CHARACTERIZING EARLY BLOOD-INDUCED JOINT DAMAGE

towards new treatment modalities of hemophilic arthropathy

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Astrid Pulles

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Characterizing early blood-induced joint damage

towards new treatment modalities of hemophilic arthropathy

Kenmerken van vroege bloedgeïnduceerde gewrichtsschade

als basis voor nieuwe behandelopties voor hemofilie arthropathie

(met een samenvatting in het Nederlands)

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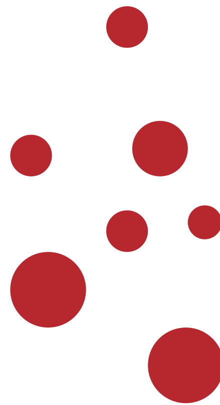

General introduction

Textbook Kelley and Firestein's Textbook of Rheumatology, 11th ed 2020, chapter 126 Hemophilic Arthropathy

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Introduction

Spontaneous joint bleeding is a characteristic manifestation of the inherited coagulation disorder hemophilia,[1] but such bleeding also can occur as a consequence of other bleeding disorders such as von Willebrand disease,[2] as a complication of anticoagulant treatment,[3] or upon experiencing trauma[4] or undergoing major joint surgery.[5] Irrespective of the underlying cause, hemarthrosis can lead to significant joint tissue damage and subsequent major morbidity.

Hemophilia is an X-linked recessive coagulation disorder caused by deficiency of coagulation factor VIII (hemophilia A) or factor IX (hemophilia B; Christmas disease). Hemophilia has an estimated frequency of 1 in 5000 to 10,000 male births,[6,7] with approximately 80% to 85% representing hemophilia A. The lack of clotting factor activity leads to inadequate thrombin generation and a tendency for bleeding to occur. The clinical phenotype is strongly correlated with the amount of clotting factor present, expressed as a percentage compared with normal clotting factor activity in blood. Patients with severe hemophilia have less than 1% clotting factor activity, whereas patients with moderate and mild hemophilia have clotting factor activity of 1% to 5% and 5% to 40%, respectively.[8] Spontaneous bleeding mainly affects patients with severe hemophilia; it occurs less frequently in individuals with moderate hemophilia, and patients with mild hemophilia experience bleeding only after sustaining a major trauma or when undergoing surgery. The phenotype is also affected by several other mechanisms, including genetic factors, age at the first episode of joint bleeding, environmental factors, and concomitant thrombotic factors and fibrinolytic activity.[9-11] In individuals with severe hemophilia, 70% to 80% of all bleeding events occur in the joints, spontaneously as well as in response to stress or trauma.[12,13]

The predilection for bleeding in the joint compared with other tissue sites is caused by an altered balance in the coagulation cascade. The expression of tissue factor, which is the initiator of the coagulation cascade,[14,15] is relatively low, while together with a highthe level of tissue factor pathway inhibitor is relatively high in the normal joint.[16] As a consequence, thrombin generation in the joint is more dependent on the intrinsic tenase complex, in which factor VIII and factor IX play an important role. Moreover, thrombin generation is required for the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) in the joint.[17] Inadequate TAFI activation in severe hemophilia impairs protection against urokinase-type plasminogen activator-mediated fibrinolysis, in addition to already increased local fibrinolysis in the hemophilic jointand increased local fibrinolysis in the hemophilic joint.[18] On the other hand, mechanical factors damaging the richly vascularized synovial tissue, specifically when the process of neovascularization has been after being triggered after a first joint bleed, might be causative in this respect.[19]

Musculoskeletal bleeding and treatment-related complications are the most important complications in hemophilia. Development of alloantibodies directed against administered clotting factor (inhibitors) is common, and the transmission of blood-borne virus infections as a result of the use of plasma-derived products in the 1980s caused major morbidity.

Clinical Features

Bleeding into the musculoskeletal system causes a spectrum of clinical features with involvement of joint, muscle, and bone. The distribution of the most affected joints and muscles is shown in Figure 1.

Acute Hemarthrosis

Nearly all patients with severe hemophilia and half of patients with moderate disease activity experience hemarthroses. The most affected joints are the large synovial joints, especially the ankles, knees, and elbows.[13] The hips and shoulders are affected to a lesser extent, and bleeds in smaller joints are rare. In individuals with severe hemophilia the first joint bleed often occurs when they begin walking or running, at a median age of 1.8 years.[10] At this age, early signs of bleeding include irritability and decreased use of the affected limb. Older children and adults frequently describe the onset of a joint bleed as a tingling sensation and tightness within the joint, followed by rapid swelling, loss of range of motion (ROM), pain, and warmth of the skin over the joint.[1,20] Flexion is the most comfortable position, and disuse (i.e., preventing movement to avoid pain) can cause secondary muscle spasm. Pain rapidly reduces after clotting factor replacement, with full recovery of joint function within 8 to 24 hours.

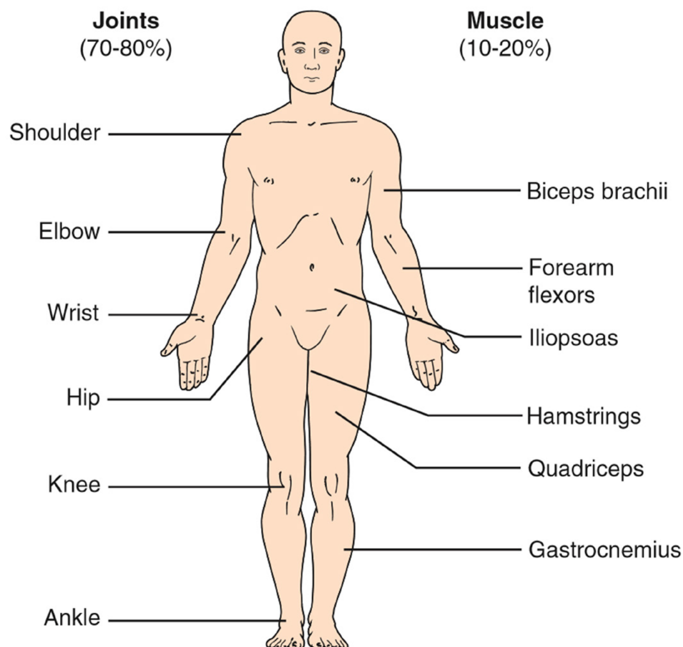

Figure 1 - Joints and muscles commonly affected by bleeding in hemophilia
Besides hemarthrosis and muscle bleeding, 5% to 10% of the episodes consist of other major bleeds (e.g., mucous membranes and gastrointestinal), and fewer than 5% of the bleeds occur in the central nervous system.

Joint bleeds often have a migratory pattern, moving from one joint to the other.[13] A so-called "target joint" develops in about 25% of patients with severe hemophilia - that is, a joint that is more susceptible to subsequent bleeds compared with other joints. It is defined as a joint in which three or more hemarthroses have occurred in the prior 6 months.[19,21] Nowadays, in youngsters with hemophilia, the target joint mostly involves the ankle joint rather than the elbow or the knee, with the latter most commonly affected before the introduction of prophylactic clotting factor replacement therapy.

Synovitis

Recurrent bleeding into a joint can lead to a vicious circle in which the synovial tissue is unable to remove blood remnants completely, triggering synovial inflammation and proliferation.[22,23] It also induces the development of a rich network of new, fragile blood vessels underneath the synovial tissue, making the joints vulnerable to subsequent (repeated) bleeding.[24,25] In this condition the joint appears swollen but usually not tense, is often painless, and is only slightly warm. In early stages of synovitis, ROM is preserved, but in chronic stages a mild limitation and flexion deformity can develop. Administration of clotting factor is considered necessary to break the self-perpetuating cycle of hemarthrosis-synovitis-hemarthrosis and to prevent the progressive degeneration of the joint. In contrast to its effect on acute hemarthrosis, clotting factor replacement does not modify the clinical findings immediately, and long-term treatment is indicated.[1]

Hemophilic Arthropathy

Ultimately, recurrent joint bleeding may lead to hemophilic arthropathy (Figure 2). The number of hemarthroses required to cause irreversible damage is unknown and is likely to differ between patients. Progressive degeneration of cartilage, synovial inflammation, and

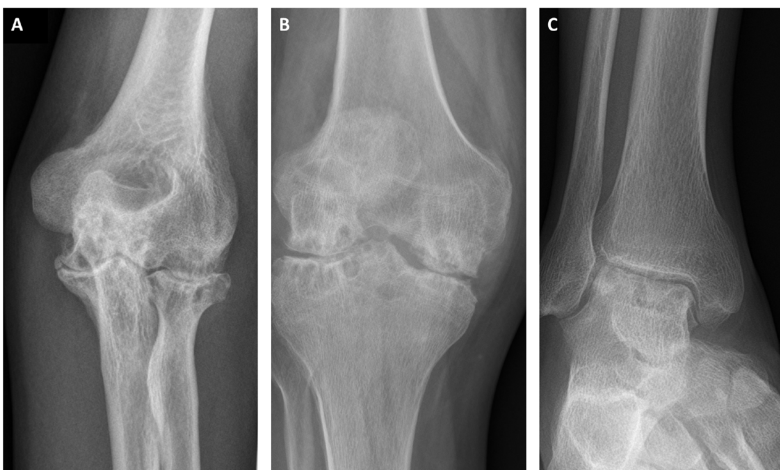

Figure 2 - Radiographic changes of hemophilic arthropathy in a 30-year old patient with severe hemophilia
A, Left elbow. B, Right knee. C, Right ankle. All of the radiographs show joint space narrowing and subchondral cysts, which are most pronounced in the elbow and knee.

bone changes cause chronic pain, joint stiffness, and a severely limited ROM. Without proper physiotherapy it is often accompanied by muscle weakness and contractures. Joint deformity, subluxation, joint laxity, malalignment, and spontaneous arthrodesis develop in the most severe cases. Physical activity and quality of life are severely affected by hemophilic arthropathy, especially in advanced disease.[26,27] Progressive synovial fibrosis decreases the frequency of hemarthroses in end-stage arthropathy.

Muscle and soft tissue hemorrhage

The muscle is the second most common site of bleeding in individuals with hemophilia, accounting for approximately 10% to 20% of bleeds.[1] The bleeding usually results from a direct blow or sudden stretch and can occur in any muscle of the body, although the most common sites are the iliopsoas muscle and the flexor compartment of the forearm.[28] The clinical features depend on the muscle involved, but overall muscle bleeding emerges more insidiously than do hemarthroses, and prodromal symptoms are rare. A tender swelling in the muscle with severe pain upon stretching or active contraction rapidly progresses into protective spasm and a flexed position. Physical examination shows a palpable tender hematoma with swelling, warmth, and bruising. Bleeding into deeper compartments can be difficult to diagnose because visible symptoms are lacking. An iliopsoas hemorrhage usually presents with pain in the lower abdomen, groin, and/or lower back, with pain on extension but not rotation of the hip.[29] A life-threatening complication of ongoing bleeding is compartment syndrome characterized by intense pain, swelling, tension, and sensory impairment. Persistence of increased pressure may result in with femoral neuropathy and and possible muscle necrosis.[30,31] Other sites of muscle bleeding associated with neuropathy are the posterior compartments of the lower leg and the flexor group of the forearm. Muscle hemorrhage can result in permanent contracture, rebleeding, myositis ossificans, infection, chronic nerve injury, and the formation of pseudotumors.[29,32,33]

Pseudotumor

The formation of pseudotumors is a rare but serious complication unique to hemophilia that mainly occurs after inadequate replacement therapy or after development of an inhibitor.[34] The prevalence in severe hemophilia is 1-2%, and even up to 10% in hemophilic patients with inhibitors,[35] although these numbers depend highly on the availability of clotting factor concentrate. Repeated and unresolved muscle bleeding or a progressive subperiosteal hemorrhage causes an painless, expanding encapsulated and calcified hematoma with radiographic features mimicking tumor-like conditions; hence, they are called pseudotumors (Figure 3).[1,36] Progressive enlargement of the pseudotumor can lead to erosion of the adjacent bone, compression of neurovascular structures, spontaneous rupture, fistula formation, and pathologic fractures. Pseudotumors have two distinct pathologic forms. In adults, pseudotumors occur proximally, (mainly in the pelvis or femur) expand slowly, and usually require surgical excision. In contrast, pseudotumors affecting young patients occur distal to the elbow or knee, result from direct trauma, develop rapidly, and often are amenable responsive to conservative treatment with immobilization and factor replacement.[37]

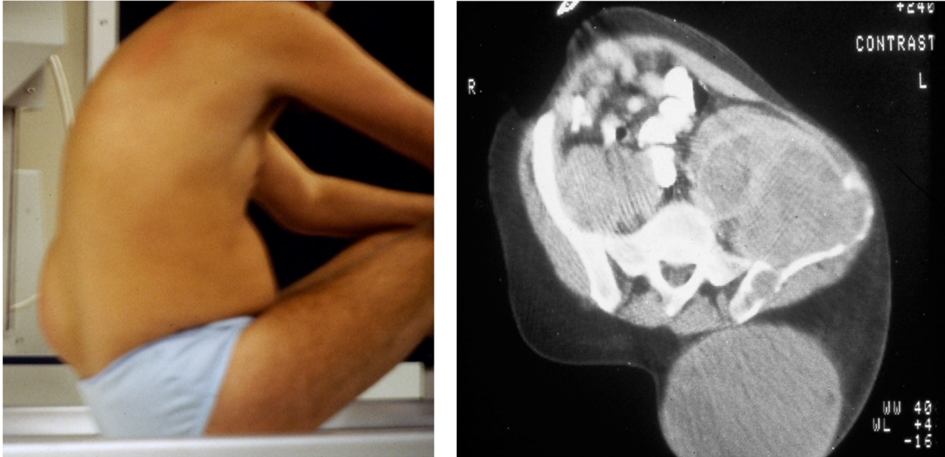

Figure 3 - A pseudotumor invading the left wing of the ilium in a patient with severe hemophilia and an inhibitor.

Osteoporosis

Patients with hemophilia have a significantly lower bone mineral density compared with age-matched control subjects, which appears to begin in childhood.[38-41] The pathogenesis is multifactorial, with several predisposing factors occurring frequently in the hemophilic population, including reduced weight-bearing activity, arthropathy, muscle atrophy, a lower body mass index, presence of an inhibitor, and the influence of blood-borne virus infections and their treatment.[42-44] To maintain bone mass, weight-bearing activities should be promoted and supplementation of calcium and vitamin D in selected cases should be considered.[1]

Diagnosis

In the majority of patients, the diagnosis "hemophilia" is made in the presence of a positive family history. Up to one-third of the patients have a negative family history, and the disease is the result of a de novo germline mutation, but somatic mosaicism in a (grand)parent also has been described.[45,46] Clinical features raising suspicion of hemophilia are easy bruising in early childhood, spontaneous bleeding (particularly into the joints, muscles, and soft tissues), and excessive bleeding after trauma or surgery. Screening tests in patients with a bleeding diathesis of unknown cause include platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT). Individuals with hemophilia characteristically have a normal platelet count, normal PT, and a prolonged aPTT, although the aPTT might be near normal in individuals with mild hemophilia. Specific clotting factor assays are performed to determine the type and severity of hemophilia and to distinguish it from von Willebrand disease. The diagnosis can be confirmed by DNA-based techniques. Genetic analysis to establish the causative mutation is important considering the role of the gene

defect in the risk of inhibitor formation, to recognize female carriers and for preimplantation genetic diagnosis.[47] Given the rarity and complexity of the disease, a doctor specializing in hemostasis should be consulted.
Frequent monitoring of musculoskeletal status is indicated in patients with hemophilia to diagnose complications at an early stage because prevention is the cornerstone of treatment. Use of physical examination assessment tools can help identify subtle early signs of joint damage, monitor joint health over time, and evaluate treatment efficacy. Several hemophilia-specific instruments have been developed and validated.[48-50]
Clinical assessment of the musculoskeletal system at the time of an acute bleed should include evaluation of warmth, bruising, swelling, tenderness, muscle tone, pain, ROM, gait, and function.[1,51] Differentiating hemarthrosis from flare-ups of hemophilic arthropathy is difficult due to overlap in symptoms.[20]

Table 1 - Pettersson Score

Joint: Elbow/Knee/Ankle		Score
Osteoporosis	Absent	0
	Present	1
Enlargement of epiphysis	Absent	0
	Present	1
Irregularity of subchondral surface	Absent	0
	Partially involved	1
	Totally involved	2
Narrowing of joint space	Absent	0
	Joint space >1 mm	1
	Joint space <1 mm	2
Subchondral cysts formation	Absent	0
	1 cyst	1
	>1 cyst	2
Erosion of joint margins	Absent	0
	Present	1
Gross incongruence of articulating bone ends	Absent	0
	Slight	1
	Pronounced	2
Joint deformity (angulation and/or displacement)	Absent	0
	Slight	1
	Pronounced	2
Total (maximum 13 points per joint)		

Radiographs of the six index joints (both knees, both ankles, and both elbows) are scored separately. The sum of these scores, with a maximum of 78 per patient, is the Pettersson score.[52]

Conventional Radiography

Conventional radiology is most frequently used for imaging in hemophilia and is mainly suitable for late osteochondral changes. Radiologic classification of hemophilic arthropathy is usually performed via the Pettersson score (Table 1).[52] This additive scoring system is based on assessment of the knees, ankles, and elbows, with eight criteria being scored: osteoporosis, epiphyseal enlargement, irregularity of the subchondral surface, joint space narrowing, subchondral cyst formation, erosion of joint margins, incongruence of the articulating bone ends, and joint deformity. The Pettersson score correlates well to function,[53-55] but is only able to diagnose late arthropathic changes. Typically it is used to evaluate progression of arthropathy and for planning of arthrodesis or joint replacement. Digital scoring systems may be more objective and have a higher interobserver variability than the Pettersson method. Digital analysis of hemophilic arthropathy of the knee is feasible,[56] but adaptation for the specific characteristics of hemophilic arthropathy is necessary.

Radiography has poor sensitivity in demonstrating early soft tissue changes that occur before irreversible cartilage and bone damage. Acute hemarthrosis shows as joint effusion and displacement of fat pads, but it is difficult to distinguish effusion from synovial hyperplasia.

Magnetic Resonance Imaging

As prophylactic treatment improves and joint damage diminishes, more sensitive MRI has obvious advantages compared with radiography in evaluating treatment efficacy. MRI enables visualization of small alterations, such as hemosiderin depositions, synovial hypertrophy, and minor cartilage damage without joint space narrowing,[57] although susceptibility artefacts may hamper interpretation of hemosiderin deposits, synovial hypertrophy, and peripheral cartilage integrity.[58] The clinical implications of small changes, especially in the absence of reported joint bleeds, remains to be established,[59] but as in osteoarthritis (OA), it may be predictive of subsequent rapid progressive damage. MRI can also provide more detailed information about advanced changes, such as erosions, subchondral cysts, and cartilage destruction. It is valuable in detailed evaluation of a pseudotumor, synovitis, or diagnosis and follow-up of a hemorrhage in deeper compartments (e.g., the abdomen and iliopsoas). Widespread use of MRI is restrained by its costs, availability, scanning time per joint, and requirement of sedation in young children, and the possible need of intra-articular contrast to depict initial osteochondral changes.[60]

Ultrasonography

With ultrasonography becoming increasingly standard in rheumatology practices, it is possible to diagnose joint effusion, synovial hypertrophy, abnormalities involving osteochondral surfaces, and pseudotumors.[57,61] Its ability to detect a bloody effusion and hemosiderin deposits has been debated.[62-65] Findings obtained by ultrasonography correlate well with MRI in evaluating hemophilic joints both with and without arthropathy, clinical findings, and functional status of the joint.[66-68] Ultrasonography is less costly than MRI, is readily available, does not require sedation in young children, and enables dynamic investigation such as assessment of vascularity of a pseudotumor. Disadvantages are the interobserver variability, complexity of image analyses, and difficulty in identifying changes in deeper structures., and a lack of validated scoring systems for hemophilic arthropathy.

Point-of-care ultrasound (POCUS), performed by trained non-radiologist physicians could be a valid and reliable tool to detect the presence of early signs of arthropathy and hemarthrosis.[69-71] Incorporation of POCUS in routine clinical examination allows for rapid diagnosis, treatment guidance, lower costs compared to comprehensive ultrasonography performed by a radiologist, and real time feedback, which could improve adherence.[72-74]

Biochemical Markers

In the field of OA and rheumatoid arthritis (RA), considerable effort is directed toward the identification of biochemical markers in blood and/or urine that can assist in diagnosis, prognosis, and response to treatment.[75] Such markers are as yet sparsely used in clinical trials and still are not used in clinical practice. Also, in the field of hemophilic arthropathy, such markers may become helpful in identifying the tissue-destructive activity of a joint bleed.[76-79]

Pathogenesis of hemophilic arthropathy

Although it is clear that the presence of blood has devastating effects on the joint, the exact pathogenetic mechanisms of hemophilic arthropathy, and in particular the earliest changes induced by acute hemarthrosis, are not completely understood. Surgical specimens of end-stage arthropathy in patients with hemophilia, together with *in vitro* experiments and animal models, have indicated that three major processes are involved: synovial inflammation, cartilage degeneration, and bone remodeling.[23,60,80,81] In this respect, hemophilic arthropathy has characteristics of both the RA and the OA joint (Figure 4).[82]

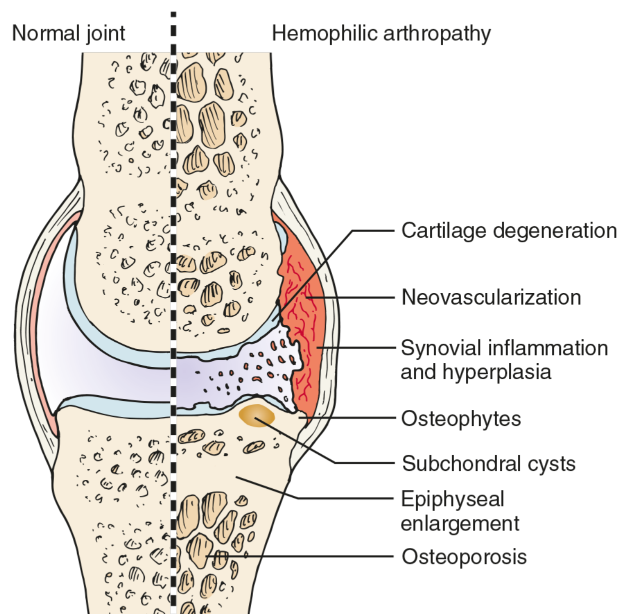

Figure 4 - Changes that occur in the synovial joint affected by hemophilic arthropathy

Synovial inflammation and proliferation

Synovial tissue is highly vascularized, and in hemophilia even minimal forces can lead to hemarthroses. Filling of the joint space with blood leads to an influx of inflammatory cells into the synovial tissue. Synoviocytes and invading macrophages evacuate blood from the joint cavity completely over a period of 3 to 4 weeks.[19] Four hours after the induction of hemarthrosis, erythrophagocytosis already can be observed.[83] Adaptive changes in the expression of iron regulation proteins in the synovium of patients with hemophilia after repeated joint bleedings are demonstrated.[84] With successive hemarthroses the synovial capacity is overloaded and blood remnants, especially iron, accumulate as hemosiderin depositions in synovial tissue and even cartilage.[22] The presence of iron triggers an inflammatory response and stimulates proliferation of synoviocytes (Figure 5).

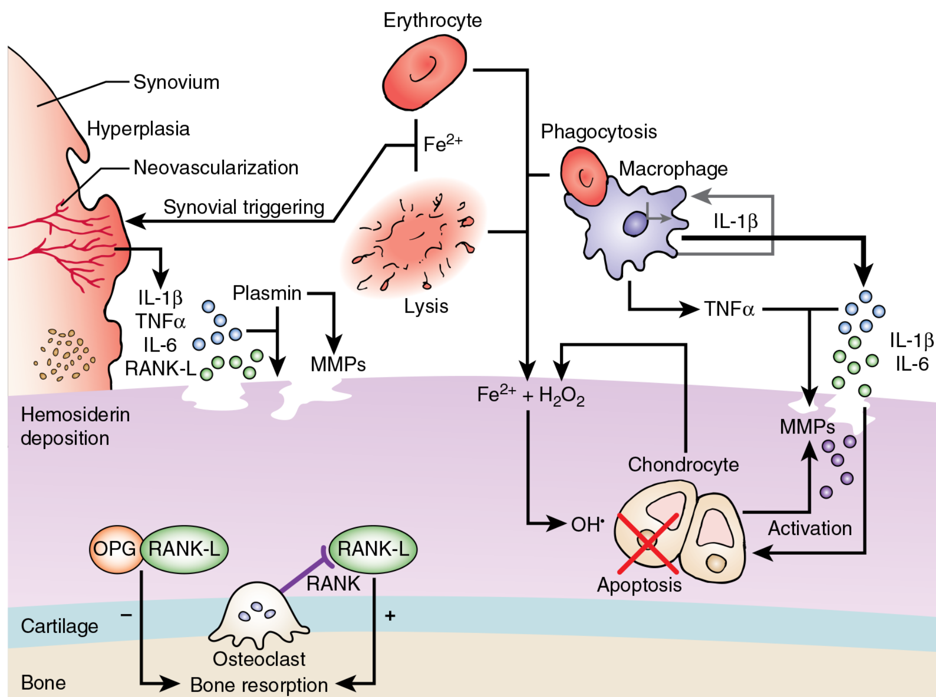

Figure 5 - Proposed mechanism of blood-induced joint damage
Joint bleeding causes synovitis, leading to synovial hypertrophy and vascular remodeling. The inflamed synovium produces plasmin, matrix metalloproteinases (MMPs) and pro-inflammatory cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNFα) that affect the cartilage. Cartilage is also directly affected by blood. Synovial and blood derived pro-inflammatory cytokines stimulate the production of hydrogen peroxide (H_2O_2) by chondrocytes. In the presence of erythrocyte derived iron(Fe2+), hydrogen peroxide is able to react according to the Fenton reaction, resulting in the generation of very toxic hydroxyl radicals (OH·) causing apoptosis of chondrocytes. Inflammation also activates the Receptor Activator of NFκB-Ligand(RANK-L)-RANK-Osteoprotegin(OPG)-pathway, resulting in bone resorption by osteoclasts. OPG protects bones from excessive resorption by binding to RANK-L instead of RANK.

Pro-inflammatory cytokines, including IL-1β, IL-6, and TNF, are produced by the iron-laden synoviocytes.[22,82] The normally thin synovial membrane becomes irregular, grossly hypertrophied, villous, friable, and highly vascular.[85,86] Synovial proliferation is postulated to result from an aberrant gene expression induced by the overload of iron.[87] It induces an overexpression of the proto-oncogene *c-Myc*, which is associated with synovial cell proliferation, and of the p53 tumor suppressor binding protein *mdm2*, leading to the abrogation of apoptosis of synovial cells.[88,89] An increased oxygen demand of the hypertrophied synovium causes hypoxia and thus potentially induces the release of growth factors such as vascular-derived endothelial growth factor and the formation of a rich network of brittle capillaries underneath the hypertrophied synovium.[24,90] This process sets up a vicious circle because these fragile vessels are more susceptible to recurrent bleeds.[25,91] Over time chronic synovitis develops, with pannus invading and eroding marginal cartilage,[92] and ultimately the synovial tissue becomes fibrotic. Expression of connective tissue growth factor is increased in synovium of patients with hemophilia in response to the presence of blood degradation products and is thought to mediate persistent fibrosis together with high levels of transforming growth factor-β1.[93] Clearly there are similarities to the rheumatoid joint.

