemophilic mice.[37] Moreover, high levels of synovial receptor activator of NFκB (RANK) are demonstrated in patients with HA.[38] DFX is capable of reducing radical formation by binding catalytic iron,[12] scavenging the already formed ROS.[35] Moreover, DFX is able to inhibit an upregulated NFκB pathway independently from ROS reduction,[35] the latter being a characteristic unique for DFX which is not shared by other chelators. As a consequence, DFX could hypothetically protect the joint from blood-induced damage by influencing these additional pathways when locally active.

The absence of an effect in our on-demand study may be explained by the delayed availability of the chelator. The harmful process following blood exposure seems already irreversible before systemically applied DFX on demand can exert its iron-chelating effect. A local beneficial effect of DFX is anticipated, but in this study it remains unclear whether DFX has reached the joint in time and in sufficient concentration to be effective. Although DFX is considered a drug with good permeability[39] and increased vessel permeability is seen post-hemarthrosis,[40] tridentate iron chelators such as DFX are also known to form polymeric complexes that cannot easily cross cell membranes.[12] As such, the lack of data on DFX concentrations in synovial fluid and plasma may be considered a limitation of this study. The small volume of synovial fluid in mice limits the possibility to determine DFX levels locally and systemic levels show a high intra- and interindividual variability.[41–43] Also the individual intake of DFX could not be established as the animals shared a drinking bottle, because they were housed in groups according to ethical regulations. On average, the measured water consumption per cage should have led to sufficient DFX intake per animal (data not shown). The administration of DFX by oral gavage was not feasible because this would have led to undesired bleedings. Plasma ferritin may serve as a surrogate marker for the effect of DFX as it reflects iron storage, but the study design limits its use. The 5-week treatment period in our study is too short to expect a significant decrease in ferritin.[44] In addition, ferritin is an acute-phase protein susceptible to inflammation and injury,[45] so a possible decrease due to iron chelation by DFX may not be detectable.

5 | CONCLUSION

On-demand treatment with DFX did not protect the joint from the harmful effects of blood exposure in this experimental setup, probably because the irreversible damaging process is initiated before DFX can exert its effect systemically and locally. As a consequence, the application of systemic on-demand treatment with DFX as a therapeutic solution for HA seems not feasible. To achieve faster efficacy, further research is needed to evaluate the potential of an intravenously administered or locally applied iron chelator at the time of joint bleeding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.