Cartilage Degeneration

Cartilage destruction results from both synovial-dependent and synovial-independent processes. Synovial pannus invades cartilage at the periphery of inflamed joints. The inflamed hemosiderotic synovium produces cartilage-destructive pro-inflammatory cytokines and cartilage matrix degrading proteases.[22,86] These destructive properties of synovial tissue on cartilage are already present within 24 to 48 hours after a joint bleed and cause long-lasting damage.[94,95]

Hemarthroses also exert harmful effects directly on the cartilage (see Figure 5). Exposure of cartilage explants to whole blood or the combination of erythrocytes and mononuclear cells severely affects cartilage matrix turnover and results in chondrocyte apoptosis.[96,97] The pro-inflammatory mediators IL-1β and TNF produced directly after blood exposure transiently decrease proteoglycan turnover. Irreversible cartilage degradation results from chondrocyte apoptosis, which also occurs upon blood-exposure. IL-1β induces H_2O_2 production by activation of chondrocytes, which in combination with iron derived from erythrocytes, leads to oxidative stress.[98-100] Iron in combination with H_2O_2 reacts via the Fenton reaction to form hydroxyl radicals, resulting in chondrocyte apoptosis.[101] Because chondrocytes in adult cartilage hardly proliferate and have the responsibility to produce and maintain the extra-cellular matrix, apoptosis of chondrocytes will result in long-lasting impaired matrix turnover. Interestingly, these effects appear more pronounced on immature articular cartilage compared with mature cartilage,[102] and impaired cartilage is at least as susceptible to blood-induced joint damage as healthy cartilage.[103] These findings imply that it is important not only to prevent joint bleeds in children but also in patients with already affected joints. These direct effects on cartilage with chondrocyte death clearly represent characteristics of OA.

Bone changes

Bone changes after blood exposure consist of cyst formation, subchondral sclerosis, osteophyte formation, epiphyseal enlargement and osteoporosis.[104] The exact pathophysiologic mechanisms underlying these changes remain to be elucidated, but both indirect (risk factors, factor VIII deficiency, thrombin) and direct (hemarthroses) factors are involved.

Decreased physical activity and reduced weight-bearing exercise from childhood, due to hemophilic arthropathy, are important risk factors for bone loss in hemophilic patients. Moreover, experimental data suggest that factor VIII deficiency itself directly influences bone density independent of hemarthroses, differences in physical activity and medical comorbidity.[105-107] The factor VIII-vonWillebrand Factor complex is involved in inhibiting osteoclastogenesis through the activation of the receptor activator of nuclear factor (RANK)/RANK ligand/osteoprotegerin pathway, resulting in bone resorption. The role of thrombin has yet to be determined. Reduction in thrombin generation secondary to factor VIII deficiency could be a cause of increased osteoclast activity, although data on this topic are conflicting.[108] Blood exposure has a direct effect on bone density, disturbing the local equilibrium in bone turnover upon hemarthroses.[109,110] Development of bone loss is also inflammatory driven, exhibiting increased bone resorption as a more prominent feature than decreased bone formation.[111-113]

The triangle of interactions between cartilage damage, synovitis and bone changes clearly designates hemophilic arthropathy as a whole joint disease like osteoarthritis and rheumatoid arthritis.

Treatment of Hemophilia

Treatment of hemophilia consists of substitution of the deficient clotting factor. This substitution was first performed through whole blood transfusion and the use of fractions from plasma. The introduction of plasma-derived factor concentrates in the 1960s dramatically improved treatment and quality of life in patients with hemophilia. Use of these concentrates enabled prophylactic clotting factor replacement therapy, thereby converting the disease from a severe to a milder form.[114] It reduced the risk of bleeds with the setback of blood-borne infections. The introduction of highly purified, safe concentrates and recombinant products led to a marked reduction in the risk of viral transmissions.

Traditionally, treatment is provided at the time of clinically evident bleeding (known as episodic treatment or on-demand therapy). Nowadays, in developed countries, most patients with severe hemophilia are treated prophylactically, that is, via regular intravenous injection to prevent anticipated bleeding.[1] This treatment is called *primary prophylaxis* because it is initiated before the second clinically evident large joint bleed and before the age of 3 years. With secondary prophylaxis, regular treatment is started after two or more bleeds into large joints, but before the onset of evident “joint disease” (documented by physical examination and conventional radiography). Prophylactic factor replacement therapy started after the onset of overt “joint disease” is called *tertiary prophylaxis*. The optimum time to initiate prophylaxis, the optimum dosage, the frequency of injection, target factor levels, and

whether prophylaxis should be given indefinitely remain unclear.[115-118] Addressing these questions remains of utmost importance because prophylaxis is a highly expensive treatment.

Prophylactic factor VIII replacement with standard half-life products typically requires infusions two to three times a week, whereas factor IX can be given less frequently because of a longer half-life. The burden of regular venipunctures in young children is high. Therefore central venous access devices, such as Port-A-Cath and Hickman lines, are often inserted for reliable long-term venous access,[119] but come with the risk of infection and thrombosis.[120] Recently, extended half-life products entered the treatment landscape for hemophilia.[121] Improved bioavailability is achieved by PEGylation, sialylation, and fusion of the clotting factor to albumin or a fragment of an immunoglobulin. Especially for factor IX, prolongation of the half-life to up to 100 hours could be achieved, allowing substitution intervals of 1 to 2 weeks.

In patients with non-severe hemophilia A or in hemophilia A carriers, desmopressin, a synthetic analogue of vasopressin, can be administered to release endogenous factor VIII, von Willebrand factor, and tissue plasminogen activator from endothelial storage sites and possibly from platelets.[122,123] Since it is suggested that factor VIII levels of at least 30 U/dL are adequate for the treatment of spontaneous or posttraumatic bleeding and on average a threefold increase in factor VIII levels can be expected, a residual factor VIII activity of greater than 10% is required. Responsiveness to desmopressin should be tested prior to therapeutic treatment because it varies largely between patients. Desmopressin is contra-indicated in some patient groups, like young children, patients with cardiac comorbidity or epilepsy.

Antifibrinolytic therapy is useful to treat or prevent bleeds in areas of increased fibrinolysis, such as skin and mucosal surfaces (epistaxis, menorrhagia, or oral bleeding).[124] It promotes clot stability by inhibiting plasminogen activation in the fibrin clot. Tranexamic acid, the most commonly used antifibrinolytic, acts by reversibly binding to plasminogen and thereby blocking its activation and transformation to plasmin. Epsilon aminocaproic acid is similar to tranexamic acid but is less widely used because it has a shorter half-life, is less potent, and is more toxic.[125]

To improve hemophilia treatment, current research focuses on non-factor therapies (see paragraph 'inhibitors') and development of gene-based therapy.[126] Gene therapy aims at curing hemophilia by replacing the defective gene by a functional gene. In hemophilia B, expression of vector-derived factor IX levels have been achieved after adeno-associated viral (AAV) vector-mediated gene transfer to the liver. Nevertheless, only either therapeutic but short-lived circulation of factor IX or prolonged but suboptimal expression levels were established, resulting in improvement of the bleeding type without preventing spontaneous and trauma-induced bleeds.[127-131] In addition, high vector doses were accompanied by liver inflammation and hepatotoxicity, requiring transient immunosuppressive treatment with corticosteroids. Moreover, children, men with active hepatitis, and individuals who have pre-existing natural immunity to AAV were not eligible for this therapy. Recently, the transfer of low dose AAV vector expressing the factor IX Padua, a high-specific-activity factor IX variant, resulted in sustained and adequate factor levels, reduced risk of liver inflammation, termination of prophylaxis and the near elimination of bleeding and factor use in ten hemophilia patients.[128]

For a long time, the use of AAV vectors in gene therapy for hemophilia A was impeded by the large size and inefficient expression of the human factor VIII coding sequence. However, successful gene transfers in patients with hemophilia A have been reported, sustaining therapeutic levels one year after gene transfer.[132] No major side-effects were observed; hepatotoxicity did not occur and none of the participants developed neutralizing antibodies to factor VIII. Although these initial studies seem promising, a number of significant hurdles have yet to be overcome before gene therapy may become common practice.

Complications of Treatment

Inhibitors

Inhibitors are alloantibodies that neutralize clotting factor activity after infusion of factor VIII or factor IX, these antibodies are primarily of the IgG4 subclass. Risk of inhibitor development is highest in the first period of treatment, especially in the first 50 to 75 exposure days. Patients with severe hemophilia A have a 20% to 30% lifetime risk of inhibitor development, compared with a risk of 5% to 10% in individuals with moderate or mild disease.[133,134] Inhibitors are encountered much less frequently in individuals with hemophilia B, occurring in fewer than 5% of affected individuals.[135] Other risk factors are age and number of exposures to factor, family history of inhibitor development, ethnicity, the causative factor VIII genotype, polymorphisms in the immune system, and intensive factor replacement therapy related to surgery or trauma.[134,136] Development of factor IX inhibitors is associated with severe allergic or anaphylactic reactions to factor administration; however, these reactions are almost never encountered in patients with hemophilia A.[137] Inhibitor development can be suspected in patients who fail to respond to clotting factor infusion, especially if they were previously responsive. In patients with mild or moderate hemophilia, the presence of an inhibitor can result in conversion of the bleeding phenotype to a more severe form because the inhibitor may neutralize endogenously synthesized clotting factor. Patients with inhibitors are classified according to the inhibitor titer, expressed in Bethesda units. Patients with titers above five Bethesda units at any time are considered high responders and will show an increase in antibody titer after each exposure to clotting factor. In low responders, antibody titers are below five Bethesda units and do not increase after factor infusion. In these patients, the inhibitor may be transient and replacement therapy may be continued with minimal dose change.

Inhibitor eradication by immune tolerance induction (ITI) through frequent administration of clotting factor is successful in up to 70% of patients with hemophilia A and 30% of patients with hemophilia B.[138,139] ITI in patients with hemophilia B carries the risk of anaphylactic reactions and the development of nephrotic syndrome as a result of complex formation and deposition in the kidney. In patients with hemophilia A who have refractory disease, rituximab may be useful.[140,141] Other useful immunosuppressive treatments are high-dose prednisolone, cyclophosphamide, or azathioprine.[142]

Control of bleeding in patients with low-titer inhibitors could be achieved through high doses of factor concentrates or in patients with high-titer inhibitors with bypassing agents such as recombinant factor VIIa and (activated) prothrombin complex concentrates.[143-145] These

agents act by boosting thrombin generation independently of the presence of factor VIII. Another option is the recent developed porcine recombinant factor VIII, which is less susceptible to inactivation by inhibitory antibodies compared to human factor VIII due to its different structure.[146,147]
Improved understanding of the coagulation cascade has led to the development of new bypassing agents. The most advanced in clinical development is emicizumab, a bispecific antibody mimicking the function of activated factor VIII in the intrinsic tenase complex.[148] Emicizumab is approved for prophylactic use in hemophilia A patients with inhibitors, is administrated subcutaneously and is dosed once-weekly or every-other-week. Concomitant use of emicizumab and activated prothrombin complex concentrates may lead to serious adverse events like thrombotic microangiopathy and thromboembolism due to an excessive thrombin generation.[145,149] Other non-traditional products in clinical development include fitusiran, a RNA interference therapeutic that targets antithrombin, and antibodies directed against tissue factor pathway inhibitor.[150,151]

Viral Infections

The use of non–virus-inactivated plasma, cryoprecipitate, and plasma-derived factor concentrates from multiple human blood donations resulted in high mortality and morbidity as a result of transmission of blood-borne virus infections. More than 90% of patients with hemophilia who were treated with these products before 1985-1987 became infected with hepatitis C.[152-154] A chronic infection developed in the majority of these patients, and in 20% to 30%, it progressed to liver cirrhosis. The cumulative incidence of HIV infection in the United States in the peak years was 78% of the factor VIII recipients and 37% of the factor IX recipients.[155] Other viruses transmitted are hepatitis A, B, or G and cytomegalovirus.
Different measures are taken to ensure safety of plasma-derived factor concentrates, including selection of healthy donors, screening donations for the absence of relevant infectious blood-borne viruses, and screening for viral markers by serologic and nucleic acid testing. Additionally, various viral inactivation procedures are required, such as pasteurization, exposure to solvent-detergent, dry heat, chemical disruption with sodium thiocyanate, or ultrafiltration.[156,157] The introduction of recombinant products and the implementation of these measures almost completely eliminated HIV and hepatitis C virus transmission through factor concentrate.[158,159] Hepatitis A and B immunization is still recommended to reduce infection risk, although the risk of infection nowadays is low.[160]

Treatment of Musculoskeletal Complications

Comprehensive Care

Maintaining musculoskeletal health and preventing complications requires encouragement of physical activities to promote physical fitness and normal neuromuscular development.[161,162] For optimal management of musculoskeletal complications, multidisciplinary care is required. Because prevention is the cornerstone of maintaining musculoskeletal health, the care provided by hemophilia specialists is important to prevent and adequately treat bleeds and provide early recognition of musculoskeletal issues.

Physiotherapy is required for recovery after musculoskeletal bleeds and to maintain muscle strength in individuals with established hemophilic arthropathy. A physiatrist can help provide patient education to prevent disabling injuries, care during bleeding episodes, long-term rehabilitation, and orthotics and shoe adaptations.[163] In individuals with end-stage arthropathy, orthopedic surgery is often inevitable.

Acute Hemarthrosis

The primary aim of therapy for acute hemarthrosis is to stop the bleeding as quickly as possible by administering clotting factor concentrates. The efficacy of joint aspiration along with adequate factor replacement is unclear. Theoretically, shortening the exposure of cartilage to blood might limit blood-induced damage and might prevent future tissue damage.[164] One study on the long-term effects of joint aspiration combined with intra-articular steroids as treatment for acute hemarthrosis reported preservation of clinical joint health after 11 years of follow-up.[165] Nevertheless, more evidence is needed to determine the effect of joint aspiration on structural outcome in the long-term. Aspiration bears the risk of infection or repeat bleeds, and even when the joint is washed, some blood will always be left in the joint. Clearly, if the suspicion of infection is high, arthrocentesis is mandatory for diagnosis, but in general it should be avoided. According to the World Federation of Hemophilia guidelines, arthrocentesis may be considered in a bleeding, tense, and painful joint that shows no improvement 24 hours after conservative treatment and after exclusion of inhibitor development.[1] Arthrocentesis should be performed only after appropriate clotting factor replacement and under strictly aseptic conditions. In recurrent or massive bleeds that are unresponsive to appropriate clotting factor replacement, therapeutic embolization could be considered.

Pain relief could be achieved by analgesics, but PRICE (protection, rest, ice, compression, elevation) is also commonly recommended to decrease pain, inflammation, and bleeding. Immobilization and avoiding or minimizing weight bearing on the affected joint is important during active bleeding and might be helpful in protecting against cartilage damage.[166] The value of ice on bleedings is subject of debate due to the suggestion that it impairs coagulation.[167] However, hemophilia patients have reported beneficial effects of cryotherapy that include reduced pain and swelling without bleeding issues.[168] As soon as the swelling and pain subside, active rehabilitation is indicated to minimize muscle atrophy, prevent contractures, and regain functional ability.

Chronic Synovitis

In chronic synovitis the joint is vulnerable to repeated bleeding, and therefore the goal of treatment is to reduce synovial irritability. This goal might be achieved by (more intensive) prophylactic clotting factor concentrate replacement for 6 to 8 weeks combined with active physiotherapy and cooling. Use of cyclooxygenase (COX)-2 inhibitors may reduce inflammation.[169] Other NSAIDs should be avoided because of the possible impairment of platelet function. If conservative measures fail and chronic synovitis persists with frequent recurrent bleeding, synovectomy might be indicated either surgically, arthroscopically, or through intra-articular injection of radiopharmaceutical agents, or chemicals or platelet-rich plasma.[170] Radioisotopic synovectomy using a pure beta emitter (yttrium-90 or

phosphorus-32) is preferred because it is minimally invasive and requires little factor replacement and less intense rehabilitation afterward. This procedure is very successful in alleviating pain and reducing hemarthroses; 60% to 100% are reduced in the majority of patients.[171] If necessary, the procedure can be repeated after 6 months. The available safety data are reassuring, especially with regard to the risk of malignancy.[172,173] However, potential direct harmful effects to cartilage in the long term should be taken into consideration.[174] Chemical synovectomy using rifampicin or oxytetracycline chlorhydrate is painful and needs to be repeated weekly until the synovitis is controlled, but it is an appropriate alternative if a radioisotope is not available.[175,176] Surgical options are preferred in cases in which MRI reveals severe synovial hypertrophy or when bone cysts are present because they carry the risk of dispersing radioactive material outside the intra-articular space. Performing orthopedic surgery in hemophilic patients requires a large supply of clotting factor and intensive rehabilitation.[177]

Hemophilic Arthropathy

In individuals with established hemophilic arthropathy, the goal of treatment is to improve joint function, relieve pain, and assist with the continuation or resumption of normal activities of daily living. With use of conservative procedures, remarkable benefit can be achieved and surgical interventions can be postponed. For pain relief, adequate analgesic treatment, manual traction of the joint, and in some cases transcutaneous electrical nerve stimulation are indicated.[60,178] Physiotherapy is crucial for muscle strengthening and stretching, joint stability, and functional training. In patients with involvement of more than one joint, hydrotherapy enables functional training with minimal weight bearing. Orthotics and shoe adaptations provide immobilization, support, stability, compensation for deformities, and reduced weight bearing.[179,180] In individuals with advanced ankle arthropathy, immobilization with a cast can provide insight about whether arthrodesis would be helpful.

In individuals with end-stage arthropathy, orthopedic surgery is often indicated. Different surgical options could be considered depending on the joint or specific condition requiring correction. Joint replacement therapy of the knee and hip and less commonly of the elbow and shoulder and ankle arthrodesis are the most performed procedures. Joint replacement therapy is very successful in relieving pain and increasing functional activities and participation. Hip replacement usually improves ROM, whereas after knee replacement therapy, a restricted ROM may limit functional recovery.[181] Joint prosthesis have a limited life span and should be postponed as long as possible to decrease the risk for revision surgery. Ankle arthrodesis is very effective in diminishing pain and stopping recurrent joint bleeding. Drawbacks of arthrodesis are the loss of mobility of the joint and the possibility of overloading other joints of the lower limb/foot, necessitating surgical repair of other joints.[182,183] Total ankle replacement has the advantage of preserving motion with limited evidence showing a favorable clinical outcome in the long-term so far.[184] Nevertheless, higher rates of aseptic loosening and deep infections due to poor bone quality and increased risk of micro-bleeds at the prosthesis-bone interface are concerns.[184-186] In this respect, joint distraction with an external fixator is a new joint□preserving treatment option. This technique is initially successfully applied in OA[187], but seems also very promising in patients

with endstage hemophilic arthropathy of the ankle.[188,189] Patients show significant clinical and structural improvement for several years postponing the first prosthesis to an age it can last for life.
Other procedures performed in end-stage arthropathy or in case of severe contractures include soft tissue contracture release, arthroscopic débridement for intra-articular adhesions and impingement, tendon reconstruction, osteotomy to correct angular deformity, and radial head excision combined with synovectomy if enlargement and erosion of the radial head cause mechanical blockage of forearm rotation.
Major orthopedic procedures can be performed safely and successfully, even in patients with inhibitors.[190,191] Surgical interventions should always be performed at or in consultation with a comprehensive hemophilia treatment center.[191-193] Adequate quantities of clotting factor concentrates should be available, as well as blood bank support and laboratory facilities for reliable monitoring of clotting factor level and inhibitor testing. In patients who have arthropathy of more than one joint of the lower extremities, multiple procedures could be combined during one in-hospital stay, either in a single session or staged.[194] With this approach, reduced quantities of clotting factor concentrate are required and a faster rehabilitation is expected. Careful assessment by a multidisciplinary team is a prerequisite to ensure that recovery will not be compromised for any of the procedures.

Thesis outline

The mainstay of hemophilia management is the prevention of joint bleeding by clotting factor replacement therapy, although it is not possible to completely prevent hemarthroses and the consequent joint damage. Moreover, patients who are not compliant, do not have access to expensive clotting factor treatment, or develop inhibitors are also at risk of joint bleedings.
Detecting patients with joint damage in an early phase and understanding the early pathophysiological changes to identify possible targets for intervention in the harmful process of blood-induced joint damage, can contribute to reduce morbidity of end-stage HA in the long-term. The aim of this thesis is to add to the knowledge of the early pathophysiology of HA and to investigate a potential new treatment modality. For this purpose, a review of literature, a retrospective cohort study, several animal studies and an *in vitro* study were conducted.

Early identification

The severity of damage upon a joint bleeding differs between patients, as does the development of HA. Identifying patients susceptible to (fast) progressive joint damage may have therapeutic implications, especially early in the disease. Imaging techniques lack the ability to detect early changes and the information provided is static. Biochemical markers of joint tissue reflect dynamic changes in tissue turnover and detect changes in an early phase. In **chapter 2** a cohort of hemophilia patients is described investigating the predictive value of biomarkers on radiographic progression of HA.

Early pathophysiological changes

The pathophysiology of HA, and in particular the earliest changes induced by acute hemarthrosis, are not completely understood. Investigating these processes in hemophilic patients is challenging, since evaluation mainly relies on indirect parameters, the early stage of arthropathy often proceeds asymptomatically, and takes years to become clinically manifest. Rodent models have proven indispensable for studying blood-induced joint damage, because joint bleed induction as the initiation of this process can be controlled. Moreover, early changes can be studied directly at joint tissue level. However, the current models also have clear limitations. **Chapter 3** provides a general discussion of the value of animal models in studying hemophilic arthropathy. Subsequently, new parameters to improve established hemophilic animal models were studied in chapter 4 and 5. In **chapter 4**, the value of the biochemically determined proteoglycan synthesis rate is investigated in a hemophilic rat model to evaluate early blood-induced cartilage degeneration. In **chapter 5**, the often used hemophilic mouse model is refined by studying the patella in addition to the tibiofemoral compartment and by comparing single versus double joint bleed induction.

Early intervention

Early intervention in the devastating process of HA is highly desirable, but no disease modifying therapy is currently available. In **chapter 6** potential targets for treatment are identified based on pathophysiological mechanisms. Considering the pivotal role of iron and its interplay with inflammation in the process of blood-induced joint damage, iron chelation is considered a promising therapeutic approach. Prophylactic treatment with the iron chelator deferasirox (DFX) was shown earlier to attenuate cartilage damage upon blood exposure in hemophilic mice.[199] However, in hemophilia patients this approach is not opportune given the unpredictable occurrence of hemarthroses. Therefore, the effectiveness of on-demand DFX treatment, initiated immediately after joint bleed induction in hemophilic mice is evaluated in **chapter 7**. In **chapter 8** the possible immunomodulatory effect of DFX is further explored in an *in vitro* study using human tissue.
Chapter 9 summarizes and integrates the chapters of this thesis. The main findings are placed in a broader perspective and implications for future research and patient care are deliberated on.

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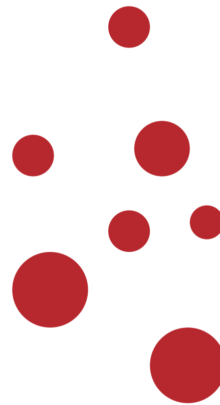
PART I

EARLY IDENTIFICATION



The combination of urinary CTX-II and serum CS-846: Promising biochemical markers to predict radiographic progression of hemophilic arthropathy

An exploratory study

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Introduction

In hemophilia patients, recurrent hemarthroses result in hemophilic arthropathy (HA), causing major morbidity. In general, the bleeding tendency is inversely correlated with the clotting factor level, so HA is mainly seen in patients with severe hemophilia. The severity of damage upon a joint bleeding differs between patients, as does the development of HA. It would be of great value to identify those patients susceptible to (fast) progressive joint damage as this might have therapeutic implications, especially early in the disease. Currently, imaging techniques such as plain radiography, ultrasonography and magnetic resonance imaging are used to monitor joint damage. The information obtained is static, providing the cumulative result of a dynamic process over time. Biochemical markers, measured in blood or urine, reflect dynamic changes in joint tissue turnover at a certain point in time. Although promising in theory, research has thus far not yielded a (set of) biochemical marker(s) with sufficient prognostic value for joint damage progression over time at the level of an individual patient. A previous study conducted by our group indicated that levels of the biomarkers uCTX-II (urinary C-terminal telopeptide of type II collagen), sC1,2C (serum cartilage cleavage product C1,2C) and sCS-846 (serum chondroitin sulphate 846) correlated with overall joint damage using the Pettersson score,[1] and with the joint space narrowing component specifically, reflecting cartilage thinning.[2] The combination of uCTX-II, sCOMP (serum cartilage oligomeric matrix protein) and sCS-846 correlated best with the degree of arthropathy. In an additional study in hemophilia patients, it was demonstrated that uCTX-II and sCS-846 increased shortly after a joint bleeding, supporting their dynamic value.[3] uCTX-II is a biomarker of type II collagen degradation considered representative for cartilage degradation,[4] as well as bone metabolism.[5] sCS-846 is an aggrecan synthesis marker.[6] In rheumatoid arthritis (RA) and osteoarthritis (OA), joint diseases sharing characteristics with HA, these two biomarkers were associated with radiographic progression.[7,8] Unfortunately, these findings on group level do not yield diagnostic or prognostic value for an individual hemophilia patient. Considering the responsiveness of uCTX-II and sCS-846 to a joint bleeding -the clear trigger to develop HA- and the results in RA and OA, the potential prognostic value of these biochemical markers was explored in this present study.

Methods

This study comprises a follow-up on our previous cross-sectional study,[2] in which eight biomarkers including uCTX-II and sCS-846, were measured in 36 hemophilia patients with various degrees of HA. Radiographs of the ankles, knees and elbows were evaluated for the degree of joint damage using the Pettersson score. As part of routine care follow-up radiographs were made every five years. Pettersson scores on both time points were assessed by a trained observer, in a blinded manner.[9] The progression rate per year was calculated by dividing the delta Pettersson score by the follow-up time in years. Fast progression of HA was defined as ≥0.4 points per year, based on the mean progression rate in a larger cohort of hemophilia patients treated in our clinic.[10] Natural log transformation of the biomarker values was performed to achieve a normal distribution. In order to give

the same weight to each biomarker in the combined score of uCTX II and sCS-846, the mean level of the biomarker at group level was calculated. For each patient, the ratio of the individual value to that mean value was determined. The combined score consisted of the mean of these ratios in each patient. To explore differences between slow and fast progressors logistic regression was performed.

Results

Of the original 36 patients, follow-up was available in 34 patients (lost to follow-up n=1, deceased n=1). Three patients were excluded due to absence of follow-up radiographs (n=1) or a Pettersson score of ≥60 points at baseline (n=2) as the high baseline score limits progression towards the maximum score of 78. The majority of patients had hemophilia A (87.1%, n=27) and had the severe type (90.3%, n=28) (Table 1). The mean age at inclusion was 35.1 years (SD ±10.1), the median delta Pettersson score was 1.0 points (IQR 0.0-2.0) and the mean follow-up was 6.5 years (range 3.9-9.7) (Table 1). The median progression rate was 0.2 points/year (IQR 0.0-0.3). Seven out of 31 (22.6%) patients showed progression of ≥0.4 points/year and were as such indicated as fast progressors. Age and degree of HA at baseline were comparable between the slow and fast progressors. All moderate patients progressed slowly.

Table 1 – Patient characteristics and results of follow-up

Type of hemophilia n (%)	Hemophilia A: 27 (87.1) Hemophilia B: 4 (12.9)
Severity n (%)	Moderate: 3 (9.7) Severe: 28 (90.3)
Age at inclusion mean (SD)	35.1 years (± 10.1)
PS at baseline median (IQR)	18.0 (6.0-36.0)
ΔPS median (IQR)	1.0 (0.0-2.0)
Follow-up mean (SD)	6.5 years (± 1.4)
Progression PS median (IQR)	0.2 points/year (0.0-0.3)
Progression >0.4 points/year n (%)	7 (22.6)

SD = standard deviation, IQR = interquartile range, PS = Pettersson score

Neither of the individual biomarkers measured at baseline predicted the presence of progression as determined by logistic regression. However, the combined index of uCTX-II and sCS-846 was significantly associated with joint damage progression (OR 8.8, 95% confidence interval (CI): 1.1-70.6, p=0.04) The discriminative ability of the prediction model

of the combined index is fair with an area under the curve (AUC) of 0.77 (95% CI: 0.60-0.95) (Figure 1A). The combined index differed significantly between slow and fast progressors (median -0.095 vs 0.33, p= 0.03; Figure 1B).

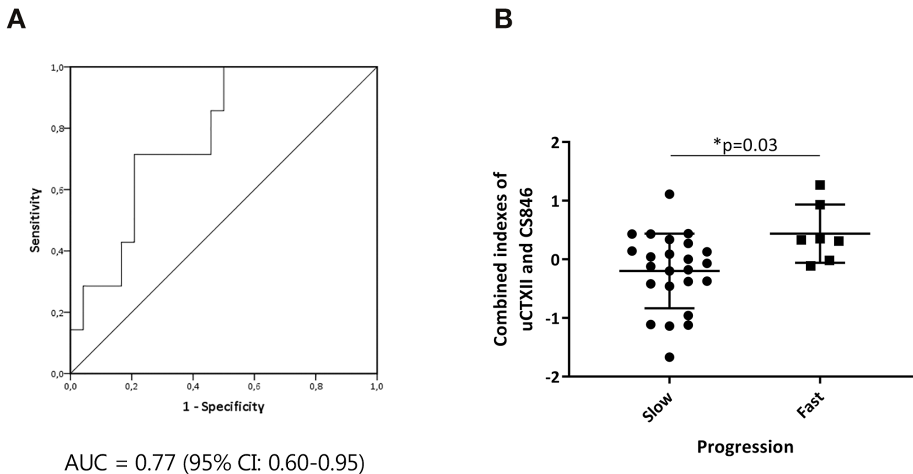

Figure 1 - Prognostic value of the combined index of uCTX-II and sCS-846
A - Area under the receiver operating characteristic curve (AUC) of the prediction model with the combined index uCTX-II and sCS-846. B - Results of the combined index uCTX-II and sCS-846 classified by presence/ absence of progression. Differences between slow and fast progressors tested by using the Mann-Whitney test.

Discussion

This exploratory study, in predominantly patients with severe hemophilia, demonstrates that the combination of uCTX-II and sCS-846 has the potential to predict radiographic progression of joint damage in HA. This finding is in line with results in RA[7] and OA[8]. Interestingly, neither of the individual biomarkers by itself has a prognostic value. This could be a reflection of the complexity of different processes contributing to joint damage after blood exposure. The combined index contains biochemical markers representing both degradation (uCTX-II) and synthesis (sCS-846). Moreover, the biomarkers are derived from the different cartilage matrix components collagens (uCTX-II) and proteoglycans (sCS-846), whereas uCTX-II might also reflect changes in bone metabolism.[5] This study is limited by its retrospective study design and small number of patients. The main drawback of a retrospective study is confounding bias, although the group of slow and fast progressors did not differ in terms of age or degree of HA at baseline. In addition, due to the lack of a control group, the biomarker levels cannot be compared to the natural variation in a healthy population. Due to the small number of patients indicated as a fast progressor, multivariate analysis could not be performed. We hypothesize that the fact that the biomarkers can still predict progression of HA is because they indicate a certain sensitivity to damage. The

biochemical markers are measured in the absence of a joint bleeding in the previous three months. The level of the biochemical markers is therefore not influenced by an acute phase following hemarthrosis, but represents the ongoing chronic processes in the joint. The combination of uCTX-II and sCS-846 discriminates between fast and slow progressors regardless of the number of joint bleedings. To date, the number of joint bleedings experienced in the past is leading in choice of treatment strategy to prevent future bleedings and development of HA. In this study, the number of joint bleedings during follow-up could not be taken into account. However, the value of self-reported joint bleedings is under debate. Nijdam et al. demonstrated that patients who stopped prophylactic treatment showed a significant greater increase in HA after 10 years compared to those who continued treatment, despite equal self-reported bleeding rates.[11] As such, self-reported bleeding rates might not be a useful tool to predict joint damage progression and thus to guide treatment. Our present study shows potential for biochemical markers in this area.

Conclusion

In conclusion, the combination of uCTX-II and sCS-846 measured in the absence of a joint bleeding is promising to predict progression of HA over time. This finding justifies studying the prognostic value of these biomarkers in a larger prospective cohort of hemophilia patients. To improve translation into clinical practice, this study should be designed to provide a threshold of the combination of uCTX-II and sCS-846 above which progression of HA is more likely to occur. If the effect is consistent, patients could benefit from a parameter indicating an increased risk of developing HA as guidance in adjusting treatment regimens.

Acknowledgements
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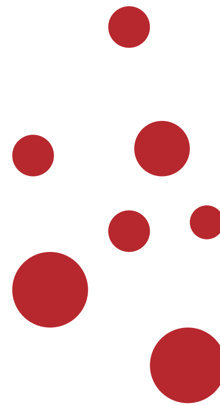
PART II

EARLY PATHOPHYSIOLOGICAL CHANGES



Models of arthropathy: what can we learn from them to improve patient's care?

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Editorial commentary

Blood-induced joint damage as seen in hemophilic arthropathy (HA) is a whole joint disease like osteoarthritis (OA) and rheumatoid arthritis (RA). It is characterized by synovial inflammation, cartilage degradation and bone changes, subsequently accompanied by muscle and ligament impairment.[1,2] Pathological processes in these tissues show differences between the aforementioned diseases in terms of their sequence, severity, relative contribution, and mutual relations.[3] HA is induced, maintained and aggravated by a clear trigger: joint bleeds. This is in contrast to RA and OA, in which the initiation is unknown and several factors contribute to the development of the disease. The process of joint deterioration in HA is considered the most aggressive and has the highest progression rate. Joint damage can be seen at a relatively young age, despite improving treatment modalities to prevent joint bleeds. Contrarily, RA and OA mostly develop later in life and, specifically the latter, progress more gradually. On the other hand, there are several similarities between the arthropathies at tissue level and knowledge from either of these diseases may add to understanding of the others.[3] The defined trigger, onset and progression rate of HA may be seen as an advantage over the other two arthropathies when it comes to identifying relevant markers (imaging and biochemical) of disease onset, progression, and treatment efficacy. In a faster progressing disease, relevant biochemical markers may be more easily detected than in slowly progressing disease.[4,5]

As in the OA and RA field, animal models are used in HA to generate new insights into the pathogenesis and to study novel treatment options. The question is: do these *in vivo* models represent the human situation and do they provide us tools to improve our understanding of human pathology? In this issue of Rheumatology Christensen et al[6] describe in detail and robustly the joint changes observed during the first week after an induced hemarthrosis in a hemophilic rat model. They investigated the impact of a joint bleed on the sequence of changes in synovium, cartilage and bone by ultrasonography, μCT scanning, histology and immunohistochemistry. Within 24 hours, synovitis develops with neutrophil and macrophage infiltration dominating T- and B-cell activity, underlying the function of synovial tissue to remove debris including blood remnants from the joint cavity, and showing a clear difference from the RA inflammatory process where T- and B-cell activity dominates.[7] Damage to cartilage and bone is detected as early as 48-96h after the bleed, with both processes developing in parallel. This finding demonstrates the direct, synovial-independent, impact of blood exposure on these tissues, known to be phenotypes of OA (inflammation driven and intrinsic cartilage driven) as well.[2] Significant and extensive bone changes are reported with the coexistence of osteoclasts and macrophages.

In their detailed study, the authors successfully mapped development from hemarthrosis to HA in their specific animal model. The developed arthropathy resembles the characteristics of severe human HA, though there are differences that require some restraint in the interpretation of the results. The assumption that their findings are representative of human HA has not been substantiated in full.
The early and extensive bone formation following a single bleed demonstrated in this rat model is unlike the human situation, but in line with the bone changes seen in other

hemophilic animal models.[8,9] In hemophilia patients, repeated intra-articular bleeding ultimately causes osteophyte formation, osteoporosis, subchondral irregularity as also seen in OA, and epiphyseal enlargement when bleeds occur before closure of the growth plate. The exact mechanism by which joint bleeds cause bone damage is largely unknown. A remarkable observation is the co-localization of tartrate-resistant acid phosphate (TRAP) activity with CD68-positive cells in areas loaded with hemosiderin or erythrocytes. This study describes the coexistence for the first time in HA, but this is reported for RA as well.[10]
Another difference between animal models of HA and the human situation is the immediate treatment and rest in the latter. In small animal models of hemophilic arthropathy, a joint bleed is induced by needle puncture, causing both intra- and extra-articular bleeding. Clotting factor replacement therapy is not performed and as such a major bleed is caused. These factors, in combination with differences in bone metabolism, higher turnover of cartilage, thicker layer of calcified cartilage and differences in biomechanics in small animals underline the importance of caution in translating findings from small animal models to the human situation.
As such the authors' statement that their rat model has a high degree of face validity and the progression of the human disease likely follows a similar albeit accelerated process, still needs further proof. Nevertheless, knowledge from the model might be of help for interpreting human disease.

It remains a challenge to translate these observations into diagnostic possibilities to detect preclinical pathology. The knowledge on sequences of events in each of the tissues is important, although the relatively unclear causative interactions between synovium, cartilage, and bone remain crucial in the pathogenesis of HA. Insight in the interaction and sequence of these processes might guide the development of early detection and targeted therapies at a more personalized patient-level to prevent (progression of) joint damage; topics of relevance to RA and OA as well. Development of biochemical and imaging markers from these animal models can be validated in the quickly progressive human HA population. The most robust ones may be validated for OA and RA subsequently.

To conclude, the study of Christensen et al[6] on HA in a rat model shows that synovial inflammation, cartilage degeneration, and bone remodeling occur immediately and in parallel upon a joint bleed. This suggests that blood directly affects each joint component, although the causative interaction between the tissues remains a subject for further research. Findings from this model will increase our knowledge on the pathological events following hemarthrosis in humans and may provide potential clinically applicable biomarkers for detection of relevant processes early in the disease. Findings may be of direct relevance for translation to understanding joint damage in other disease like OA and RA.

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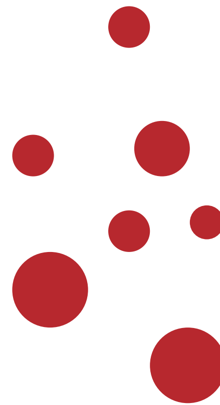

Proteoglycan synthesis rate as a novel method to measure blood-induced cartilage degeneration in non-hemophilic and hemophilic rats

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Abstract

Introduction

Hemophilic animal models are used to study blood-induced cartilage damage, but quantitative and sensitive outcome measures are needed.

Aim

To develop a novel quantitative method for detecting early cartilage degeneration in a hemophilic rat model of blood-induced joint damage.

Methods

The ^{35}Sulphate incorporation ($^{35}SO_4^{2-}$-assay) was applied to tibial and patellar cartilage of wild-type rats to quantify baseline proteoglycan synthesis and to evaluate the effect of 4 days blood-exposure *in vitro*. Next, hemarthrosis was induced in 39 FVIII-deficient rats and characterised by changes in knee joint diameter and development of bone pathology (using micro-CT). Four and 16 days post-hemarthrosis proteoglycan synthesis rate (PSR) was assessed using the $^{35}SO_4^{2-}$-assay, with the contralateral knee as control.

Results

In vitro, a decrease in PSR in tibial and patellar cartilage was demonstrated following blood-exposure. *In vivo*, joint diameter and development of bone pathology confirmed successful induction of hemarthrosis. In the blood-exposed knee, tibial and patellar PSR was inhibited 4 and 16 days after induced hemarthrosis. Interestingly, at day 16 the proteoglycan synthesis in the contralateral knee was also inhibited to an extent correlating with that of the blood-exposed knee.

Conclusion

For the first time, early changes in cartilage matrix synthesis upon blood exposure were quantified with the $^{35}SO_4^{2-}$-assay in a hemophilic rat model, establishing this assay as a novel method to study blood-induced cartilage damage.

Introduction

Joint damage upon bleeding causes significant morbidity in patients with hemophilia[1], and adds to joint degeneration after trauma[2] and major joint surgery.[3] While the synovial inflammatory response following a single joint bleeding is considered transient,[4,5] the damaging effect on cartilage is prolonged and irreversible.[4,6-] Even short-term blood exposure leads to impairment of cartilage matrix turnover due to chondrocyte apoptosis,[7,9] causing loss of proteoglycans that over time may lead to clinically evident joint damage.
Given the irreversible and severe consequences of blood exposure, targeted therapy to intervene in the process of blood-induced cartilage damage is crucial but lacking. A better understanding of the pathophysiological mechanisms, and in particular the earliest changes induced by hemarthrosis, may provide new insights to identify novel treatment options.[10] Investigating these processes in hemophilic patients is challenging, since the early stage of arthropathy often proceeds asymptomatically and evaluation mainly relies on indirect parameters such as imaging[11] and systemic biochemical markers.[12,13] Consequently, rodent models have proven indispensable for studying blood-induced joint damage, displaying translatable pathological changes in the tibiofemoral and patellar compartment following an induced hemarthrosis.[14,15] Cartilage damage is especially pronounced in the factor VIII deficient ($F8^{-/-}$) rat,[14] in which apoptotic chondrocytes and loss of proteoglycans were identified histologically within days following an induced hemarthrosis.[9] However, histological evaluation is time consuming, less sensitive for subtle (early) changes, and results are subject to interpretation despite initiatives to harmonize semi-quantitative scores.
To improve the evaluation of early blood-induced cartilage damage in the $F8^{-/-}$ rat model, a quantitative outcome parameter able to detect the very early cellular changes is needed. Blood exposure results in chondrocyte apoptosis and induces permanent disturbances in cartilage matrix turnover, causing loss of proteoglycans essential for resilience of cartilage tissue.[22] Determining the proteoglycan synthesis rate (PSR), by incorporation of radioactive ^{35}Sulphate in cartilage ($^{35}SO_4^{2-}$-assay), is an eligible method to detect early signs of cartilage degeneration[16] and is measurable before histological alterations manifest.[17]
Although not commonly used, in rodent models of other degenerative joint diseases, the $^{35}SO_4^{2-}$-assay has been used to measure proteoglycan synthesis in cartilage from the tibia[18,19] and the patella.[20,21] Moreover, this method has previously been applied in human cartilage explants and larger animal models to evaluate blood-induced cartilage damage,[8,22] but has not been used in a hemophilic rodent model before.
The aim of this study was to develop a novel method to detect early cartilage degeneration in a hemophilic rat model of joint damage by experimentally inducing a joint bleed. Therefore, the $^{35}SO_4^{2-}$-assay was first applied to tibial and patellar cartilage of wild-type (WT) rats and the effect of *in vitro* blood exposure was evaluated. Secondly, an *in vivo* study in hemophilic rats was conducted to evaluate the effect of an induced hemarthrosis on the PSR in the short (after 4 days) and longer (after 16 days) term.

Materials and Methods

Study design

Healthy cartilage from WT rats was used for *ex vivo* studies to determine baseline PSR and to measure the *in vitro* effect of 4 days blood exposure (Table 1). The effect of blood exposure *in vivo* was measured in $F8^{-/-}$ rats 4 and 16 days after induction of a single hemarthrosis. After euthanasia, the hind legs were transported to the University Medical Center Utrecht (UMCU, the Netherlands), and cartilage proteoglycan synthesis was assessed within 24 hours of euthanasia. All *ex vivo* and *in vitro* studies were performed at the UMCU. *In vivo* studies were conducted in $F8^{+/-}$ rats at Novo Nordisk A/S (Maaloev, Denmark). Experiments were approved by the Danish Animal Experiments Committee under the Danish Ministry of Environment and Food, as well as the Novo Nordisk Animal Welfare Body. All procedures were performed according to the Danish Animal Experimentation Act and the EU Directive 2010/63/EU. In all studies rats of both genders and aged approximately three months were included.

Table 1 – Overview experiments

Group	Rats	n	Joint bleed induction	Euthanasia DK	µCT	Euthanasia NL	Culture blood -/+	PSR
Baseline	WT	6				Day 0		Day 1
***In vitro* culture**	WT	9				Day 0	Day 1	Day 5
4-day group	F8-/-	14	Day -4	Day 0	Day 0			Day 1
16-day group	F8-/-	18	Day -16	Day 0	Day 0			Day 1

— performed at Novo Nordisk, Denmark (DK) — performed at UMCU, the Netherlands (NL)
PSR = proteoglycan synthesis rate

Cartilage isolation

Keeping the knee flexed at 90 degrees, the patella tendon was cut at the level of the joint space and removed including the patella. Subsequently, the lateral, medial and posterior boundaries of the joint cavity were cleaved, thereby disengaging the femur and tibia. The tibia was fixated in a bench vice to slice full thickness cartilage fragments from the tibial plateau by use of a scalpel (surgical blade size 15; Swann Morton). A maximum of two slices were combined to represent the tibial cartilage of a single joint and a weight of more than 0.8 mg was considered sufficient for further analysis. The weight of the patellar cartilage was not measured due to the interference of the attached tissue. Instead, equally sized samples were provided by punching the central part of the patella using the cannula of a bone marrow biopsy set (T-Lok Bone Marrow Biopsy Needle, 8Gx4", Argon Medical Devices). Next, the cartilage explants from tibia and patella were separately transferred to culture medium in a 96-wells round-bottomed microtiter plate. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco), glutamine (2mM), penicillin (100IU/mL), streptomycin sulphate (100µg/mL; all Paisley, UK), and ascorbic acid (85µM; Sigma).

Determination of proteoglycan synthesis rate

Proteoglycan synthesis in cartilage explants was determined *ex vivo* or after *in vitro* culture by adding 4μCi $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) to the explants for 4 hours during which radioactive labelled sulphate is incorporated in newly synthesized proteoglycans. After 4 hours of pulse labelling, the explants were washed twice in cold phosphate buffered saline. The patellae were decalcified in 1ml 0.5M ethylenediaminetetraacetic acid (EDTA) overnight. Subsequently, all samples were digested for two hours at 65°C with 2% papain (Sigma), and stored at -20°C. Next, the glycosaminoglycans (GAGs) were precipitated by 0.3M hexadecylpyridinium chloride monohydrate (CPC; Sigma) in 0.2M NaCl and dissolved in 3M NaCl. The amount of radioactivity was measured by liquid scintillation analysis and normalized to the specific activity of the pulse medium, labelling time, and cartilage weight in case of the tibial explants. As a measure of cartilage matrix PSR, sulphate incorporation rate is expressed as nanomoles of sulphate incorporated per gram weight of tibial cartilage tissue (nmol/h.g) and as nanomoles of sulphate incorporation (pmol/h) per patella.

Baseline proteoglycan synthesis in WT rats

Tibial and patellar cartilage from WT rats (3 males, 3 females; Sprague Dawley, Taconic, Lille Skensved, Denmark) not subjected to an induced hemarthrosis (n=6) was obtained *post mortem* and used to determine baseline PSR.

Proteoglycan synthesis in WT rats after 4 day blood exposure in vitro

Patellar and tibial cartilage from 9 surplus WT rats (all females, Sprague Dawley, bred at UMCU, The Netherlands) were obtained and cultured in culture medium for 4 days in a 96-wells round-bottomed microtiter plate at 5% CO2 in air, 37°C, and 95% humidity. Patellar (n=5) and tibial (n=4) explants obtained from the left knee were cultured in the presence of 50% volume/volume (v/v) whole blood, drawn in a BD vacutainer heparin tube from a healthy WT rat, to mimic a joint bleed.[22] The samples derived from the contralateral knee were cultured in medium only. After 4 days of culturing, the explants were washed by two 20-minute incubations in culture medium to remove blood components, and the PSR was determined, as described above.

Assessment of cartilage degeneration in a hemophilic rat model of joint damage by experimentally inducing a joint bleed

The *in vivo* study included 39 $F8^{-/-}$ rats (Sage: SD-$F8^{tm1sage}$; 18 males, 21 females) on a Sprague-Dawley background, bred at Novo Nordisk A/S (Maaloev, Denmark). As the study was exploratory, the number of animals used considered that up to 20% of the rats could be euthanised due to humane endpoints, and that sufficient cartilage isolation could be unsuccessful in up to 20% of the knee joints. The rats were housed and monitored daily as detailed in Supporting Information. All invasive procedures were performed under inhalation anaesthesia (5% isoflurane, 0.7L/min O_2, 0.3L/min N_2O for induction; 2% isoflurane, 0.7 L/min O_2, 0.3L/min N_2O for maintenance).

In brief, anaesthetised rats received a subcutaneous (SC) dose of buprenorphine analgesia (0.05mg/kg, Temgesic, Indivior UK Limited, Berkshire, United Kingdom) and hemarthrosis was induced by needle-puncture as previously described[14,15] and detailed in Supporting

Information. To confirm successful induction of hemarthrosis, joint swelling was characterized by measuring the knee diameters before induction and 24 hours after induction, as previously described.[23] At both time points, the diameter of each knee joint was measured five times with a digital calliper (Mitutoyo Corporation, Kawasaki, Japan). The delta diameter was calculated as the difference of the mean diameter of the blood-exposed knee minus the mean diameter of the contralateral knee.

Four ('4-day group') and 16 ('16-day group') days after induction of hemarthrosis, animals were euthanised by intracardial injection of 1ml pentobarbital (Mebumal, SAD, Amgros I/S, Copenhagen, Denmark, 50mg/ml) while in general anaesthesia.

Knees were excised *post mortem* and subsequently imaged with micro-computed tomography (micro-CT, Quantum FX, Perkin Elmer, Waltham, MA, USA) at field of view 20mm, 90kV, 160μA with 4.5 minutes acquisition time. Micro-CT scans were blindly evaluated for pathological bone remodelling with Quantum FX 2.2 (Perkin Elmer, Waltham, MA, USA) by reviewing 2D images in the coronal, sagittal, and transverse plane. Each scan received a score from 0-7 based on the presence/absence of seven pathological bone changes: osteophytosis (femur, patella, tibia), periosteal bone remodelling (femur, patella, tibia) and/or subchondral cyst, as previously described[24].

Following micro-CT imaging, the hind legs including the skin were stored in a 50mL tube in a foam box at a constant temperature of approximately 4ºC and transported to UMCU by airplane. Within 24 hours of euthanasia, tibial and patellar cartilage from both knees was obtained and the PSR measured *ex vivo*, as described above.

Statistical analysis

Differences in PSR, joint diameter, and micro-CT scores between paired samples (left and right knee of the same animal) were analysed using the paired t-test or the Wilcoxon signed rank test, when appropriate. Differences in PSR between groups were analysed using the Mann-Whitney test. Correlation between the level of PSR in the blood-exposed and contralateral knee was analysed using the Pearson correlation coefficient. Results were considered significant if $p<0.05$. Graphic presentation and statistical analyses were performed using GraphPad Prism (Version 8.0.1; GraphPad Software Inc, San Diego, CA, USA).

Results

Blood exposure decreases PSR in tibial and patellar cartilage of WT rats in vitro

First, cartilage of healthy bleeding-naïve control animals (n=12 knees) was obtained to establish baseline PSR directly after isolation. On average, a total of 1.2mg ± 0.4 (mean ± standard deviation (SD)) tibial cartilage per joint was obtained. Tibial and patellar baseline PSR was 12.9 nmol/h.g±8.4 and 22.3 pmol/h±5.1, respectively.

Next, the effect of blood exposure on cartilage was evaluated *in vitro.* After 4 days of culturing *in vitro* the PSR of healthy tibial cartilage (n=4) was 31.2 nmol/h.g±7.1, compared to 3.1 nmol/h.g±0.3 upon blood exposure, corresponding to a 90% decrease (Figure 1A, p=0.004). In patellar cartilage (n=5), the PSR was decreased by 81% (Figure 1B, healthy (10.7 pmol/h±3.3) *versus* blood-exposed cartilage (2.1 pmol/h±2.0), p=0.004).

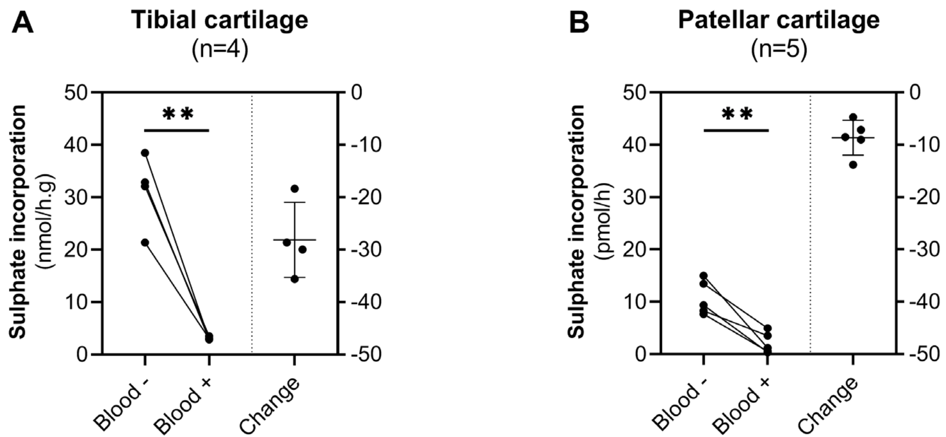

Figure 1 – Proteoglycan synthesis after four day culture in vitro in wild type rats
Four day blood exposure *in vitro* resulted in a significant decrease in sulphate incorporation rate in both tibial (A, n=4; expressed as nmol of sulphate incorporated per hour per gram of tissue) and patellar (B, n=5; expressed as pmol of sulphate incorporated per hour) cartilage, resulting in a negative change (blood-exposed knee minus contralateral knee, depicted as mean ± standard deviation). Paired t-test for the difference in proteoglycan synthesis between cartilage exposed to blood (blood +) or culture medium only (blood -), ** p=<0.01.

Induction of hemarthrosis causes joint swelling and pathological bone remodelling in F8$^{-/-}$ rats

In total, 32 out of 39 animals subjected to hemarthrosis completed the study (4-day group n=14, 16-day group n=18). Successful induction of hemarthrosis was confirmed by measuring the diameter of the knees; the day after induction, the diameter of the injured knee was significantly increased compared to the contralateral side (4-day group: median 2.78mm vs -0.02mm, p<0.001; 16-day group, median 2.65mm vs -0.02mm, p<0.001, Figure 2).
After euthanasia, blood-induced joint damage was confirmed by scoring the knees for presence of pathological bone remodelling on micro-CT (Figure 3A). The blood-exposed knees had a significantly higher bone pathology score than the contralateral knees in the 16-day group (median score of 5 vs 0, p=<0.001), but no difference in the bone pathology score was observed between the injured and contralateral knee in the 4-day group (median score of 0 vs 0, p=0.22). In the 4-day group, 3 out of 14 rats developed bone pathology in the blood-exposed knee (score 1-2), whereas in the 16-day group, 13 out of 18 rats developed bone pathology in the blood-exposed knee (score 3-7, Figure 3B). In the latter group, one contralateral knee had a score of 1. Bone changes were equally distributed between the femur, patella, and tibia (data not shown).

Hemarthrosis results in decreased tibial PSR

Equal amounts of tibial cartilage were obtained from the contralateral and blood-exposed knees (4-day group: 1.4mg ± 0.6 vs 1.4mg ± 0.6, 16-day group: 1.3mg ± 0.6 vs 1.5mg ± 0.4). Samples not meeting the minimum weight were excluded (4-day group n=1/14, 16-day group n=4/18).

Four days after induction of hemarthrosis, the PSR of tibial cartilage from the contralateral knee was 5.1 nmol/h.g (4.0-16.7) (median with interquartile range (IQR)), compared to 2.5 nmol/h.g (1.5-8.1) in the blood-exposed knee (Figure 4A). This corresponds to a significant decrease of 52% in PSR of the blood-exposed knee compared to the contralateral knee (p=0.007) and 81% compared to bleeding-naïve control animals (p=0.005). One statistical outlier was excluded from this analysis.

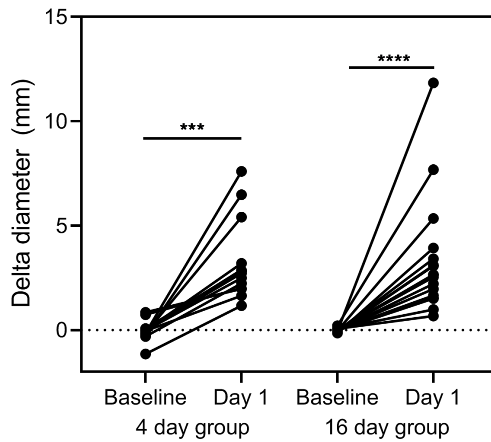

Figure 2 – joint swelling confirms induction of hemarthrosis in F8$^{-/-}$ rats
The diameter of the induced and contralateral knee was measured before and one day after induction of hemarthrosis. The difference between the injured and contralateral knee, the delta diameter, increased significantly on Day 1 in both the group euthanised 4 and 16 days after injury. ***p<0.001, ****p<0.0001, Wilcoxon signed rank test.

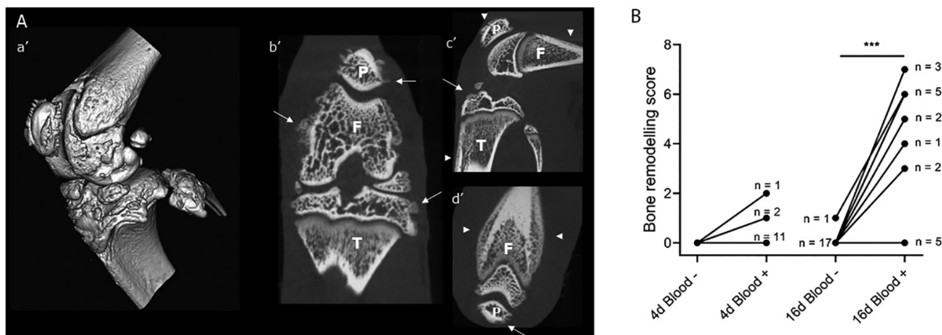

Figure 3 – Bone pathology after induced hemarthrosis
Knee-injured F8-/- rats were euthanized 4 or 16 days (d) after injury, and pathological bone remodelling scored from 0-7. A: Injured knee of F8-/- rat displaying extensive bone remodelling. a′: 3D volume of the injured knee. b′, c′, d′: Coronal (b′), sagittal (c′) and transverse section of the injured knee showing osteophytosis (arrows) and periosteal bone remodelling (arrowheads) on femur (F), patella (P) and tibia (T). B: Bone remodelling score was significantly increased in the injured (blood +) vs contralateral (blood -) knees of F8-/- rats euthanized 16 days after injury. ***p<0.001, Wilcoxon signed rank test.

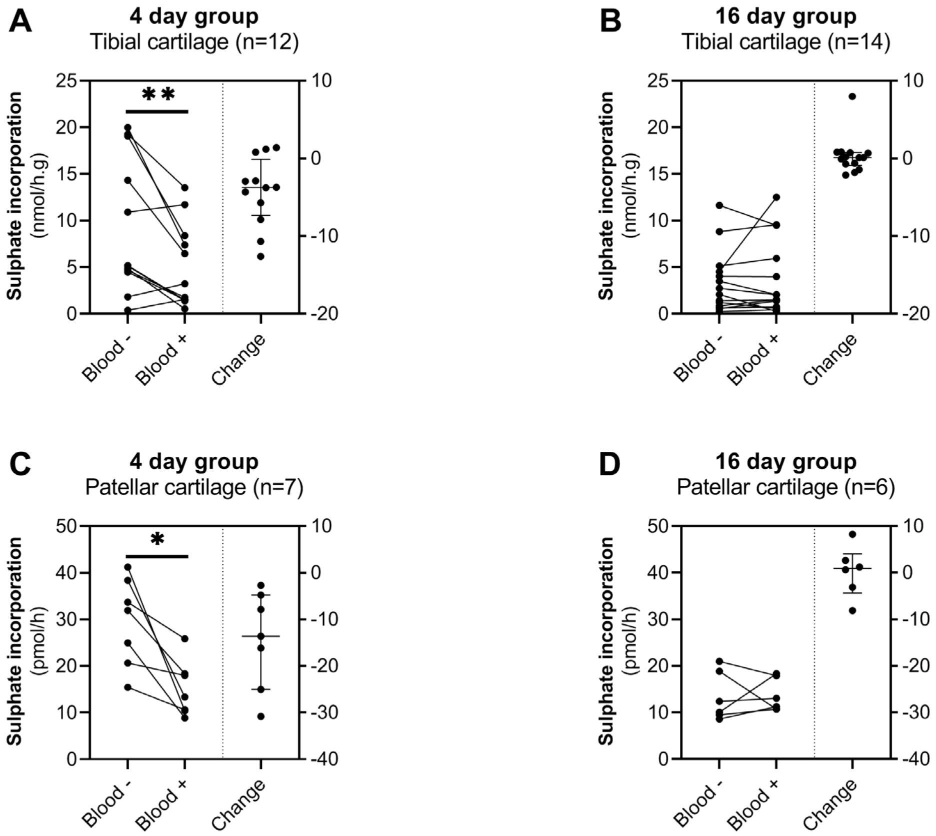

Figure 4 – Proteoglycan synthesis rate 4 and 16 days after induced hemarthrosis in F8-/- rats
Sulphate incorporation was measured 4 and 16 days (d) after an induced joint bleed, in tibial (A; 4d, C; 16d; normalised to tissue weight) and patellar (B; 4d, D; 16d) cartilage. At 4 days, the proteoglycan synthesis rate was significantly decreased in the blood-exposed compared to the contralateral knee, *p=<0.05, ** p=<0.01, Wilcoxon signed rank test. Change (blood-exposed minus contralateral knee) is depicted as median ± interquartile range.

Sixteen days post-hemarthrosis a low proteoglycan synthesis was not only noted in the blood-exposed knees (2.0 nmol/h.g (1.1-6.6)), but also in the contralateral knees (2.7 nmol/h.g (0.8-4.5)) (Figure 4B; p=0.855). Blood exposure resulted in a decrease of 85% in PSR compared to the control group (p=0.003).

Hemarthrosis results in decreased patellar PSR

In a subgroup of animals (4-day group n=7, 16-day group n=6) the patellar PSR was assessed, demonstrating similar results as seen in the tibia. Induced hemarthrosis led to a decreased PSR of 60% compared to the contralateral knee after 4 days (Figure 4C, contralateral knee 31.9 pmol/h (20.7-38.4) *vs* blood-exposed knee 13.4 pmol/h (10.4-18.4), p=0.016) and a decrease of 40% compared to bleeding-naïve controls(p=0.005).

Sixteen days after the induced hemarthrosis, comparable low PSR were seen in both knees (Figure 4D, contralateral knee 11.2 pmol/h (9.3-19.4) *vs* blood-exposed knee 12.2 pmol/h (10.7-18.3), p=0.844), and blood exposure led to a significant decrease in PSR of 45% compared to the control group(p=0.003).

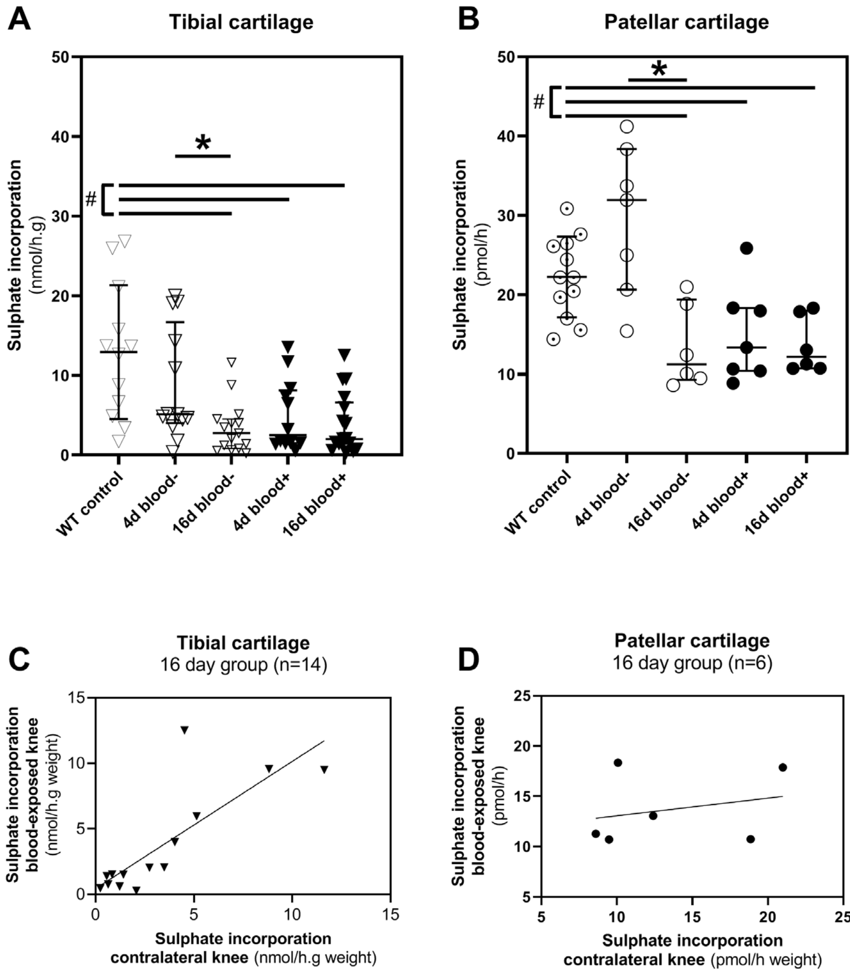

Figure 5 – Evaluation of proteoglycan synthesis rate in the contralateral knee
A and B: Sulphate incorporation in the tibia (A) and patella (B) of bleeding naive control wild type (WT) rats and blood-exposed (blood+) and contralateral (blood-) knees of F8-/- rats, 4 and 16 days after hemarthrosis induction. Sulphate incorporation in the tibia and patella of the contralateral knees was significantly decreased in rats euthanised 16d after induced hemarthrosis compared with rats euthanised 4 days after induced hemarthrosis, *p<0.05, Mann Whitney test. A significant difference compared to control animals was seen in all blood-exposed joints, as well as the contralateral joints of the 16-day group, #p<0.05, Mann Whitney test. C and D: Sulphate incorporation was significantly correlated between the injured and contralateral knee regarding the tibia (C; r=0.803, p=<0.001), but not the patella (D; r=0.259, p=0.620) 16 days after hemarthrosis.

Induced hemarthrosis affects the cartilage of the contralateral knee

Sixteen days after induced hemarthrosis, the PSR rate in the contralateral knees also appeared to be decreased. To analyse this, we compared the PSR across the 4- and 16-day group (Figure 5A and B). While there was no difference in the PSR between the injured knees of the 4- and 16 day groups (tibia p=0.491, patella p=0.624), the PSR in the contralateral knees was significantly lower in the 16-day group compared to the 4-day group (tibia p=0.013, patella p=0.008). In addition, no statistically significant differences in PSR were found between the contralateral knee at day 4 and the control group (tibia p=0.225, patella p=0.100), whereas the PSR in the contralateral knees in the 16-day group were significantly different from the control group (tibia p=<0.001, patella p=0.007). Moreover, in the 16-day group we found a significant correlation between the PSR in the blood-exposed *vs* the contralateral knee for the tibia (Figure 5C and D, r=0.803, p=<0.001), but not the patella (r=0.259, p=0.620).

Discussion

In the present study, we demonstrate that proteoglycan synthesis can be quantified with the $^{35}SO_4^{2-}$assay in healthy tibial and patellar cartilage of the rat, and that it is affected by blood exposure *in vitro*. Further, we demonstrate for the first time in a hemophilic animal model that induced joint bleeds lead to decreased proteoglycan synthesis, establishing the $^{35}SO_4^{2-}$-assay as a novel method for detecting early blood-induced cartilage damage.

The proteoglycan synthesis found in healthy cartilage of WT rats after the 4 day culture was clearly higher than in human and canine cartilage cultured *in vitro* under the same conditions.[4,6,7,22,25] This might be explained by a higher turnover of cartilage in small animals.[26]

The relative inhibition of proteoglycan synthesis after 4 days 50% v/v blood exposure *in vitro* in tibial (90%) and patellar (81%) cartilage was comparable to data from human and canine explants (74-99%).[4,6,7,22,25]

After confirmation of the use of the $^{35}SO_4^{2-}$-assay to study blood-induced cartilage damage in an *in vitro* model, feasibility of the assay was tested in an *in vivo* design. Whereas previous studies have measured a blood-induced decrease in proteoglycan synthesis either following *in vitro* blood exposure[6,7,25] or following intra-articular injection of blood in WT animals,[8,27,28] this is the first study in which a decrease in proteoglycan synthesis has been demonstrated in hemophilic animals. Upon induced hemarthrosis in hemophilic rodent models, the entire joint is rapidly filled with blood,[15] and the bleeding remains unresolved for several days.[9,29] In contrast, intra-articular injection of autologous blood is cleared quickly (<1 day) in WT animals.[30] Nonetheless, our findings of decreased proteoglycan synthesis in the blood-exposed knee 4 days after injury corroborates with reports from WT animals injected intra-articularly with autologous blood, but with a more pronounced relative decrease in proteoglycan synthesis (52% in tibia and 60% in patella *versus* up to 36% in literature.[4,8,28,31] This may reflect a higher degree of blood-exposure in hemophilic animals.

Surprisingly, we did not find any difference in PSR between the blood-exposed and contralateral knee 16 days after injury. This observation did not reflect an enhanced synthesis in the blood-exposed knee, as seen in the canine model,[8] but rather a decreased

synthesis in the contralateral knee. The persistent, low proteoglycan synthesis observed in the blood-exposed knee may be due to the relatively large and untreated bleed induced in the hemophilic model, in combination with the previously discussed slower clearance of blood from the joint. Secondly, the decreased proteoglycan synthesis in the contralateral knee appears to be related to the degree of cartilage damage in the blood-exposed knee, a finding described in rodent models of degenerative joint disease as well.[32] This correlation was demonstrated for tibial, but not patellar cartilage. It is possible that shifted weight-bearing following an induced joint bleed[29,33] increases the mechanical load on the contralateral leg,[34] potentially damaging the cartilage.[32] Further, hemophilic rats are susceptible to spontaneous (micro)bleeds that could lead to direct cartilage damage,[35] a phenomenon which could be enhanced by increased weight-bearing in the contralateral joint. In addition, previous studies have shown that in hemophilic rodent models, an induced joint bleed elicits a local and systemic angiogenic stimulus and upregulation of pro-inflammatory cytokines.[5,29,36] Thus, it cannot be ruled out that contralateral cartilage damage is induced by systemic modulators induced by the joint bleed. Moreover, neurogenic inflammation has been suggested as a cause of contralateral damage.[37,38] Local inflammatory processes induce local neurogenic stimulation, which may cause a bilateral response of the nervous system resulting in bilateral cartilage degeneration.[39]
On histology, cartilage and bone changes in the $F8^{-/-}$ rat occur simultaneously,[9] whereas we observed a significant decrease in proteoglycan synthesis before pathological bone remodelling became evident (on day 4). Possibly, this reflects the high sensitivity of the $^{35}SO_4^{2-}$-assay for detecting early cartilage damage.[17] In contrast to the cartilage, the bone of the contralateral knee was not affected after 16 days, as evaluated by micro-CT. Pathological bone remodelling as well as upregulation of a signalling pathway leading to osteopenia have been shown to correlate with the amount of blood in the joint,[15,40] and thus it is possible that spontaneous microbleeds, systemic changes induced by the bleed or increased weight-bearing in the contralateral knee are not sufficient to elicit bone changes.
Our study has some limitations. The induced joint bleeds were only characterized by joint diameter, and therefore a possible correlation between the joint bleed volume and degree of cartilage damage could not be investigated. Proteoglycan synthesis was assessed within 24 hours of euthanasia, and although *in vitro* experiments found no differences when measurements were done immediately or 24 hours after euthanasia (data not shown), we cannot completely rule out that time and transportation may have affected the chondrocyte activity. Also, patellar cartilage was only obtained from a subgroup of rats, and thus conclusions are made from a relatively small number of animals (4-day group: n=7, 16-day group, n=6). Finally, baseline proteoglycan synthesis should ideally be assessed in $F8^{-/-}$ rats, which was not feasible due to availability of the rats. While this does not affect the conclusion that the PSR in the contralateral knees was significantly lower in the 16-day group compared to the 4-day group, the contralateral effects of hemarthrosis on PSR in this model could not be fully addressed since the baseline measurements were done in WT rats (on the same genetic background) that potentially could have a different baseline PSR. However, WT rats have the advantage that the baseline PSR was measured in the absence of potential subclinical bleeds.

Conclusion

In conclusion, we show that the well-established $^{35}SO_4^{2-}$ assay can be used to detect blood-induced cartilage damage after a single induced hemarthrosis in the $F8^{-/-}$ rat. As no current treatment specifically targets early blood-induced cartilage damage in hemophilia, this assay combined with the hemophilic rat as a pharmacological model could be used to test novel therapies. However, the use of the contralateral knee as control is questionable, as contralateral cartilage damage was seen 16 days after induced hemarthrosis.

Acknowledgements

Novo Nordisk A/S partially funded the study.

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Supporting Information

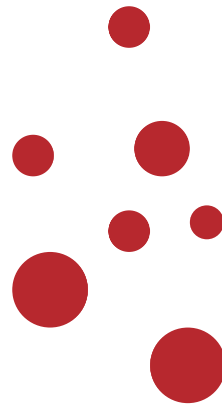
In vivo study: animals

The rats were group-housed under standard conditions (12h/12h light-dark cycle, *ad libitum* food and water, 20-23ºC), and subjected to daily welfare assessments and monitoring of spontaneous bleeds. Rats that met predefined humane endpoints or experienced spontaneous bleeding unrelated to the induced hemarthrosis were excluded.

In vivo study: hemarthrosis

Rats were placed in dorsal recumbency. Both knees were shaved with an electronic shaver and cleansed with an alcohol swab. The left knee was mildly flexed and a 30-gauge needle (BD Micro-fine 30 gauge, BD, Berkshire, United Kingdom) inserted approximately 7mm into the knee joint through the patellar ligament, as described.

Six hours after induction of hemarthrosis, buprenorphine was re-supplied (0.05mg/kg SC). For the remainder of the study, analgesia was provided through the water (6mg/L buprenorphine), and food was placed in the bottom of the cage for easy access.



Evaluation of tibiofemoral and patellar joint damage following a single and double injury in a murine model of hemophilic arthropathy

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Abstract

Background

Hemarthrosis in hemophilia patients result in arthropathy, predominantly observed in larger synovial joints. Recurrent joint bleeds eventually lead to hemophilic arthropathy (HA), causing major disability. The hemarthrosis model in hemophilic mice is valuable to improve our insights in the pathogenesis of HA.

Objectives

To advance knowledge of the hemarthrosis model in hemophilic mice by evaluating the patella in addition to the tibiofemoral compartment, comparing a single versus double joint bleed, and investigating the influence of induced arthropathy on the contralateral joint.

Methods

Factor VIII-deficient mice were subjected to a single (one-hit model; n=34) or double (two-hit model; n=42) joint bleed by infra-patellar needle puncture. Five weeks after the last joint bleed, tibiofemoral and patellar inflammation and cartilage damage were histologically assessed in both knee joints. Ten joint-bleed naive hemophilic mice were included as controls.

Results

Induced hemarthrosis resulted in inflammation comparable for the patellar and tibiofemoral compartment. While minor cartilage damage was demonstrated in the tibiofemoral compartment, patellar cartilage damage was substantial. No clear aggravation of joint damage was observed upon the second bleed, while the survival rate significantly decreased. Moreover, the severity of cartilage damage in the injured joint was associated with development of cartilage damage in the weight-bearing area of the contralateral knee.

Conclusion

Assessment of the patella provides additional information on the damaging process upon hemarthrosis in this hemophilic mouse model, while a second induced bleed had no clear surplus value. Importantly, contralateral damage resulting from arthropathy should be considered when using an internal control.

Introduction

Hemarthrosis is the most characteristic manifestation in patients with hemophilia,[1] but also occurs as a consequence of other bleeding disorders such as von Willebrand disease,[2] as a complication of anticoagulant treatment,[3] or after trauma[4] or major joint surgery.[5] Over time, recurrent joint bleeds result in progressive joint tissue damage and hemophilic arthropathy (HA). Although prophylactic clotting factor replacement therapy is effective in reducing the annual bleeding rate and severity of arthropathy, development of HA cannot be entirely prevented and still is a main complication of hemophilia.[6,7] HA is characterized by synovial inflammation, cartilage degradation, and bone changes, subsequently accompanied by muscle and ligament impairment.[8] Although the presence of blood in the joint is the clear trigger for the development of HA, the exact pathogenetic mechanisms still need further study. As there is an unmet need for treatment of HA, a better understanding of the pathogenesis may eventually reveal new targets for therapy.[8]

In medical research, genetically engineered laboratory animals are utilized to mimic characteristics of human genetic disease. By knockout technology hemophilic mice are created, which are supposed not to bleed spontaneously.[9] An artificially induced joint bleed results in joint tissue damage as seen in human disease.[10,11] While HA takes years to develop in humans, the joint destructive process is accelerated in mice and already visible after a few days to weeks, which is considered an advantage in studying the pathogenesis. An additional advantage is that mechanisms can be studied directly at joint tissue level, while in humans evaluation relies on indirect parameters, like imaging techniques[12] and biochemical marker analyses.[13-15] Moreover, in humans the early stages of the disease are often asymptomatic, while in mice the initiation of the process is known. As such, mouse models are valuable for evaluation of pathogenetic mechanisms and preclinical assessment of possible novel therapeutics.[16,17]

On the other hand, the use of the hemophilic mouse model also has its limitations. A joint bleed is most commonly induced by needle puncture of the knee. Because no clotting factor replacement therapy is given, not only a major intra-articular bleed is induced but often also peri-articular bleeding occurs. The latter may have its drawback on animal welfare and survival. Furthermore, multiple joint bleed induction has been applied to mimic the human situation of recurrent hemarthroses.[18-21] The additional value of a subsequent bleeding in this specific model, however, has not been studied. Moreover, detailed evaluation of the impact of joint damage in the blood-exposed joint on the contralateral joint has hardly been reported.

Quantifying joint damage is another challenge. Semi-quantitative scoring systems specific for mice are utilized. The often used Valentino score[22] predominantly evaluates inflammation, whereas the Osteoarthritis Research Society International (OARSI) score is specifically developed to grade osteoarthritis related cartilage damage.[23] However, the relatively mild manifestations of blood-induced cartilage damage in the hemophilic mouse model are not properly reflected by the OARSI score.[24,25] Therefore, this scoring system has been adjusted enabling evaluation of more subtle changes in cartilage damage.[26,27] The modified OARSI score is quite sensitive and detects early alterations, even in presumed healthy, unaffected joints.[21,26,27] This scoring system was originally applied to the tibiofemoral joint, neglecting

the patellar compartment. Previous mouse studies performed by our group indicated that also the patella was affected following a joint bleed. This is supported by the fact that blood spreads throughout the joint cavity and ascends to the patella upon hemarthrosis, as seen on quantitative *in vivo* contrast-enhanced micro-CT imaging in hemophilic mice[28]. Morphological bone changes of the patella as observed in rodent models, also show the involvement of this compartment in the overall joint pathology.[10,11,29-31]
To gain new insights in the hemarthrosis model in hemophilic mice, in the present study the patella in addition to the tibiofemoral compartment is evaluated, joint damage induced by a single (one-hit) and double (two-hit) injury model is compared, and the impact of induced arthropathy on the presumed healthy contralateral joint is evaluated.

Methods

Animal care

Factor VIII (FVIII)-deficient mice (B6;129S4-F8tm1Kaz/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in the central animal facilities of the University Utrecht. Mice were housed in filter-top cages in low density (maximum of 5 animals per cage) in order to minimize the risk of combat-related bleedings. They were fed a standard diet and water ad libitum. This study was performed according to the European Convention on Animal Care and is approved by the institutional and national animal ethical committee (project number AVD115002016451).

Study design

Skeletally mature animals between three and four months of age (male n=36, female n=40) were anesthetized with isofluran/O2 and hair of both knee joints was shaved off to facilitate direct observation. In the first group, a single joint bleed was induced in the right knee (experimental joint) on day 0 by insertion of a 30-Gauge needle through the infra-patellar ligament, according to the method previously described (one-hit model, n=34, 16 males and 18 females).[10] In the second group, hemarthrosis was induced twice, on days 0 and 14 (two-hit model, n=42, 20 males and 22 females). The left knee of each animal served as an untouched internal control. The joint diameter (JD; mean of three measurements using a micrometer caliper[10]) and visual bleeding score (VBS)[32] were assessed at baseline, 2 and 14 days after induction of the bleed, and at euthanasia (35 days after the last bleed). An increase in JD less than 0.5mm in combination with a maximum VBS lower or equal to 1 was considered as an unsuccessfully induced bleed and these animals were excluded from further analysis. In addition, ten female joint-bleed naive hemophilic mice (comprising 20 joints) of the same age were included as controls.

Blood analysis

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

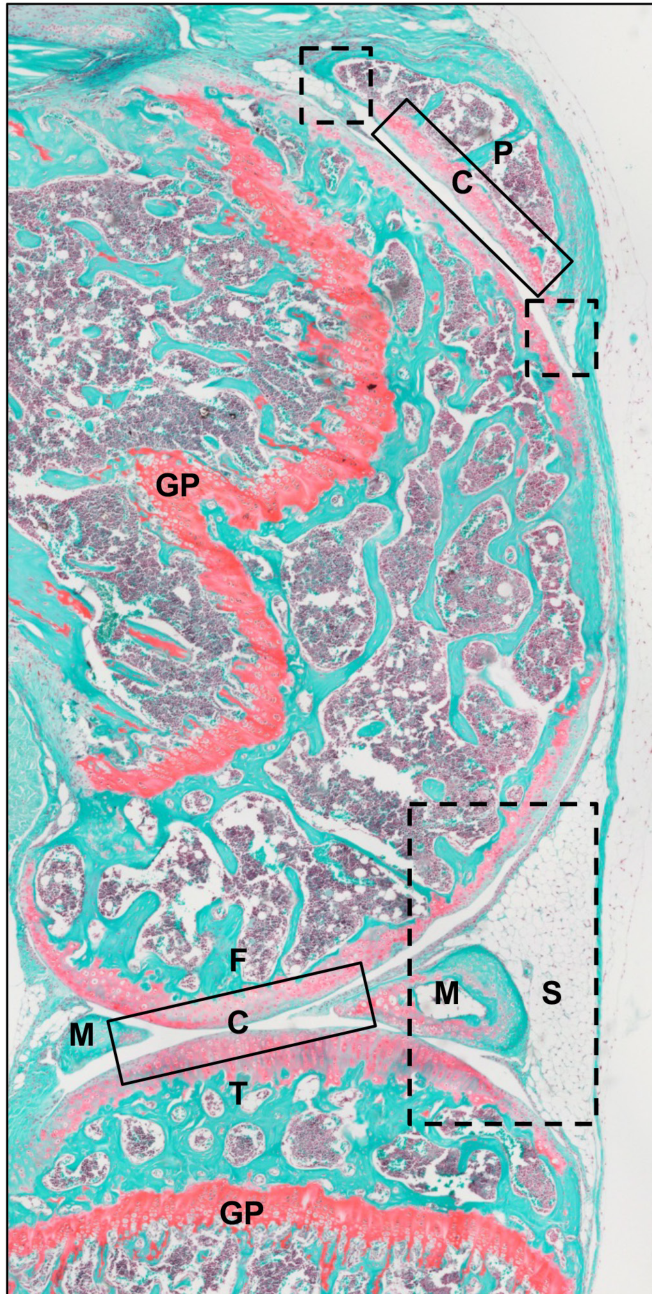


Figure 1 - Locations where joint damage is scored
Image stained by Safranin-O. Patellar and tibiofemoral joint damage was scored within the indicated rectangles (dashed line: inflammation, continous line: cartilage damage). For detailed images of the tibiofemoral and patellar compartment, see Figure 7 panel A and B. C= cartilage, F= femur, GP= growth plate, M= meniscus, P=patella, S= synovium, T= tibia.

Histopathological evaluation

All animals were euthanized by cervical dislocation five weeks after the last joint puncture (one-hit: day 35, two-hit: day 49). In addition, the ten joint-bleed naive mice were processed the same way. The hind legs were removed, the knee joints isolated, fixed in 4% formaldehyde for at least 48 hours, decalcified in EDTA 12.5% and embedded in paraffin for histological examination. Sagittal tissue sections (4 μm) of the joint were made to assess the tibiofemoral joint surface. In case the patella was not visible, additional sections were cut aiming for the patella only.
Tibiofemoral synovial inflammation was scored on hematoxylin-eosin (H&E)-stained sections according to the Valentino score.[22] 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).[33] Tibiofemoral and patellar cartilage damage was evaluated on Saf-O-stained sections using the modified OARSI score as previously described.[21,26,27] See Figure 1 (and 7) for specific scoring localizations and see supplementary files for the detailed description of all scoring methods. 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. For further calculations the mean of the observers' scores were used.

Statistical analysis

Assessed by histograms and Shapiro-Wilk normality test, none of the continuous data were normally distributed and therefore these data were analyzed using the Mann-Whitney test. Categorical data consisting of five or more categories were considered continuous and as such analyzed the same way. Paired data were analyzed using the Wilcoxon signed rank test. Correlation was analyzed using the Spearman Rank Correlation. Results were considered significant if $p<0.05$. Statistical analyses and graphical presentation were performed using GraphPad Prism (Version 8.0.1; GraphPad Software Inc, San Diego, CA, USA).

Results

Joint bleed, survival and histologic processing

After a single joint bleed 26 out of 34 animals could be included (survival rate 76%), while after two injuries 24 out of 42 animals completed the study (survival rate 57%). A significant increase in both the diameter of the injured joint (Figure 2) and the VBS (data not shown) was observed at two days following induction of the single as well as the double joint bleed (all $p<0.001$). Animals with unsuccessfully induced bleeds (see Methods: one-hit model: n=3, two-hit model: n=0) were excluded from further analysis. Hemarthrosis resulted in a decrease in Hb level measured five weeks after the last bleed in both the one-hit model (median 8.07 mmol/L (interquartile range (IQR) 7.33-8.40), $p=0.018$) and the two-hit model (7.95 mmol/L (7.54-8.50), $p=0.013$), compared to the control animals (8.50 mmol/L (8.15-8.80)). Except for two animals in the one-hit model, levels of individual animals did not drop below the reference value (6.21-10.55 mmol/L)[34].

From the 23 mice included in the one-hit model, 20 paired histological sections of the tibiofemoral compartment could be evaluated (contralateral joints n=23, experimental joints n=20) and in all but one animal (n=22 out of 23) the sections of the patellar compartment of both joints were of sufficient quality (contralateral joints n=22, experimental joints n=23). In the two-hit model, 22 paired histological sections derived from 24 mice were eligible to study the tibiofemoral compartment (contralateral joints n=23, experimental joints n=23). Unfortunately, in the two-hit model the patellar compartment could be studied histologically in both joints in only 13 out of 24 animals (contralateral joints n=17, experimental joints n=18). Because of the relatively low number of available paired patellar compartments in the two-hit model, a subgroup analysis was performed to assess whether the loss of samples had resulted in selection bias. The modified OARSI score applied to the tibiofemoral compartment of the experimental joint was compared between the 13 animals and the overall group of 22 animals. There were no statistical differences (p=0.738) present and thus the subgroup of 13 is considered representative in this respect. In the ten joint-bleed naive mice, all 20 tibiofemoral compartments and 18 patellar compartments could be evaluated (in two animals one of the patellar compartments was of sufficient quality for evaluation).

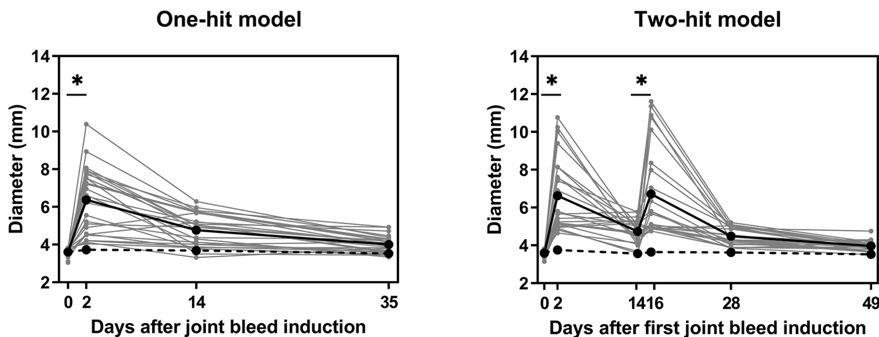


Figure 2 - Joint diameter in the one and two hit model
Joint diameter of the injured joint increases significantly two days post-injury in both models. Grey lines represent the diameter of the injured joint of individual animals (one hit n=26, two hit n=24), the mean is indicated by the black line. The dotted line indicates the mean of the contralateral joint. *p= <0.001, two tailed Wilcoxon test.

Hemarthrosis triggers inflammation in the tibiofemoral and patellar compartment

On average, increased inflammation was present in the experimental joints compared to the contralateral joint after a single bleed (Figure 3A and C, Valentino score contralateral versus experimental joint: median 2.5 (IQR 2.0-3.0) vs 5.5 (4.0-6.5), adapted Koizumi score: 0.0 (0.0-0.0) vs 3.0 (1.8-3.0), for both compartments p=<0.001). Upon two sequential bleeds, a similar trend was noticed (Figure 3B and D, Valentino score: 2.5 (2.0-4.0) vs 4.0 (3.0-5.5), p=0.077, adapted Koizumi score 0.0 (0.0-1.5) vs 2.5 (1.0-3.0), p=0.107), although only nearing statistical significance.

In both the one- and two-hit model, the injured joints showed an inflammatory response (Figure 4; dotted bars) as compared to the joints of the bleeding naive animals (Figure 4; dashed bars, controls: Valentino score 2.0 (1.0-2.0), adapted Koizumi score 0.0 (0.0-0.0), all p=<0.001). Surprisingly, also the contralateral joints (Figure 4; white bars) of the experimental animals demonstrated an inflammatory response (all p=<0.05, except for the patellar compartment of the one-hit model) compared to the control animals.
Comparison of the one- and two-hit model showed no statistically significant differences in the degree of inflammation between the experimental joints (Figure 4, Valentino score p=0.106, adapted Koizumi score p=0.524). In contrast, the contralateral joints demonstrated more inflammation in the two-hit model as compared to the one-hit model, statistically significant for peri-patellar inflammation (Figure 4B, adapted Koizumi score p=0.004).

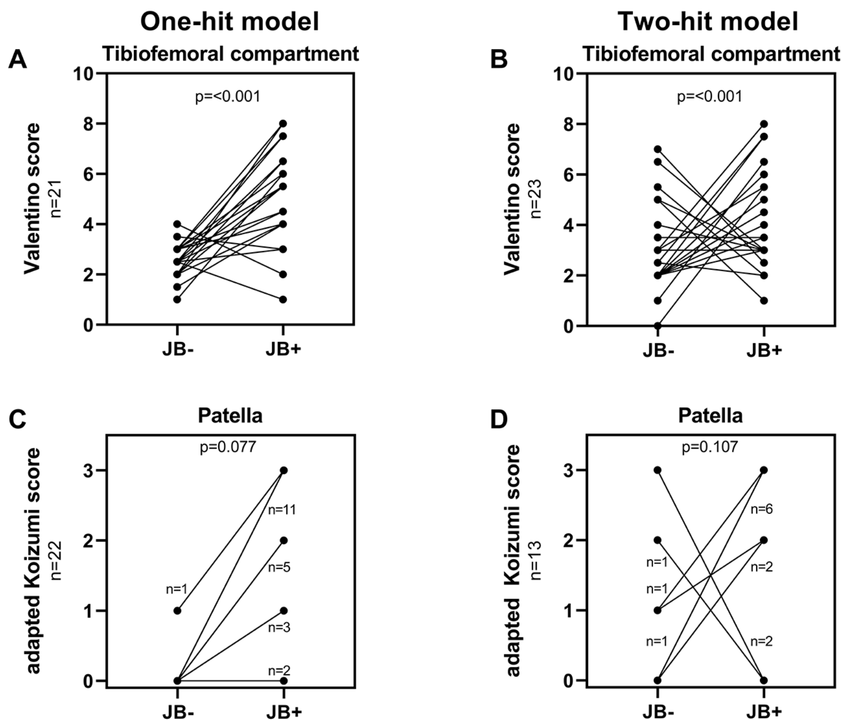


Figure 3 - Tibiofemoral and patellar inflammation scores
A single injury clearly triggers both synovial and patellar inflammation (A and C). Upon two sequential bleeds, synovial inflammation according to the Valentino score (0-10) follows the same trend (B), whereas patellar inflammation using the adapted Koizumi score (0-3) demonstrates no significant difference (D). N-values indicate the number of animals represented by the black line. P-values for the difference between the experimental joint (joint bleed (JB)+) and contralateral joint (JB-) are given at the top of each graph (two tailed Wilcoxon test).

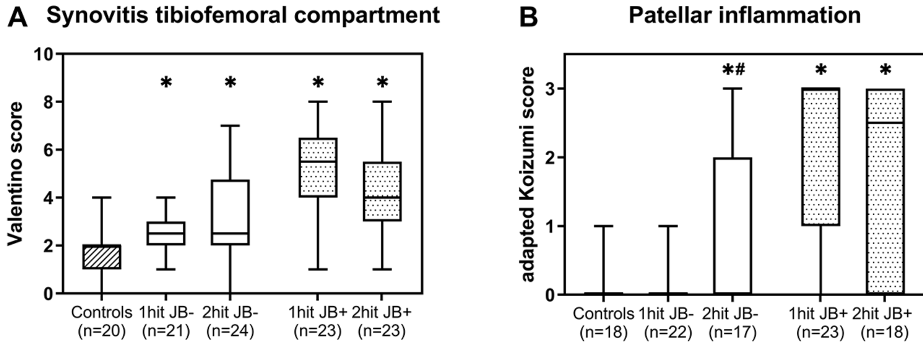


Figure 4 - Comparison of inflammatory response in the one and two hit model
The inflammatory response in the contralateral joint (joint bleed (JB)-; white bars) and experimental joint (JB+; dotted bars) upon hemarthrosis in the tibiofemoral (A) and patellar (B) compartment is compared to bleeding naive control animals (dashed bars) (*p=<0.05, two tailed Mann-Whitney). The contralateral joints showed more inflammation in the two-hit model as compared to the one-hit model, statistically significant for peri-patellar inflammation (#p=0.004, two tailed Mann-Whitney). No enhanced inflammatory response was demonstrated in the experimental joints following a second bleed for both the tibiofemoral and patellar compartment. Scores are depicted as median with interquartile range (whiskers min to max).

Cartilage damage dominates in the patellar compartment

In both the one- and two-hit model, no statistically significant increase in cartilage damage in the tibiofemoral compartment of the experimental joint compared to the contralateral joint could be detected (Figure 5A and B, one-hit contralateral vs experimental joint: median modified OARSI 1.6 (IQR 0.4-3.5) vs 0.6 (0.2-1.8), two-hit: 2.4 (0.9-3.3) vs 1.7 (0.3-3.1)). In the patellar compartment an increase in cartilage damage could be demonstrated in the experimental joints as compared to the contralateral joints, both after a single and double joint bleed (Figure 5C and D, one-hit: modified OARSI 0.5 (0.0-2.6) vs 5.0 (3.8-6.0), p=<0.001, two-hit: 2.0 (0.0-6.0) vs 6.0 (4.0-6.0), p=0.016). Unexpectedly, although similar as for inflammation, not only the experimental joints (Figure 6; dotted bars) but also the contralateral joints (Figure 6; white bars) demonstrated a statistically significant increase in cartilage damage in both the tibiofemoral and patellar compartment and in both the one-hit and the two-hit model compared to control animals (Figure 6; dashed bars, modified OARSI tibiofemoral 0.0 (0.0-0.1) and patella 0.0 (0.0-0.5), all p=<0.01). Although not statistically significant, tibiofemoral and patellar cartilage damage tended to increase after a second bleeding in the experimental joint (Figure 6A and B; p=0.168 and p=0.295, respectively), as well as cartilage damage in the contralateral joints (p=0.560 and patella p=0.165, respectively) (see also Figure 7 for representative images). The latter suggests a relation of the damage between the experimental and the contralateral joint.

Severity of damage in the experimental joint relates to severity of damage in the contralateral joint

To provide an overall cartilage damage severity score for the whole joint, the scores of the tibiofemoral and patellar compartment of the joint exposed to blood were summed into a combined total joint score (TJS) for both the one- and two-hit model, weighing both compartments equally. The TJS of the experimental joints correlated to the degeneration of the weight bearing surface (tibiofemoral compartment) of the contralateral knee joint (Figure 8, Spearman's correlation: r=0.411, p=0.012). This was found for damage in the femur and tibia of the contralateral joint separately as well (see supplementary files: Spearman's correlation r=0.346, p=0.036 and r=0.428, p=0.007, respectively).

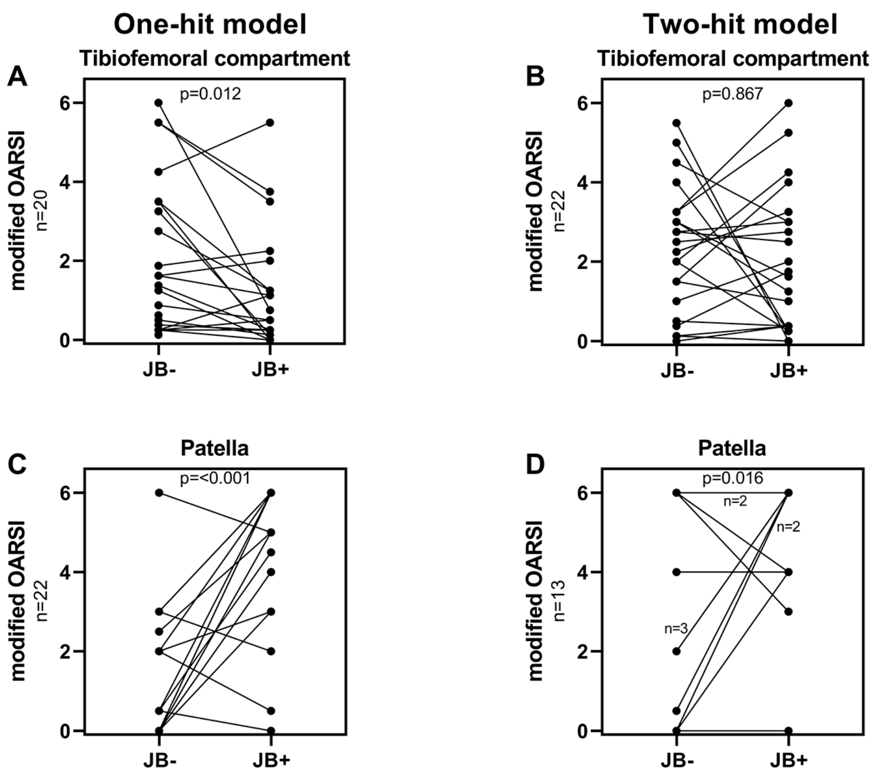
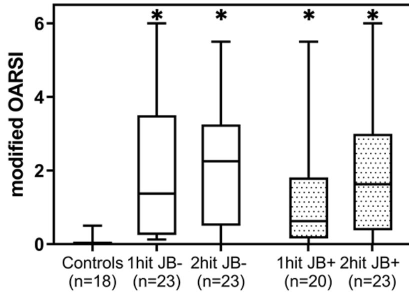


Figure 5 - Tibiofemoral and patellar cartilage degeneration
The modified Osteoarthritis Research Society International (OARSI) score (0-6) applied to the tibiofemoral compartment (A and B) and patella (C and D) five weeks after a single (one-hit) and double injury (two-hit). Lines representing more than one animal are indicated by the n-value. P-values for the difference between the experimental joint (joint bleed (JB)+) and contralateral joint (JB-) are given at the top of each graph (two tailed Wilcoxon test).



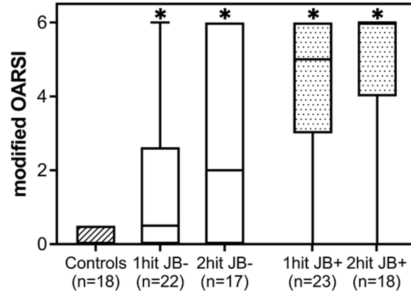


Figure 6 - Comparison of cartilage damage in the one- and two-hit model
Cartilage damage in the tibiofemoral (A) and patellar (B) compartment of the contralateral joint (joint bleed (JB)-; white bars) and experimental joint (JB+; dotted bars) is compared to bleeding naive control animals (dashed bars) (*p=<0.01, two tailed Mann-Whitney). Cartilage damage in the two-hit model tended to be higher compared to the one-hit model, not only in the experimental joints but also in the contralateral joints, although not statistically significant. Scores are depicted as median and interquartile range (whiskers min to max).

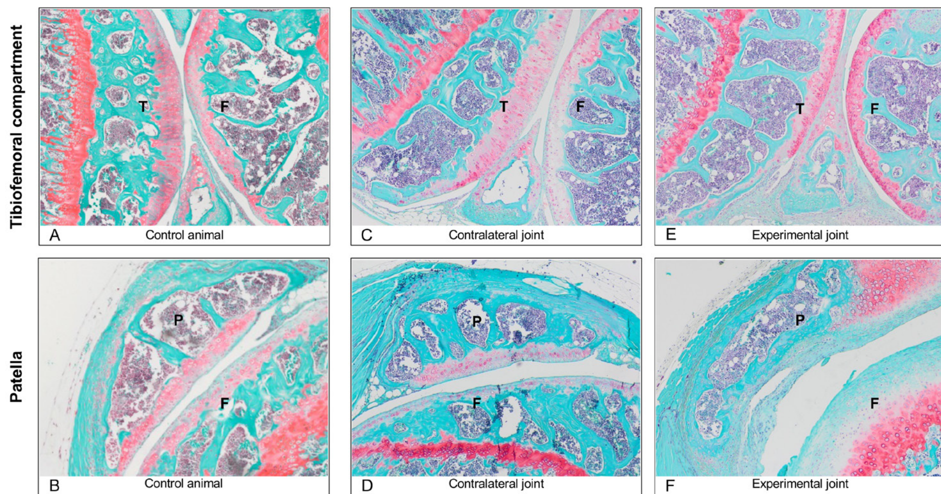


Figure 7 - Representative images of histological changes upon hemarthrosis
Representative micrographs (magnification 4x) of Safranin-O staining used to quantify tibiofemoral and patellar cartilage damage. Panel A and B illustrate the joint of a hemophilic mouse without an induced joint bleed (control animal). The tibiofemoral and patellar compartment of the contralateral (C and D) and experimental joint (E and F) belonging to one animal five weeks after experiencing a single bleed are displayed. Severe patella deterioration (F) upon hemarthrosis is demonstrated, while the loss of proteoglycans is more pronounced in the tibiofemoral compartment of the contralateral knee (panel C) than the injured knee (panel E). F= femur, P= patella, T= tibia

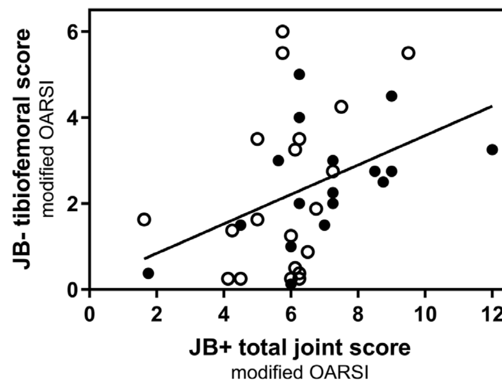


Figure 8 - Correlation modified OARSI score
Tibiofemoral and patellar modified Osteoarthritis Research Society International (OARSI) scores of the blood-exposed joint were summed into the total joint score for both the one- (open symbols) and two-hit model (filled symbols), weighing both compartments equally. The total joint score of the experimental joint is significantly correlated to the weight-bearing tibiofemoral cartilage damage of the contralateral joint (Spearman's correlation: r=0.411, P=0.012). JB= joint bleed.

Discussion

In this study we enhance our knowledge of the hemophilic mouse model to assess blood-induced joint damage by providing insight in the role of the patellar compartment with regards to inflammation and cartilage damage, by comparing a single to a double injury, and by evaluating the impact of blood-induced arthropathy in the experimental joint on the contralateral joint.

For the first time, the patellar compartment is assessed systematically demonstrating clear inflammation and cartilage damage after joint bleed induction. This is in line with subcortical bone changes and osteophyte formation in the patella in other hemophilic rodent models.[10,11,29-31] Cartilage damage was clearly more pronounced in the patellar compartment as compared to the tibiofemoral compartment. Being quadruped, rodents have different biomechanics compared to humans. So, the question raises how to translate this finding to human hemophilic arthropathy. Morphological changes in the patella develop in patients with HA as well, although this is mainly described as a separate entity in the less recent literature.[35-39] The patellar compartment is also frequently involved in other joint degenerative disease like osteoarthritis.[40] Yet patellar complaints do not often dominate in patients with hemophilia, which contrasts with this mouse model.

The two-hit injury model appears to have no clear added value over the one-hit model and comes at the price of reduced survival. A second bleed did not clearly result in more joint damage in the affected joint on group level, which is consistent with findings in hemophilic rats.[41] Also in hemophilia patients the number of hemarthrosis are associated with radiographic joint damage at group level,[42] while there is no clear relation between the (observed) bleedings and arthropathy on an individual level.[43-45] On the other hand, the used scoring systems for the patella might have reached its plateau in the one-hit model already,

limiting the detection of more severe damage in the two-hit model. Thus, a potential further increase following a second joint bleed cannot be reflected in these scoring systems applied to the patella. Additionally, the two-hit model comes at the price of lower survival rates. This might have led to a selection bias due to inclusion of only the animals with less severe damage, which may underestimate the damage in the two-hit model. Survival rates following one or two injuries without clotting factor administration in our study (76% and 57%, respectively) are comparable to previous published data (one hit: 75-90%,[46] two hits: 55%,[21] three hits: 35%[19]). Thus, without (observable) additional joint damage and with increased mortality the two-hit model is considered of no additional value.

Since intra-articular blood volume is related to the subsequent degree of joint damage,[28] characterizing the extent of the induced joint bleed is relevant to our findings. However, in our study only indirect parameters (JD, VBS, Hb) were available, which did not correlate with the severity of inflammation nor cartilage damage (data not shown). Histological evaluation to prove the presence of abundant blood in the joint is not applicable to our study either, since the period between the (last) joint bleed and euthanasia exceeds the two weeks it takes to completely remove blood from the murine joint.[10,25] However, Vøls et al demonstrated that 100% of the needle-induced joint bleeds resulted in the presence of intra-articular blood in hemophilic mice.[28] In our study, the development of synovitis in the tibiofemoral compartment is in accordance with literature.[21,25-27,47] However, we found no difference in cartilage damage between the experimental joint and the contralateral joint, while in previous studies this relative difference was demonstrated. However, these studies differed in the technique of inducing the bleeding,[26,27,47] the scoring system,[19,24,25] and/or time until evaluation of the joint damage after the last joint bleed.[21]

Notable are the high scores for inflammation and cartilage damage in the contralateral joints. In most previous studies evaluating blood-induced cartilage damage in this hemophilic mouse model, either sham / wild type animals[19,28,30] or the supposedly unaffected joint served as a control,[21,24-27,47,48] without specifying the alterations in the contralateral joint.[10,25] In three studies, manifestations of inflammation in the contralateral leg upon hemarthrosis are explicitly mentioned. Mejia-Carvajal et al. described the development of synovitis in the contralateral joint in some mice after inducing blunt force trauma in the other,[48] Cooke et al. demonstrated synovial upregulation of IL-6 and reparative (M2) macrophage response in the uninjured joint,[49] and Bhat et al. reported signs of neo-angiogenesis in the contralateral knee.[24] Cartilage damage in the contralateral joint was only disclosed by Nieuwenhuizen[26,27,47] and Van Vulpen.[21] In none of these studies data are depicted in pairs, so relating their mutual relationship is not possible.

The clear inflammation and cartilage destructive activity in the contralateral joints in our study seemed even more pronounced in the two-hit as compared to the one-hit model. The overall severity of cartilage damage in the affected joint is indeed associated with more cartilage deterioration in the unaffected joint. This observation clearly controverts the use of the contralateral joint as an internal control. There are a number of possible explanations for the alterations in the contralateral joint. First of all, no tibiofemoral damage is seen in the control mice, so the changes must arise from the induced joint bleed. Due to pain, swelling and limited motion, weight-bearing may have shifted from the injured leg to the uninjured hind leg,[24,50] so the disturbances in the contralateral joint can be caused by an

altered mechanical loading. This may be accompanied by mechanically induced (micro-) bleeds in the unaffected joints, especially since recent evidence shows that FVIII-deficient mice can develop spontaneous bleeding.[51] Providing twice as much burden as a result of the hemarthroses, this could also explain why contralateral alterations are more clearly seen in the two-hit model than in the one-hit model. The relatively long follow-up in our study allows for develop of this contralateral mechanically induced damage. Mice interrupt their activities only temporarily after a joint bleed,[48] in contrast to the human situation, in which an acute hemarthrosis is treated with rest and clotting factor replacement therapy. In addition, a systemic response affecting the contralateral joint, cannot be ruled out. There is evidence of upregulation of pro-inflammatory cytokines and systemic angiogenic stimuli after a joint bleed in rodent models.[24,49,52,53] This might have a repercussion on the (forced loaded) contralateral joint. Moreover, neurogenic inflammation can lead to contralateral damage in rodent models of degenerative joint disease,[54,55] by inducing a bilateral response of the nervous system upon local inflammation resulting in bilateral cartilage damage.[56,57]

In conclusion, this study adds to our understanding of blood-induced joint damage in hemophilic mice, which can be used to optimize its significance as a preclinical model. While joint damage is most pronounced in the patellar compartment, its translational value seems limited. Moreover, the double injury model appears to have no clear added value over the one-hit model. Due to the occurrence of contralateral changes the use of an internal control in studies requiring a longer follow-up is at least debatable.

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Supplementary files

Supplementary Tables 1-3

Scoring systems for histopathological evaluation of inflammation and cartilage degeneration

Table 1 - Valentino Score

Characteristic	Grade	Description
Synovial hyperplasia	0	Normal, less than four cell layers
	1	Four to five cell layers
	2	Six to seven cell layers
	3	More than seven cell layers
Vascularity (400x)	0	None
	1	Less than one-third of the field
	2	One-third to two-third of the field
	3	More than two-third of the field
Discoloration by hemosiderin	0	Absent
	1	Present
Blood (erythrocytes) present	0	Absent
	1	Present
Villous formation	0	Absent
	1	Present
Cartilage erosion	0	Absent
	1	Present

Table 2 - Adapted Koizumi Score

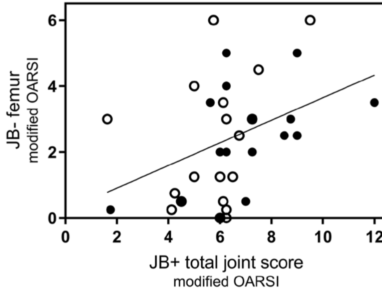
Grade	Peri-patellar inflammation
0	None
1	Slight
2	Moderate
3	Marked

Table 3 - Modified Osteoarthritis Research Society International (OARSI) Score

Grade	Cartilage damage
0	Normal
0.5	Loss of some superficial Safranin O
1	Superficial fibrillation without loss of cartilage
2	Loss of some Safranin O down to the layer immediately below the superficial layer
3	Loss of Safranin O on < 25% of the articular surface
4	Loss of Safrain O on 25–50% of the articular surface
5	Loss of Safranin O on 50–75% of the articular surface
6	Loss of Safranin O on >75% of the articular surface

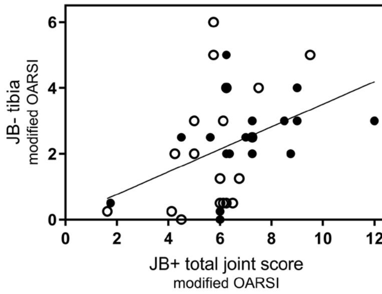
Supplementary Figure

A



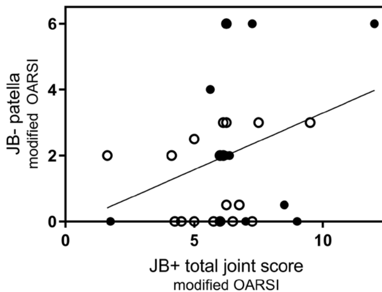
Spearman's correlation
r=0.346, p=0.036

B



Spearman's correlation
r=0.428, p=0.007

C

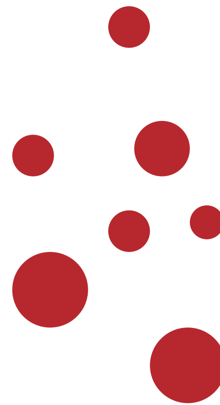


Spearman's correlation
r=0.287, p=0.112

Supplementary Figure 1 - Correlation modified OARSI score
Tibiofemoral and patellar modified OARSI scores of the blood-exposed joints for both the one- (open symbols) and two-hit (filled symbols) model were summed into the total joint score to represent the overall cartilage damage upon hemarthrosis, weighing both compartments equally. The total joint score is significantly correlated to the femoral (A) and tibial (B) cartilage damage of the contralateral leg, while no such correlation is demonstrated for the patella (C). JB= joint bleed.

PART III

EARLY INTERVENTION



Pathophysiology of hemophilic arthropathy and potential targets for therapy

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Abstract

Hemophilia is a congenital clotting factor deficiency characterized by spontaneous and trauma-related bleeding. Spontaneous bleeding shows a predilection for joints, and repeated hemarthroses lead to a disabling condition called hemophilic arthropathy. Treatment of this condition consists of preventing joint bleeding on the one hand and orthopedic surgery as a last resort on the other. Up till now, there is no disease modifying therapy available to fill the gap between these extremes. This review provides an overview of the pathogenesis of hemophilic arthropathy in order to identify potential targets for therapy.

Joint bleeding induces synovial inflammation, cartilage degeneration and bone damage. These processes interact with each other and result in a vicious circle. Hemarthrosis promotes synovial hypertrophy and neoangiogenesis, increasing the susceptibility to mechanical damage and subsequent bleeding. The inflamed synovium affects the cartilage, while cartilage is also directly affected by blood via the release of cytokines and metalloproteinases, and via hydroxyl radical formation inducing chondrocyte apoptosis. Apart from the inflammatory pathways, iron plays a pivotal role in this process, as does the fibrinolytic system.

Considering its pathogenesis, potential targets for disease modifying therapy in hemophilic arthropathy are iron, inflammation, vascular remodeling, hyperfibrinolysis, bone remodeling and cartilage regeneration. So far, iron chelators, anti-inflammatory therapy, anti-fibrinolytics and bone remodeling agents have demonstrated beneficial effects, predominantly in a preclinical setting. There is still a long way to go before these interventions will translate into clinical practice. The most important challenges are: establishing a universal outcome measure to predict efficacy in humans, and determination of the optimal route and timing to administer disease modifying therapy.

Introduction

Hemophilia is an X-linked congenital bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII) in hemophilia A or factor IX (FIX) in hemophilia B. The prevalence of hemophilia A is 1 in 5000-10.000 males,[1] while hemophilia B is estimated at 1 in 25.000-30.000 males.[2] In general, the severity of bleeding is inversely correlated with the clotting factor level. Patients with severe hemophilia (factor level <1%) suffer from spontaneous bleedings, whereas in mild hemophilia (factor level >5%) bleeding only occurs after major trauma or surgery. Spontaneous bleedings occur mainly in the large synovial joints.[3]
The use of prophylactic clotting factor concentrates (CFCs) considerably diminishes the risk of hemarthrosis.[4,5] By factor substitution therapy disease severity changes from a severe in a milder form.[6] Nevertheless, joint bleeding is still a major concern for several reasons. First, the use of CFCs is limited to developed countries due to high costs of treatment. For the majority of patients worldwide regular treatment with CFCs is impossible. Additionally, about one third of patients develop antibodies (inhibitors) against the administered CFCs.[7] Bypassing agents like activated prothrombin complex concentrates or recombinant FVIIa are available, but this require high amounts of resources.[8] So a significant proportion of patients with inhibitors are likely to encounter repetitive joint bleeding after all. Finally, current prophylactic regimens are not able to prevent joint bleeds completely. It has been shown that joint bleeding and joint damage still occur despite adequate prophylaxis.[5,9]
Recurrent hemarthroses ultimately lead to hemophilic arthropathy (HA), a condition in which the synovium, cartilage and subchondral bone are damaged (Figure 1).

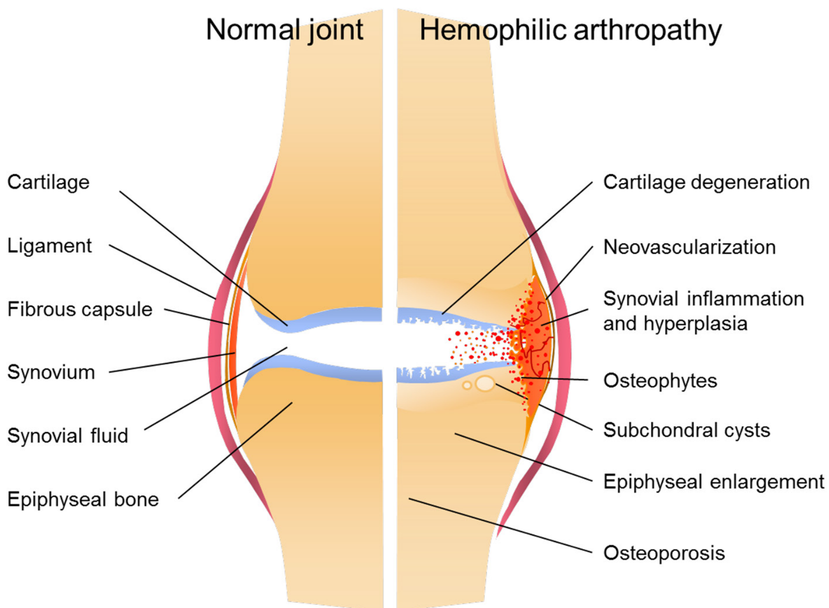

Figure 1 - Schematic representation of a healthy joint and hemophilic arthropathy
Left: healthy joint. Right: structural changes due to recurrent hemarthrosis resulting in hemophilic arthropathy.

CFCs can reduce the risk of developing HA, especially when started at an early age[10,11] and in case of good compliance,[12] but cannot entirely prevent it. In up to half of patients with severe hemophilia signs of HA are present.[13] HA is a disabling condition associated with chronic pain, joint impairment and reduced quality of life, often starting at an early age.[14] In case of established HA a conservative approach is preferred. Up till now, there is no disease modifying therapy available to intervene the perpetuating process of HA. In short, treatment of HA consists of preventing joint bleeding on the one hand and orthopedic surgery (especially joint replacement or arthrodesis) as a last resort on the other. New modalities to fill the gap between these extremes are urgently warranted (Figure 2). To explore possible targets for therapy, understanding the pathogenesis of HA is necessary. In this review an overview of the current literature on this topic is given, focusing on potential targets for therapy.

Joint bleedings

Joint bleeding is the distinctive manifestation of hemophilia, but can also occur in the context of Von Willebrand disease,[15] as a complication of anticoagulant treatment,[16] upon trauma[17] or major joint surgery.[18] Irrespective the underlying cause, hemarthrosis can lead to significant joint damage and subsequent major morbidity. In hemophilia, musculoskeletal bleeding episodes account for 80% of all bleeds and most commonly the elbow, knee and ankle are involved[3]. Synovial joints are susceptible to spontaneous bleeds because the synovium is well-vascularized. Mechanical stress is an important factor as suggested by the onset of hemarthrosis with weight bearing. Moreover, local hemostasis in a joint differs from other tissues.[19,20] Initiation of the coagulation cascade in joints is restrained and this imbalance is even more reflected in joint tissue of hemophilia patients.[21] In addition, there

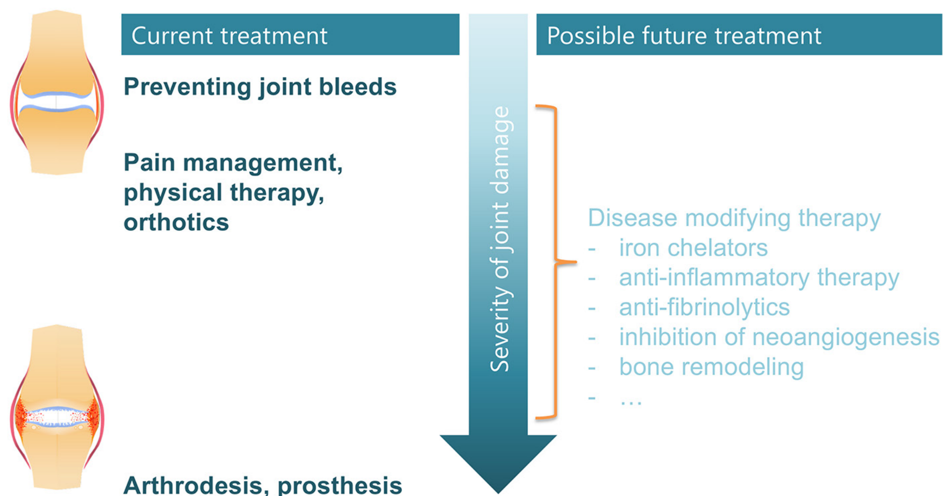

Figure 2 – Current en possible future treatment of hemophilic arthropathy
Current treatment of hemophilic arthropathy consists of preventing joint bleedings on the one hand and orthopedic surgery as a last resort on the other. New modalities to intervene in the process of development of hemophilic arthropathy are urgently warranted.

is a pronounced activation of the synovial fibrinolytic system following hemarthrosis.[22] Joint bleeding in hemophilic mice induces the expression of synovial urokinase-type plasminogen activator (uPA) and the levels of active uPA and plasmin are increased compared to healthy controls.[23] This hyperfibrinolysis results in an accelerated degradation of blood clots in an area very vulnerable to mechanical stress, and as such in prolongation or recurrence of the joint bleed.

Pathogenesis of hemophilic arthropathy

Traditionally, development of HA is characterized by two major processes: synovial inflammation and cartilage degeneration (Figure 3).[24,25] In this respect, HA has certain features in common with both inflammatory joint disease such as rheumatoid arthritis (RA), as well as degenerative joint disease like osteoarthritis (OA).[26,27] Another well-known but less investigated manifestation of blood-induced joint damage is bone damage. As HA progresses, an imbalance between osteoclastic bone resorption and osteoblastic bone formation alters bone remodeling.[28]

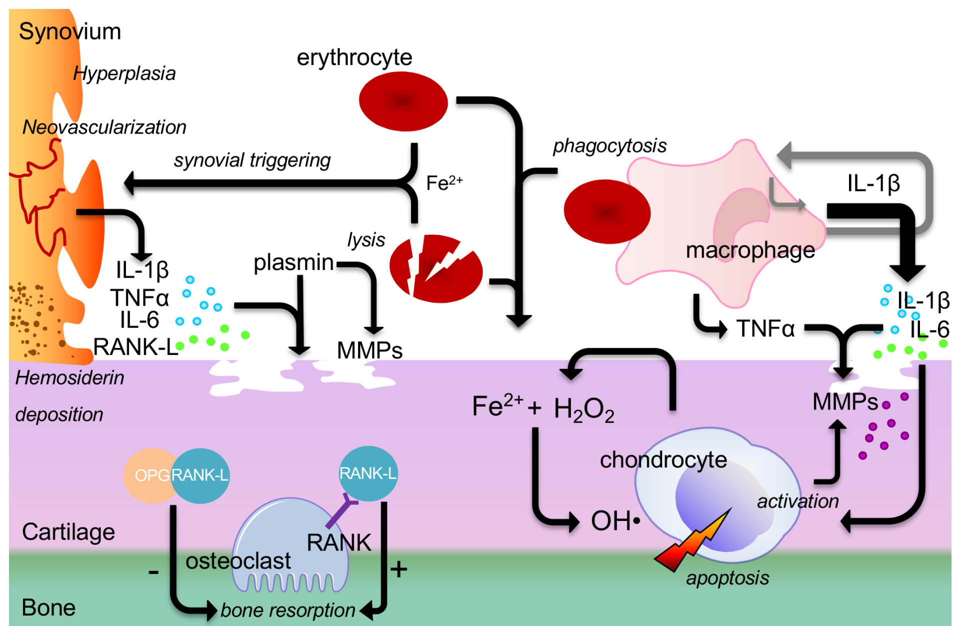

Figure 3 – Schematic representation of the major processes involved in hemophilic arthropathy
Synovitis and cartilage degeneration. Joint bleeding causes synovitis, leading to synovial hypertrophy and vascular remodeling. The inflamed synovium produces plasmin, matrix metalloproteinases (MMPs) and pro-inflammatory cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNFα) that affect the cartilage. Cartilage is also directly affected by blood. Synovial and blood derived pro-inflammatory cytokines stimulate the production of hydrogen peroxide by chondrocytes. In the presence of erythrocyte derived iron (Fe2+), hydrogen peroxide (H2O2) is able to react according to the Fenton reaction, resulting in the generation of very toxic hydroxyl radicals (OH·) causing apoptosis of chondrocytes. Inflammation also activates the Receptor Activator of NF-κB Ligand(RANK-L)-RANK-Osteoprotegin(OPG)-pathway, resulting in bone resorption by osteoclasts. OPG protects bones from excessive resorption by binding to RANK-L instead of RANK.

6

Synovitis

Following an acute episode of hemarthrosis, it takes about a week before the blood is cleared from the joint cavity by the synovial lining cells.[29] Macrophages and other inflammatory cells migrating to the joint contribute to this removal process.

In case of repeated extravasations or ongoing bleeding, the amount of blood exceeds the synovial removal capacity. Erythrocyte derived iron accumulates as synovial hemosiderin deposits,[30] which triggers synovial inflammation.[31,32] It is demonstrated that macroscopically hemosideritic synovium contains considerably more inflammatory cytokines than normal tissue.[30] Nuclear factor kappa B (NF-κB)-associated signaling pathways are key in inflammation, and also in a murine hemophilia model its up regulation is demonstrated. A joint bleed led to up regulation of several genes of the NF-κB pathway and their responsive pro-inflammatory cytokines like interleukin (IL)-1β, IL-6, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα).[33] This is in line with observations of the role of NF-κB in synovitis[34] and cartilage degeneration in osteoarthritis[35] and rheumatoid arthritis.[36] In addition, patients with HA exhibit high expression of synovial levels of Receptor Activator of NF-κB (RANK).[37]

The presence of iron transforms the thin synovial membrane into a hypertrophic, villous membrane, via induction of DNA-synthesis and cell proliferation. Iron stimulates the amplification of c-myc, a proto-oncogene associated with cell proliferation,[38] and mdm2, a protein that targets the p53 tumor suppressor gene, thereby inhibiting synovial cell apoptosis.[39]

The inflamed and hypertrophic synovium has an enhanced oxygen demand stimulating the release of growth factors like vascular-derived endothelial growth factor (VEGF). VEGF promotes neoangiogenesis, both locally and systemically.[40,41] Systemic angiogenic factors released in response to joint bleeding can also lead to hypervascularity in otherwise unaffected joints.[42]

The combination of iron, inflammation, hypertrophy and neovascularization can result in a vicious circle. Hemarthrosis leads to synovitis; the affected synovium thickens, making it more susceptible to mechanical damage and via vascular remodeling more vulnerable to subsequent bleeding. In this way a so called target joint can develop, which occurs in about 25% of patients with severe hemophilia. A target joint is clinically defined as a joint in which 3 or more spontaneous bleeds occur within a consecutive 6-month period.[43] Over time, recurrent hemarthroses lead to chronic synovitis and ultimately the synovium becomes fibrotic.

Cartilage degeneration

Cartilage degeneration following a joint bleed results both from synovial dependent and independent mechanisms. First, hemophilic synovitis forms an invasive and destructive layer (pannus) over the cartilage surface.[44] Pannus tissue is composed of aggressive macrophage- and fibroblast-like mesenchymal cells and other inflammatory cells that release collagenolytic enzymes.[45] Further degradation of the cartilage matrix is provoked by synovial derived pro-inflammatory cytokines, plasmin and matrix metalloproteinases

(MMPs).[23,28,30,46] Pro-inflammatory cytokines cause cartilage degradation via MMPs and aggrecanases. Plasmin contributes to cartilage damage directly by inducing proteoglycan release in human cartilage,[23,47] or indirectly via activation of pro-MMPs.[48] MMPs are endopeptidases involved in the degradation of extracellular matrix components, like collagen and proteoglycans.[49] In addition, plasmin is able to influence cell signaling via proteinase activated receptors (PARs) resulting in synovitis and cartilage degradation.[50] Upon joint bleeding an increased expression of PARs is found in chondrocytes and synovium.

Besides this synovium dependent mechanism, blood also exerts a direct harmful effect on cartilage. Cartilage is a rather inert tissue, consisting of chondrocytes and extracellular matrix. Chondrocytes are responsible for matrix synthesis, and rely on synovial fluid for nutrients as cartilage lacks blood supply. Blood exposure causes extracellular matrix degradation as well as chondrocyte apoptosis. Short exposure to a low amount of blood already leads to prolonged and irreversible disturbances in matrix turnover,[51,52] still present ten weeks after initial blood exposure.[53] These permanent disturbances in matrix turnover are the result of chondrocyte apoptosis induced by oxidative stress. Synovial and blood derived pro-inflammatory cytokines stimulate the production of hydrogen peroxide by chondrocytes. In the presence of erythrocyte derived iron, hydrogen peroxide is able to react according to the Fenton reaction, resulting in the generation of very toxic hydroxyl radicals and subsequent apoptosis of chondrocytes.[54]

Bone damage

As the development of synovitis and cartilage degeneration progresses, the underlying bone becomes affected. Bone changes result from a disturbed equilibrium in bone resorption and bone formation, leading to a decrease in bone mineral density (BMD) and osteoporosis.[55-57] A decreased BMD is found both in children[58-60] and older hemophilia patients,[61-63] and local osteoporosis is also a feature of arthropathy. Other bone changes in patients with hemophilia are cyst formation, subchondral sclerosis, osteophyte formation and epiphyseal enlargement.[64] While bone damage in hemophilia patients is considered a late phenomenon, both in hemophilic rats and mice excessive bone remodeling was seen as early as two weeks after induced joint bleedings.[65,66] These severe bone changes in animal models might be an enhanced reflection of the human situation, due to differences in matrix turnover rate, cartilage thickness and joint biomechanics.[67]
The exact mechanism by which joint bleeds cause bone damage is largely unknown. One can discuss whether bone damage is an indirect or direct effect of hemarthrosis. Acute hemarthrosis and chronic HA lead to local disuse and generalized decline in physical activity. As such, it may negatively affect BMD by decreasing peak bone mass as well as increasing bone resorption.[68] Moreover, infection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) and their corresponding treatments may affect BMD negatively.[63,69-71]
Local changes in bone turnover upon hemarthrosis are hypothesized to result from changes in the RANK-Ligand(RANK-L)/RANK/osteoprotegin(OPG)-pathway, an important pathway in bone resorption induced by inflammation.[72-75] RANK-L is mainly expressed on osteoblasts/stromal cells and is synthesized by reactive lymphocytes and synovial cells.[72] By binding to

its receptor RANK, it stimulates bone resorption by osteoclasts.[28] OPG acts as a decoy receptor and competes with RANK for the binding to RANK-L. By preventing the interaction between RANK-L and RANK, OPG protects bones from excessive resorption. In synovial tissue of hemophilia patients with severe arthropathy an increased expression of RANK and RANK-L and a decreased expression of OPG is demonstrated, which favors osteoclastic differentiation and thus bone resorption.[37]
The question remains whether joint injury is the primary cause of bone loss in hemophilia or a contributing factor. In FVIII deficient mice a decrease in BMD was demonstrated despite having experienced joint bleedings.[76] Several molecular mechanisms are hypothesized to directly impact bone density in FVIII deficiency. A decreased thrombin production[77] results in less thrombin induced PAR-1-mediated proliferation of osteoblasts.[78] Moreover, FVIII deficient mice were more likely to have undetectable levels of two important bone regulating cytokines, IL-1α and interferon-β, thereby theoretically inducing bone resorption.[79]
In summary, hemarthrosis leads to iron deposition, inflammation, synovial proliferation, cartilage degradation, neoangiogenesis, and fibrinolysis, making the joint vulnerable to repeated bleeds and as such inducing a vicious cycle. Moreover, bone damage results from a multifactorial process, of which hemarthrosis, amongst others, is a major contributor.

Targets for treatment

Based on current knowledge of the pathogenesis of HA, potential targets for treatment can be identified. New treatment options preferably interrupt the vicious circle and are directed against iron deposition, inflammation, hyperfibrinolysis or bone remodeling (Table 1).

Iron chelating agents

As described above, a pivotal role for iron in blood-induced joint damage is suggested, so an approach of defusing iron would be of interest. So far, only two studies were conducted to evaluate the effect of iron chelating agents. In non-hemophilic rabbits, homologous blood was injected in the knee accompanied by deferoxamine, a parental iron chelator. Deferoxamine reduced synovial and cartilage degeneration assessed by histopathology.[80]
In another study, hemophilic mice were randomized to receive placebo or oral deferasirox prophylactically 8 weeks before and 5 weeks after an induced joint bleed. Although deferasirox had no effect on synovitis, it limited cartilage damage significantly (Figure 4).[81]
Interestingly, besides its iron chelating properties, deferasirox acts as a potent NF-κB inhibitor in leukemia cells exhibiting hyperactivity of the NF-κB pathway. This is an iron-independent mechanism not relying on the reactive oxygen species scavenging properties of the drug.[82] As the NF-kB pathway is induced in hemophilic arthropathy, deferasirox may have two underlying pathways to exhibit its beneficial effect, a phenomenon not shared by other chelators.

Table 1 – Potential targets for therapy based on the pathogenesis of HA and their stage in research

Target	Intervention	Administration	Stage in research	Effect			Refs
				Cartilage	Synovium	Bone	
Iron	Iron chelators						
	• Deferoxamine	i.a.	*In vivo*	+	+		[80]
	• Deferasirox	p.o.	*In vivo*	+	-		[81]
Inflammation	Anti-inflammatory therapy						
	• IL-4+IL-10	i.a.	*In vitro + in vivo*	+	-		[83, 85]
	• IL-1β	-	*In vitro*	+			[87]
	• TNFα	-	*In vitro*	-			[87]
Fibrinolytic system	Anti-fibrinolytics						
	• Antiplasmin	i.a.	*In vivo*	+	+		[93]
	• Amiloride	i.a., p.o.	*In vivo*	-	-		[93]
	• PAR-inhibitor	i.a.	*In vitro + in vivo*	+	+		[50]
Bone remodeling	Bone remodeling agents						
	• Bisphosphonates	p.o.	*In vivo*			+	[94]

i.a. = intra-articular, p.o. = per os, + = beneficial effect, - = no beneficial effect. IL = interleukin, TNFα = tumor necrosis factor-alpha, PAR = protease activated receptor

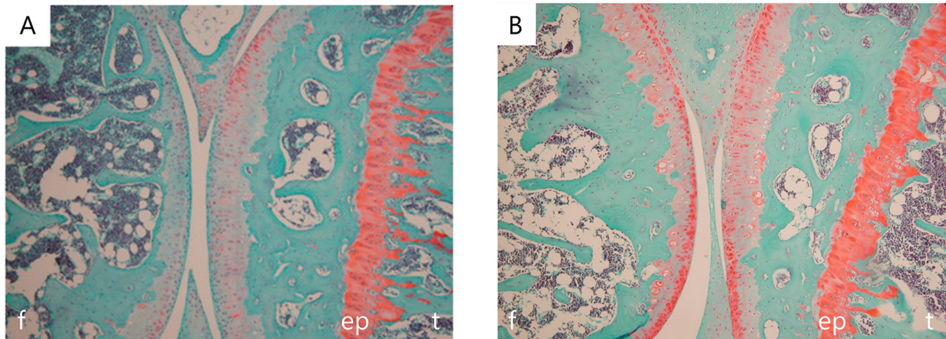

Figure 4 – Representative photomicrographs of knee joints of hemophilic mice
Hemophilic mice were treated for five weeks after joint bleed induction with control (A) or deferasirox (B).[81] The intensity of Safranin O staining (red color) is directly proportional to the proteoglycan content in the cartilage and is a measure of cartilage damage. In the deferasirox group the intensity of Safranin O staining is more intense, indicating that deferasirox prevents cartilage damage. F = femur, t = tibia, EP = epiphysis

Anti-inflammatory therapy

More research is conducted regarding the effect of anti-inflammatory therapy. IL-4 and IL-10 are cytokines with well-known suppressive effects on pro-inflammatory cytokine production and an experimentally demonstrated cartilage-protective and analgesic efficacy. The combination of IL-4 and IL-10 completely prevents cartilage damage upon blood-exposure *in vitro.*[83] Even when added 8 hours after blood exposure the outcome is favorable.[84] In the previously mentioned hemophilic mouse model, an intra-articular injection with the combination of IL-4 and IL-10 after induction of hemarthrosis, is able to alleviate

cartilage damage,[85] but without a clear effect on synovitis. Synovitis can be limited by an IL-6 receptor antagonist added to clotting factor replacement after joint bleeding.[86] A significant decline in synovial hyperplasia, hemosiderin deposits and infiltration of macrophages is demonstrated.

Recently, the role of IL-1β and TNFα was studied *in vitro*.[87] Antagonizing IL-1 by recombinant human IL-1β monoclonal antibody or IL-1 receptor antagonist had a protective effect on blood-induced cartilage damage in a dose- and time-dependent manner. Cartilage matrix proteoglycan turnover nearly normalized when higher concentrations were used. On the contrary, addition of a TNFα monoclonal antibody did not ensue cartilage protection.

Despite the clinical availability of several anti-inflammatory agents, translation into clinical trials regarding blood-induced joint damage is still awaited. Blocking IL-1 has been successfully applied in other joint diseases. Conditions effectively treated vary from rheumatoid arthritis and gout to auto-inflammatory syndromes.[88] In addition, in two cases of iron-induced arthropathy of the hand due to hemochromatosis, a disorder sharing several characteristics with HA,[89] therapeutic efficacy of an IL-1 receptor antagonist (IL-1RA) was demonstrated.[90] There is less evidence of blocking IL-1 in degenerative disease, although some beneficial effects were seen in small numbers of patients with erosive osteoarthritis[91] and posttraumatic osteoarthritis.[92]

Interference with the fibrinolytic system

Reducing the effect of plasmin through inhibition of the synovial fibrinolytic system could be another target for therapy. An intra-articular injection with antiplasmin in hemophilic mice prevented synovitis and cartilage degeneration.[93] On the contrary, treatment with amiloride, a specific uPA-inhibitor, was ineffective. The effect of plasmin can also be reduced by silencing PARs. In a murine hemophilia model intra-articular treatment with small interfering RNA targeted against PAR1-4 attenuated synovitis and cartilage damage upon joint bleeding.[50] *In vitro*, silencing of PAR1-4 reduced plasmin-induced cartilage damage of human tissue explants.

The effect of tranexamic acid, an oral inhibitor of plasminogen activation, has not been studied in this perspective.

Bone remodeling agents

Bone damage is a late phenomenon of hemophilic arthropathy. Bisphosphonates diminish the activity of osteoclasts, resulting in maintained or increased BMD.[57] In one relatively small prospective study, ten hemophilia patients with an increased fracture risk were monthly treated with the oral bisphosphonate ibandronate.[94] After a 12 months follow-up, ibandronate significantly improved spinal BMD and reduced bone resorption.

Inhibition of RANK-L could be an attractive alternative for treating hemophilia related osteoporosis. In postmenopausal women with low bone mass, the RANK-L inhibitor denosumab was equally effective compared to bisphosphonate therapy.[95] To our knowledge, this treatment modality has not been studied in hemophilia patients.

Other approaches

In theory, inhibition of neoangiogenesis using VEGF-inhibitors could be effective to prevent

the formation of new brittle blood vessels that can give rise to new bleeds. Monoclonal antibodies directed against VEGF are clinically available and used to treat certain metastatic cancers[96-98] and ocular diseases characterized by neoangiogenesis.[99,100] So far, no preclinical or clinical research has been conducted focusing on HA.
A different approach is to pursue cartilage regeneration via bone marrow stimulation, osteochondral auto-/allograft transplantation, administration of mesenchymal stem cells and growth factors, and joint distraction.[101,102] Although interesting, these techniques are beyond the scope of this review.

Discussion

Our knowledge of the pathogenesis of HA has dramatically increased in the past decennia. Inflammation and the presence of iron are key factors in the processes of synovitis, cartilage degeneration and bone remodeling. Currently, treatment to abort or turn around these processes is lacking. As clotting factor substitution is a very expensive treatment and not ubiquitally available, there is an urgent need for affordable treatments abrogating the vicious circle in the pathogenesis of HA.

In this review several promising targets have been identified with beneficial effects on cartilage, synovium or bone. Often it is assumed that synovitis and cartilage degeneration develop in parallel, as an expression of the same process. However, based on clinical observations in minimally treated patients a dissociation between these two manifestations is hypothesized.[103] Some patients develop severe cartilage degeneration without the synovium being affected to the same degree, or the other way around. Possibly, the emphasis on several pathways involved in the development of HA varies amongst patients. This two-compartment model can explain why some interventions, like deferasirox and IL-4 plus IL-10, only limit cartilage damage and not synovitis, in the homogenous group of hemophilic mice.[81] This model underlines the importance of targeted therapy on an individual level.

It is still a long way before targeted therapies will be translated into clinical practice and embedded in standard care. It has been estimated that it takes 17 years on average for research evidence to find its way to clinical practice.[104] Up till now some targets are just conceptual thoughts, whereas others have been investigated in a preclinical setting. To our knowledge, the only therapy tested clinically is bisphosphonate therapy. Of the interventions studied in animal models and demonstrating a beneficial effect, only the iron chelators already have several approved applications which can accelerate translation. IL-1 blocking agents are clinically available, but its therapeutic efficacy in blood-induced damage has only been demonstrated *in vitro*.

Assessing the viability of new targets remains a challenge. First, research so far is mainly conducted in *in vitro* and animal models. The main limitation of *in vitro* work is studying joint tissue, e.g. cartilage, lacking the context of surrounding structures, as well as the inability

of resembling an ongoing or repeated bleed. For *in vivo* studies, caution is needed when extrapolating results from experimental animal models to the human situation. The murine studies are based on a homogeneous population, while patients form a heterogeneous group with many different factors modifying disease outcome. A major complicating factor for conducting therapeutic studies in hemophilia patients is the lack of a universal and reliable outcome measure to detect small changes in joint status.

A second obstacle to overcome is the route of administration. In most murine studies, anti-fibrinolytics and anti-inflammatory modalities are administered via intra-articular injection. With clotting factor substitution, HA is more and more a disease restricted to a single joint. As such, it makes sense to administer the therapy locally ensuring the affected site is reached and adequate levels of the active compound are achieved. Conversely, an intra-articular injection is not preferred considering the bleeding tendency of hemophilia patients and the risk of infection.

Finally, the issue of determining the optimal time frame to administer disease modifying treatment has to be addressed. Current investigational treatment modalities have only been applied before or shortly after joint bleeding induction *in vivo* or blood exposure *in vitro*. As a consequence continuous treatment is required since joint bleeding cannot be predicted. Treatment "on demand" would be more convenient and ameliorates compliance. A related dilemma is the treatment of established HA, will the aforementioned targeted therapies still be beneficial? In order to answer this, special attention is required to improve non-invasive diagnostics methods to prospectively monitor joint damage, and to detect early stage HA. Since a single bleeding already causes joint damage, quantifying early changes is essential to evaluate possible treatment strategies. Further research must address these issues.

Conclusion

Disease modifying treatment for HA to fill the gap between factor replacement therapy and orthopedic surgery is urgently warranted. Based on the pathophysiological processes discussed in this review iron chelating, anti-inflammatory therapy, anti-fibrinolytics and bone remodeling agents seem to be the most promising targets in this respect. However, we still have a long way to go for use in clinical practice, as most options are only tested in a preclinical setting.

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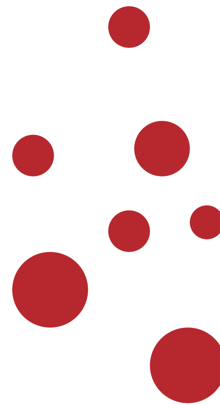

On-demand treatment with the iron chelator deferasirox is ineffective in preventing blood-induced joint damage in hemophilic mice

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Abstract

Introduction
Early intervention in the devastating process of hemophilic 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 hemophilic mice demonstrated that treatment with the iron chelator deferasirox (DFX) 8 weeks before joint bleed induction, attenuated cartilage damage upon blood exposure. However, in hemophilia 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 hemophilic mice served as controls.

Results
A joint bleed resulted in significant inflammation and cartilage damage in the blood-exposed joint compared to those of control animals, in both the placebo and DFX group (all $p=<0.05$). No differences in tibiofemoral or patellar inflammation ($p=0.305$ and $p=0.787$, respectively) nor cartilage damage ($p=0.265$ and $p=0.802$, 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 hemophilic mice. DFX seems unable to reach the joint in time to exert its effect before the irreversible harmful process is initiated.

Introduction

Spontaneous joint bleeding is a characteristic manifestation of the inherited coagulation disorder hemophilia. 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 of 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 hemophilia patients encounters recurrent joint bleeding, ultimately leading to the disabling condition hemophilic 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 (Fe2+) 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 hemophilic mice to study the effect of DFX on blood-induced joint damage.[15] Mice were treated prophylactically with DFX, systemically administered eight weeks prior to induction of a joint bleed. Treatment was continued for an additional five 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, hemophilic patients without systemic iron overload should use DFX chronically, exposing them to undesirable side effects like renal insufficiency.[14] Therefore, the present study evaluated the effectiveness of on-demand DFX treatment initiated immediately after joint bleed induction in hemophilic mice.

Materials and methods

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 hemophilic 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 four 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).

Joint bleed induction

Mice were anesthetized 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 micrometer caliper.[18] An increase in JD less than 0.5mm 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.

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 0.2mg/ml, within the range of attainable concentrations reported in literature.[20] Adjusted for the average weight of a mouse [30g] and daily water intake [15ml/100g], 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 five weeks of the experiment. To prevent precipitation, a new solution was prepared and provided three times a week.

Blood analysis

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

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] is 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 hemophilic mice.[27] Therefore, evaluation of the patellar compartment is included in this study.

Perls prussian blue staining was performed to evaluatie the presence of synovial iron deposits. Synovial inflammation in the tibiofemoral compartment was scored on hematoxylin-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 was 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 were used.

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 $p<0.05$. Graphic presentation and statistical analyses were performed using GraphPad Prism (Version 8.0.1; GraphPad Software Inc, San Diego, CA, USA).

Results

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=0.246). A clear increase in the diameter of the experimental joint was seen two days after joint bleed induction as compared to the baseline value (Figure 1A and B; both groups p=<0.001). The VBS also increased after the joint bleed (Figure 1C and D; both groups p=<0.001). No differences in JD or VBS of the experimental joint were found between the treatment groups at day 2 (p=0.364 and p=0.322, 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 to 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=0.018 and p=0.057, respectively). The decrease was not statistically significantly different between both treatment groups (p=0.208).

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 to the contralateral joint in the placebo group (Table 1 and Figure 3A; median score +3, p=<0.001), as well in the DFX group (median score +2.3, p=<0.001). The contralateral joint of the placebo and DFX group showed comparable tibiofemoral inflammation (p=0.842), but this was significantly increased compared to the bleeding naive control animals (p=0.005 and p=0.018, respectively). The Valentino score in the experimental joint did not differ between the placebo and DFX group (p=0.305), 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

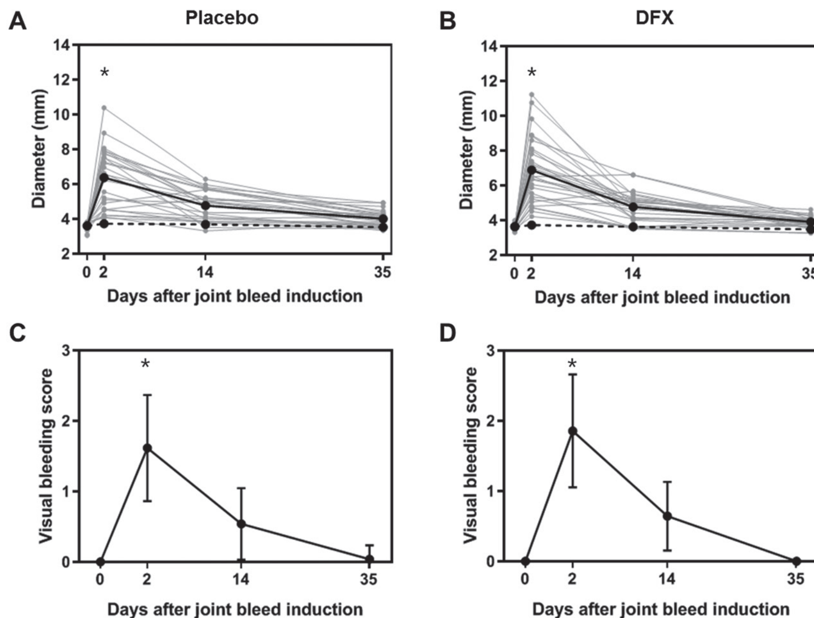

Figure 1 - Joint diameter and visual bleeding score increased 2 days post-hemarthrosis
Change in joint diameter (JD) in the blood-exposed joint over time, in the placebo (panel A) and deferasirox (DFX; panel B) treated group, as well as visual bleeding score (VBS) in the placebo (panel C) and DFX group (panel D). A and B: JD was measured using a micrometer caliper 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= <0.001 for comparison with baseline (day 0), Wilcoxon signed rank test.

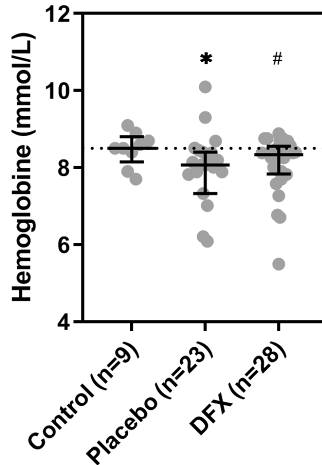

Figure 2 - Hemoglobin values slightly decreased at day 35
Hemoglobin (Hb) values are assessed in whole blood at the day of euthanasia (day 35). Two data points in the placebo group: Hb 2.0 and 3.5 mmol/L were outside the axis limits, but included in the analysis. Dotted line represents the median of the control group. Data depicted as median with interquartile range, *p=0.018, #p=0.057, one-tailed Mann-Whitney test. The levels of both treatment groups were not statistically significantly different (p=0.208).

treatment groups (Figure 3B; p=0.506). 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 inflammation, an increase in peri-patellar inflammation was observed in the experimental joint compared to the contralateral joint in the placebo and DFX group (Table 1 and Figure 3C; both groups median score +3, p=<0.001). Comparison between the treatment groups demonstrated no differences between the contralateral nor the experimental joints (p=0.214 and p=0.787, respectively). Also, the change in adapted Koizumi score did not differ between both groups (Figure 3D; p=0.794).

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 to the joints of the bleeding naive control animals (Table 1; both p=0.003). Also, the contralateral joints of the placebo and DFX group demonstrated a significant increase in tibiofemoral cartilage degeneration compared to the control animals (Table 1; p=<0.001 and p=0.002, 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=0.265 and 0.802, 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=0.143).

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=<0.001) and DFX group (median score +5.5, p=<0.001). No differences in the contralateral (p=0.802) or the experimental joint (p=0.882) 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

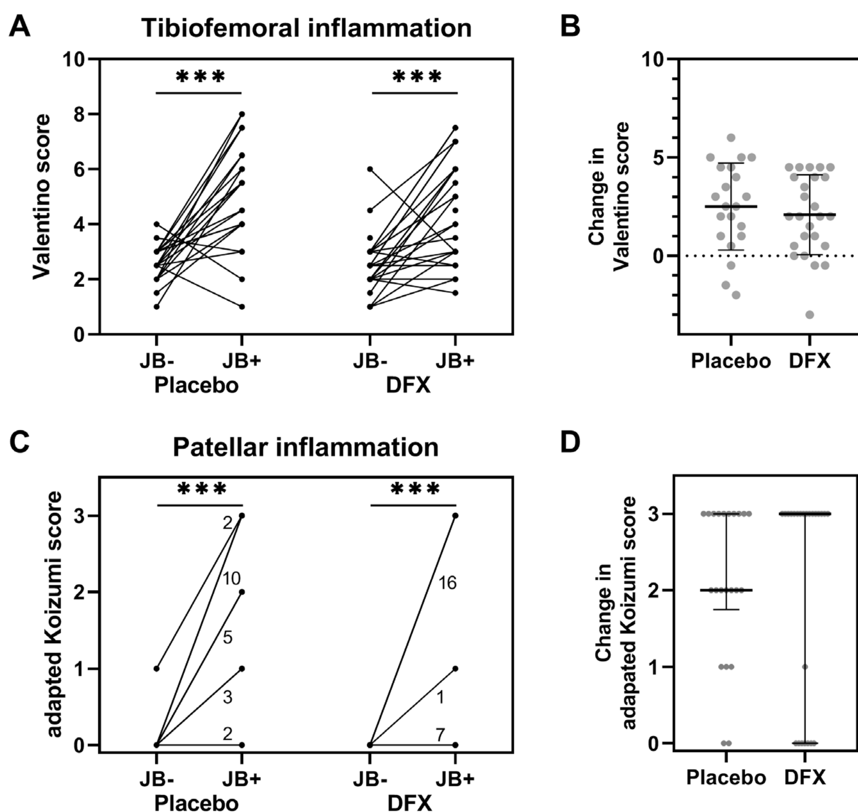

Figure 3 - No effect of DFX on tibiofemoral and patellar inflammation
Clear inflammation was demonstrated in the tibiofemoral compartment (A/B; Valentino score) and patella (C/D; adapted Koizumi score, numbers indicate how many animals are represented by a black line) of the blood-exposed joint 35 days 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=<0.001 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.

contralateral joints of both treatment groups were significantly increased compared to the bleeding naive control animals (p=0.001 and p=<0.001, respectively). The change in patellar cartilage degeneration is comparable between the treatment groups (Figure 5D; p=0.536).

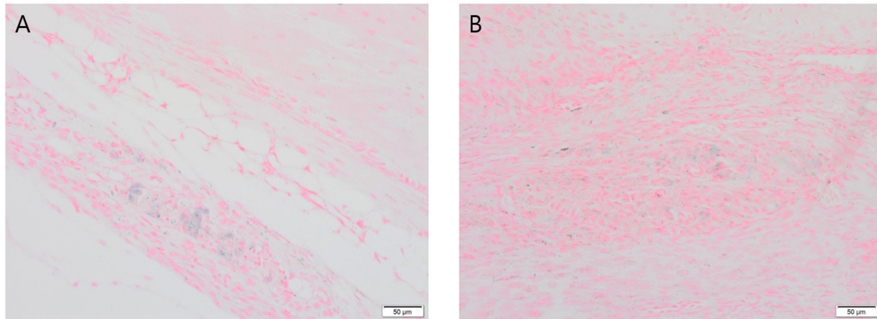

Figure 4 - On-demand treatment with DFX does not affect synovial iron staining
Representative photomicrographs of the synovial Perls Prussian blue staining of experimental knees, demonstrating similar presence of iron (indicated by the blue color) in the control (A) compared to the defersirox (DFX) group following a joint bleed. Magnification: 10x.

Table 1 - Histological joint damage

	Control group	Placebo group			DFX 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)	<0.001	2.5* (2.0-3.0)	4.8* (3.0-6.0)	<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)	<0.001	0.0 (0.0-0.0)	3.0* (0.0-3.0)	<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)	0.012	0.5* (0.3-3.5)	0.5* (0.1-2.5)	0.449
Patellar cartilage damage (modified OARSI)	0.0 (0.0-0.5)	0.5* (0.0-2.5)	5.0* (3.0-6.0)	<0.001	0.5* (0.4-2.3)	6.0* (2.0-6.0)	<0.001

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. Synovial (in the tibiofemoral compartment) and patellar inflammation was assessed according to the Valentino score (0-10) and adapted Koizumi score (0-3), respectively. Tibiofemoral and patellar cartilage damage was 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=<0.05) compared to control (bleeding naive) animals (Mann-Whitney test).

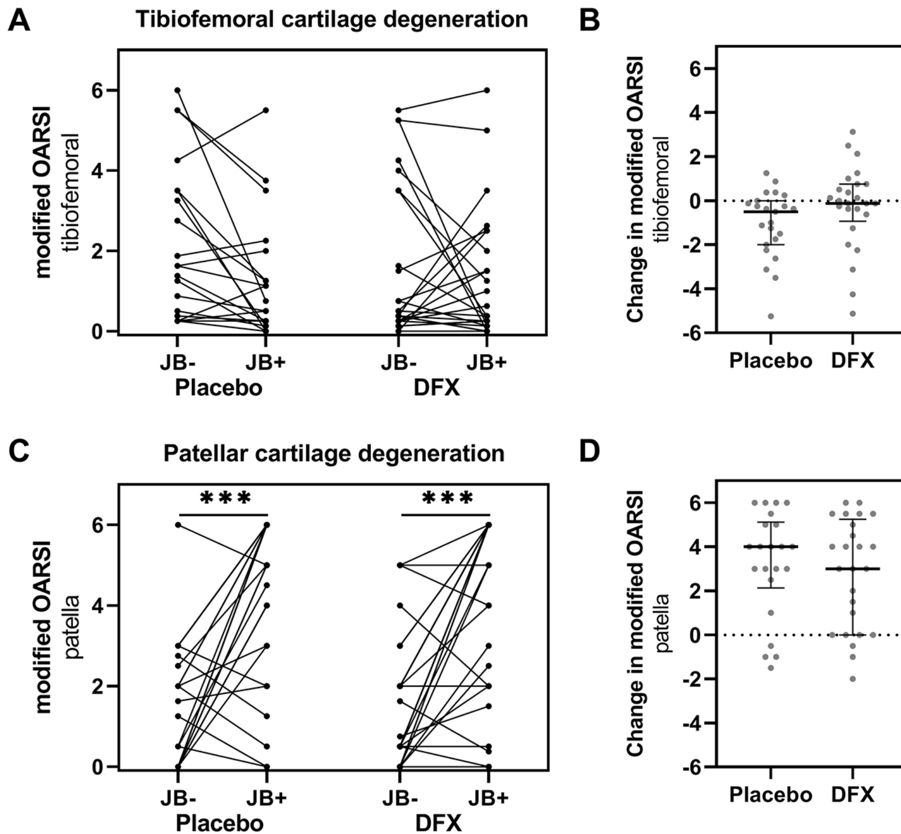

Figure 5 - No effect of DFX on tibiofemoral and patellar cartilage degeneration
The modified OARSI score was applied to the tibiofemoral (A/B) and patellar (C/D) compartment 35 days following hemarthrosis. Comparison of the change in modified OARSI (score of blood-exposed minus contralateral joint, depicted as median with interquartile range) is presented for the tibiofemoral (B) and patellar (D) compartment. ***p=<0.001 for the difference between the blood-exposed and contralateral joint. No statistical differences between both treatment groups were found. JB= joint bleed.

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 hemophilic 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, 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 hemophilic 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 (e.g. hemosiderin), 3. Reducing / scavenging radical formation 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-hemophilic 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 signaling pathways,[36] which have been demonstrated to play an important role in blood‐induced inflammatory and cartilage degenerative processes in hemophilic 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 like 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 five 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.

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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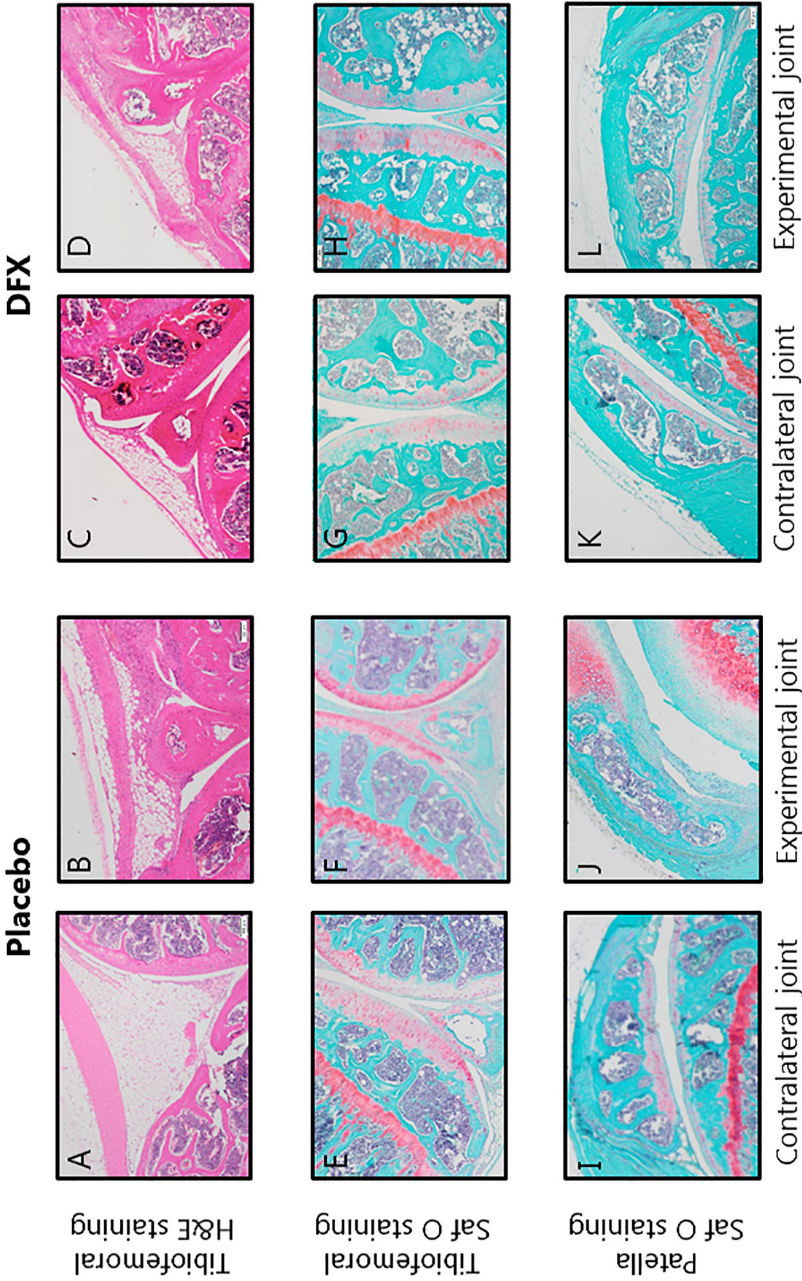
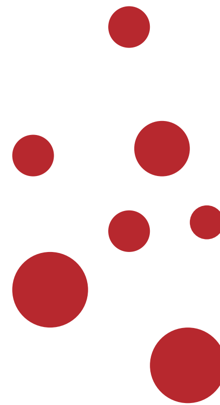
Supplementary data



Supplementary file - Representative images of histological changes upon hemarthrosis
Representative micrographs (magnification 4x) of hematoxylin-eosin (H&E) staining (panel A-D) used to quantify tibiofemoral inflammation and Safranin-O staining (panel E-L) utilized to assess tibiofemoral and patellar cartilage degeneration and patellar inflammation. The contralateral and experimental joint belong to one animal five weeks after experiencing a single bleed.



The iron chelator deferasirox has chondroprotective capacity in blood-exposed cartilage degeneration by reducing nuclear factor-κB expression *in vitro*

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Abstract

Background

In blood-induced joint damage, the interplay between inflammation (with nuclear factor (NF)-κB-pathway considered a major regulator) and iron is pivotal. Deferasirox (DFX) is an interesting therapeutic agent considering its well-established capacity as iron chelator and unique characteristic to inhibit the NF-κB-pathway.

Aim

To evaluate the effect of DFX an *in vitro* model of blood-induced cartilage damage and its possible immunomodulary capacity.

Methods

The effect of different concentrations of DFX on the production of cytokines as downstream products of the NF-κB-pathway was studied in peripheral blood mononuclear cells (PBMC) and whole blood (WB) cultures. Cell viability was assessed by a lactate dehydrogenase assay. The potential direct toxicity of DFX was also tested on cartilage tissue, cultured either in medium or medium with 50% v/v blood, by establishing proteoglycan turnover and evaluating apoptosis. NF-κB expression in cartilage was visualized by immunohistochemistry.

Results

DFX inhibits cytokine production in PBMC and WB culture. In the absence of iron / blood, DFX induces irreversible cytotoxicity in both PBMC and cartilage. However, DFX 0.1 μg/μl limits blood-induced cartilage damage by 9%. Upregulation of NF-κB in cartilage tissue is observed upon blood-exposure and reduced by DFX 0.1 μg/μl.

Conclusion

DFX is able to diminish blood-induced cartilage degeneration *in vitro*, with a role for the inhibition of NF-κB upregulation, though with a narrow therapeutic range due to the observed cytotoxicity in the absence of blood.

Introduction

Joint bleedings occur in the context of the bleeding disorders hemophilia[1] and Von Willebrands disease,[2] as a complication of anticoagulant treatment,[3] after (sports) trauma[4] or major surgery.[5] While a single bleed triggers transient synovial inflammation, the damaging effect on cartilage is profound and long-lasting.[6] The pathophysiology of blood-induced cartilage damage is not fully understood, but the interplay between inflammation and iron is pivotal.[7]
The nuclear factor (NF)-κB pathway is considered a major regulator of the inflammatory response following a joint bleed.[8] Upregulation of this pathway leads to production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)α by blood-derived mononuclear cells,[9-11] the subsequently activated synoviocytes,[12,13] and stimulated chondrocytes.[14,15] In this pro-inflammatory state, the presence of iron triggers oxidative stress and radical formation resulting in permanent cartilage damage.[6,16] Activated mononuclear cells and chondrocytes generate reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2).[17] H_2O_2 reacts according to the Fenton reaction, resulting in the formation of highly toxic hydroxyl radicals. These radicals cause chondrocyte apoptosis and disturb cartilage matrix turnover, resulting in loss of proteoglycans essential for resilience of cartilage tissue.[16] Specifically in an inflammatory environment, the cartilage becomes more vulnerable to subsequent mechanical damage independently from blood exposure, ultimately leading to progressive joint damage.
Targeting iron is an interesting therapeutic approach in blood-induced joint damage.[18] The chondroprotective effect of the iron chelator deferasirox (DFX) has been studied in hemophilic mice in which joint bleeds were induced. Systemic DFX treatment eight weeks prior to joint bleed induction limited cartilage damage upon blood exposure.[19] In contrast to this prophylactic treatment, on-demand treatment was ineffective: no chondroprotective effect was demonstrated when treatment was initiated at the moment of joint puncture.[20] A possible explanation is a difference in DFX concentration in the joint at the moment of the bleed, suggesting that local availability is essential to prevent blood-induced cartilage damage.
The way DFX exerts its chondroprotective effect is unclear and several mechanisms have been suggested.[20] DFX is able to mobilize iron from various tissues,[21,22] eliminate iron systemically,[23] prevent the formation of ROS by binding catalytic iron and scavenges already formed ROS.[24] Interestingly, DFX inhibits an upregulated NF-κB-pathway independently of ROS reduction,[25] a characteristic unique for DFX not shared by other iron chelators. This finding suggests that DFX, besides its well-established iron chelating capacity, may have an immunomodulatory effect that could be used to prevent blood-induced cartilage damage. As such, the aim of the current study is to evaluate the possible immunomodulatory effect of DFX *in vitro* in more detail using peripheral blood mononuclear cells (PBMC), whole blood (WB), and cartilage cultures.

Methods

Blood culture

To study the effect of DFX on the production of cytokines as downstream products of the NF-κB pathway, cultures of PBMC and WB (50% v/v) were performed. The experiments were designed based on the methods described in a paper by Banerjee, demonstrating that TNFα is inhibited by DFX.[26] Peripheral blood samples from healthy adult donors (n=5, 2 male/3 female) were collected in heparinized vacutainer tubes.
PBMC were isolated by Ficoll-Paque (GE healthcare life Sciences, Little Chalfont, United Kingdom) density gradient centrifugation. For WB cultures 50% v/v fresh blood was used. DFX was dissolved in dimethyl sulfoxide (DMSO) and added in a concentration of 0.01, 0.1 and 1 μg/μl for 24 hours. These concentrations were based on a range in plasma concentration between 14-170 μmol/L (0.005-0.068 μg/μl) described in literature.[27] DFX was kindly and unrestricted provided by Novartis Pharma AG 124 (Basel, Switzerland).
Next, PBMC or WB cultures were stimulated by 0.1 μg/ml of soluble anti-CD3 for a subsequent 24 hours. This period was selected because of the fast NF-κB induced inflammatory response in the first 24 hours upon hemarthrosis demonstrated by Sen.[8] Finally, samples of these cultures were collected and centrifuged at 1500g for 10 minutes, and supernatants were stored at −80°C. Pro-inflammatory cytokines TNFα and IL-6, as representative inflammatory mediators for blood-induced cartilage damage,[28] were measured with commercially available enzyme-linked immunosorbent assays (ELISA) (Invitrogen) and analyses were performed according to the manufacturer's instructions.

Cytotoxicity assays

To study whether the effect of DFX on cytokine production was inhibitory or cell toxic, cell viability in PBMC and WB cultures was assessed by a lactate dehydrogenase (LDH) assay (Sigma-Aldrich) according to the manufacturer's instructions. Cytotoxicity is expressed as a percentage of viable cells measured in the control condition without DFX exposure. PBMC and WB experiments were performed as described above.
To investigate a potential protective effect of the presence of iron in WB in contrast to the lack of iron in PBMC cultures, a concentration range of ferric ammoniac citrate (FAC, 0-3-10-30 μM) as a source of catalytic iron was added to the PBMC cultures with the highest concentration DFX (1 μg/μl) present. After 24 hours, the LDH assay was performed.

Cartilage culture

The potential direct toxicity of DFX was also tested on cartilage tissue. Human articular cartilage tissue was obtained either within 24 hours post mortem from the humeral head of donors without known history of joint disorder (healthy cartilage, n=2, mean age 80.5 year ± 9,, all male) or from patients undergoing knee arthroplasty (osteoarthritic (OA) cartilage, n=5, mean age 66.6 year ± 6.7, all male) to mimic affected cartilage observed in patients with hemophilic arthropathy (HA). Collection of cartilage tissue was performed according to local medical ethical regulations.
Full thickness cartilage slices were cut aseptically, excluding the underlying bone, and kept in phosphate buffered saline (PBS), pH 7.4. Within 1 hour after dissection, slices were cut

in cubic explants and cultured individually in a 96-wells round-bottomed microtiter plate at 5% CO2 in air, 37°C, and 95% humidity for 24 hours. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% heat inactivated pooled human male AB serum (Invitrogen), glutamine (2mM), penicillin (100IU/mL), streptomycin sulphate (100μg/mL; all Paisley, UK), and ascorbic acid (85μM; Sigma). After 24 hours, the cartilage explants were weighed aseptically (range 5-15mg; accuracy ±0.1mg) and medium was refreshed.
Explants were exposed for four days to the same dose range of DFX as used for the blood cultures. To differentiate a permanent from a transient effect, the experiment was repeated as described above but followed by a 12-day recovery period in culture medium only without DFX, refreshing the culture medium every four days.
In a subsequent experiment, the effect of DFX on blood-exposed cartilage was tested. Explants were exposed for four days to 50% volume/volume (v/v) WB drawn from healthy adult donors (n=7, heparin tubes (BD Vacutainer®)) and DFX in different concentrations (healthy cartilage: 0.01-0.1-1 μg/μl, OA cartilage: 0.03-0.1-0.3 μg/μl or 0.1 μg/μl only). Four days represent the natural evacuation time of blood from the joint cavity.[29,30] After blood exposure, the explants were washed twice by 20-minutes incubations in culture medium to remove all blood components. Subsequently, a 12-day recovery period in culture medium only without WB or DFX followed, refreshing the culture medium every four days.

Identification of apoptosis in chondrocytes

Apoptosis of chondrocytes was detected using an anti-single-strand deoxyribonucleic acid (ssDNA) monoclonal antibody (clone F7-26/apostain; Alexis Corporation, The Netherlands). Cartilage tissue was permeabilized with 0.2mg/ml saponin (sigma) and 20ug/ml Protease K (Ambion) and heated to 56°C in formamide to denature unstable DNA and then transferred to ice-cold PBS. Sections were incubated with 3% hydrogen peroxide to 1 quench endogenous peroxidase and subsequently blocked with 3% non-fat dry milk. The sections were incubated with anti-ssDNA antibody and the antibody complex was visualized using 3,3'-diaminobenzidine (DAB; Vector). The tissue was then counterstained with hematoxylin (Merck).

Determination of proteoglycan turnover

Each experiment was performed with cartilage from a single donor. To correct for possible biological variation between samples, the mean value of 6-8 individually handled cartilage explants per experimental condition per donor, was taken as a representative value. Proteoglycan synthesis rate was evaluated at the end of each experiment. Sulphate incorporation rate into glycosaminoglycans (GAGs) was determined by addition of Na235SO4 (NEX-041-H carrier free; DuPont; 74kBq per well) for four hours. Subsequently, cartilage explants were washed twice in cold PBS, digested for 2 hours at 65°C with 2% papain (Sigma), and stored at -20°C. GAGs were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (CPC; Sigma) in 0.2M NaCl and dissolved in 3M NaCl. The amount of radioactivity in the GAGs was measured by liquid scintillation counting, normalized to the specific activity of the medium, labelling time, and wet weight of the cartilage explant. Results are expressed as nanomoles of sulphate incorporated per hour per gram wet weight of cartilage tissue (nmol/h*g).

NF-κB expression in cartilage by immunohistochemistry

One explant per condition was embedded in paraffin, cut in sections of 5 μm, and NF-κB expression was visualized by immunohistochemistry. The sections were pretreated using heat-mediated antigen retrieval with sodium citrate buffer (pH 6.0) and blocked for non-specific binding with 5% normal goat serum (Sigma-Aldrich). Next, the sections were incubated overnight with the primary antibody (ab16502 pAb to NF-κB p65, 1ug/ml; Abcam) diluted in 2% normal goat serum at 4°C. Subsequently, the antibody was visualized by addition of horseradish peroxidase conjugated immunoglobulins (DAKO) for 30 minutes at room temperature, following by a 5 minute conversion of diaminobenzidine (DAKO). Sections were counterstained by Mayer's haematoxylin (Merck). NF-κB positive cells in the superficial, middle and deep zone of the cartilage were counted per grid by two independent observers and expressed as a percentage of the total cell count per zone.

Statistical analysis

Differences in cytokine level, cytotoxicity, proteoglycan synthesis and NF-κB expression between control and treatment conditions of blood or cartilage samples from the same donor were analyzed using non-parametric and parametric tests when indicated. P-values <0.05 were considered statistically significant. Graphic presentation and statistical analyses were performed using GraphPad Prism (Version 8.3.0; GraphPad Software Inc, San Diego, CA, USA).

Results

Reduction of cytokine levels by DFX in PBMC and WB cultures

In the first set of experiments the effect of DFX on TNFα and IL-6 production in PBMC and WB cultures was measured. DFX 1 μg/μl reduced TNFα and IL-6 levels in PBMC cultures and TNFα levels in WB cultures significantly (all p=<0.05) and the effect on IL-6 in WB is approaching significance (p=0.08) (Figure 1A and B). DMSO up to 1% tested as a control had no effect on the cytokine production (data not shown).

DFX induces cytotoxicity in the absence of iron

To evaluate whether the observed cytokine reduction was caused by inhibition or cytotoxicity, cell viability assays were performed. In PBMC cultures, DFX induced cytotoxicity in a dose-dependent manner, reaching statistical significance compared to control at the highest concentration tested (1 μg/μl; p=0.002, Figure 2A). Cell viability was not affected by the dissolvent DMSO alone (data not shown). Interestingly, no significant cell death was observed in WB cultures (Figure 2A).

We hypothesized that this difference in cell viability between isolated cells and whole blood was caused by the absence of iron in the PBMC culture compared to the WB culture. Adding iron in the form of FAC to the PMBC culture with DFX 1 μg/μl resulted in a decline of cytotoxicity in a dose-dependent manner (Figure 2B). This effect was significant for the two highest concentrations compared to the control condition without FAC (both p=<0.05), approaching normal cell viability at a FAC concentration of 30 μM.

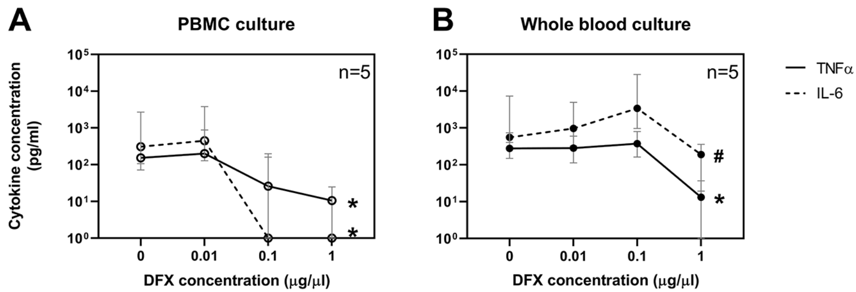


Figure 1 – DFX inhibits cytokine production in PBMC and WB culture
Deferasirox (DFX) in increasing concentrations was added to peripheral blood mononuclear cells (PBMC; n=5, panel A) and whole blood (WB; n=5, panel B) cultures for 24 hours. Subsequently, the cells were stimulated by soluble CD3 and the cytokine release of TNFα (black line) and IL6 (dashed line) were measured in the supernatant after another 24 hours. Data are depicted as median ± inter quartile range. *p=< 0.05, #p=0.08 compared to control condition without DFX, Friedman test.

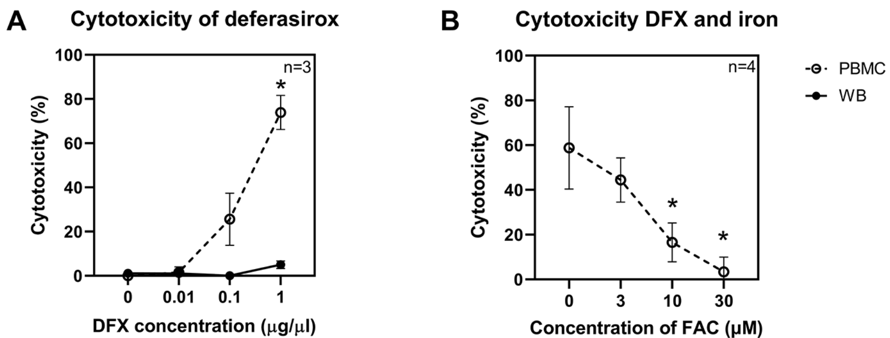
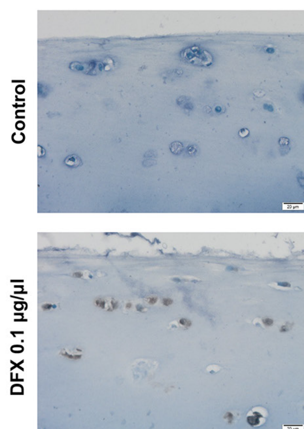


Figure 2 – Cytotoxic effect of DFX
Panel A (n=3): lactate dehydrogenase (LDH) as a measure of cellular cytotoxicity was determined in peripheral blood mononuclear cells (PBMC; dashed line) and whole blood (WB; black line) cultures exposed to Deferasirox (DFX) in increasing concentrations for 24 hours and stimulated for another 24 hours by CD3. Cytotoxicity is expressed as a percentage of viable cells in the control condition without DFX exposure. *p=<0.05 for comparison with control condition without DFX, paired t-test.
Panel B (n=4): ferric ammonium citrate (FAC) and DFX 1 µg/µl were added to PMBC cultures and after 24 hours LDH was measured. *p=<0.05 for comparison with control condition without FAC, paired t-test.
Data are depicted as mean ± SD.

Cartilage is irreversibly damaged by DFX

Apoptosis of chondrocytes is detected in cartilage exposed to DFX 0.1 µg/µl, while cartilage cultured in medium only is unaffected (Figure 3A for representative images). In addition, cartilage cultured in medium without blood and exposed to DFX for four days resulted in a decrease in sulphate incorporation rate, which hardly recovered after 12 additional days of culturing in medium without additives (Figure 3B). The highest concentration of DFX inhibited the proteoglycan synthesis almost completely.



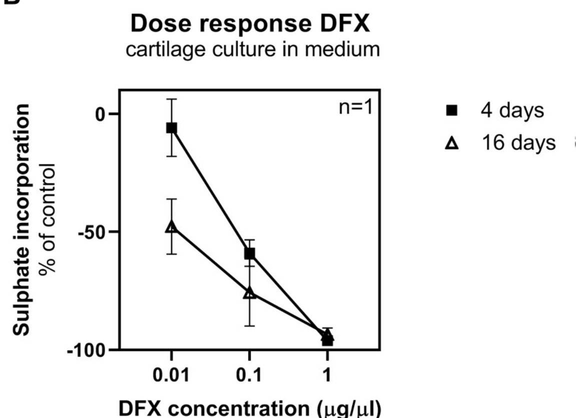


Figure 3 – Irreversible damage by DFX on cartilage cultured in medium
Panel A: representative images of an anti-single-strand deoxyribonucleic acid (ssDNA) stain to visualize apoptosis of chondrocytes after a four day cartilage culture with either medium or medium plus Deferasirox (DFX) 0.1 µg/µl, followed by a 12 day recovery phase in culture medium only. Panel B: Healthy human cartilage was cultured in standard culture medium and a concentration range of DFX was added for four days. Proteoglycan synthesis rate reflected by sulphate incorporation was directly measured after four days (squares, n=1) or after an additional recovery phase of 12 days (triangles, n=1). Sulphate incorporation is expressed as a percentage of the control condition without DFX exposure. Data are depicted as mean ± SD of 8-10 cartilage cubes per condition.

DFX limits blood-induced cartilage damage at 0.1 µg/µl specifically

The potential chondroprotective effect of DFX as shown in hemophilic mice[19] was evaluated in an established and well-validated *in vitro* model of blood-induced cartilage damage. Cartilage exposed to 50% blood resulted in an expected decrease in proteoglycan synthesis, and the addition of DFX in a range of 0.01 to 1 µg/µl for four days had no effect except for the concentration of 0.1 µg/µl. In healthy blood-exposed cartilage a 10% recovery of proteoglycan synthesis by DFX 0.1 µg/µl was observed compared to control without DFX) (Figure 4A).
Repeating the experiment with a narrower concentration range of DFX (0.03 to 0.3 µg/µl) in degenerated cartilage still demonstrated DFX 0.1 µg/µl as the dose that could protect cartilage from blood-induced proteoglycan synthesis inhibition (Figure 4B). To verify this effect of DFX 0.1 µg/µl, blood-exposed osteoarthritic cartilage of 5 donors was used and revealed a significant 9% recovery of proteoglycan synthesis ($p=<0.01$, Figure 4C).

Upregulated NF-κB in blood-exposed cartilage is reduced by DFX

Considering the beneficial effect of DFX in blood-induced cartilage damage, the involvement of the NF-κB pathway in this process, and the known capability of DFX to inhibit NF-κB, prompted us to finally study NF-κB expression (p65) upon blood exposure in cartilage. Blood exposure resulted in a clear upregulation of p65 positive cells in all cartilage zones (surface, middle, and deep zone) compared to cartilage cultured in medium only (all $p=<0.001$) (Figure 5). Addition of DFX 0.1 µg/µl reduced the expression of NF-κB to values nearing those of the control condition without blood nor DFX.

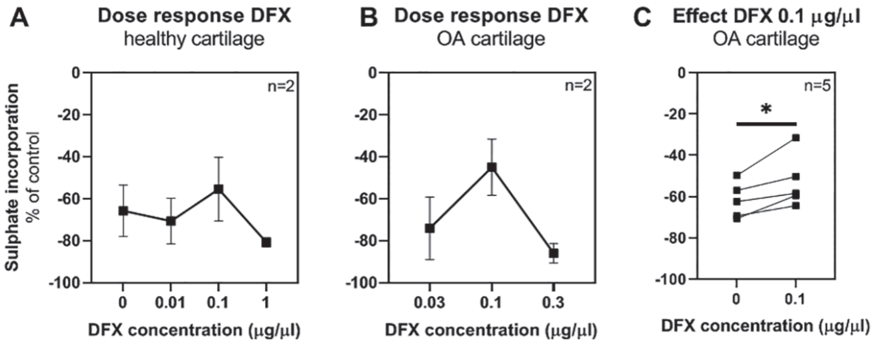


Figure 4 – Protective effect of DFX 0.1 µg/µl on blood-induced cartilage damage
Healthy human cartilage was exposed to blood 50% v/v and a broad concentration range (0.01-1) of Deferasirox (DFX) for four days and cultured in medium only for an additional 12 days (panel A, n=2). Because a slight improvement in proteoglycan synthesis at the dose of 0.1 µg/µl, the experiment was repeated in affected osteoarthritic (OA) cartilage using a narrower concentration range (0.03-0.3) (panel B, n=2) and DFX 0.1 µg/µl as only condition (panel C, n=5).
Sulphate incorporation is expressed as a percentage of the control condition without blood exposure. Data are depicted as mean ± SD. *p=<0.05, paired t-test.

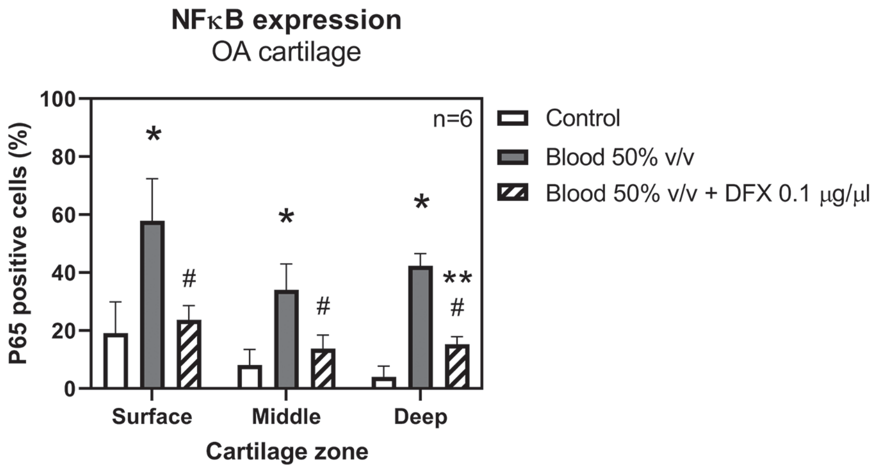


Figure 5 – Upregulation of NFκB in blood-exposed cartilage, reduction by DFX 0.1 µg/µl
NFκB expression reflected by P65 positive cells was measured in cartilage cultured for four days in the absence (control) or presence of 50% blood (with and without addition of DFX 0.1 µg/µl), followed by a 12 day recovery phase. *p=<0.001 control compared to blood exposed cartilage without DFX. #p=<0.05 blood exposed cartilage with and without DFX. No statistical differences between control group compared to blood exposed cartilage with DFX, except for the deep layer (**p=<0.001).

Discussion

This exploratory study aimed to evaluate the therapeutic potential of DFX in blood-induced cartilage damage and gain insight in its workings mechanisms. DFX limits blood-induced cartilage damage *in vitro*, which is in line with previous studies demonstrating that iron chelators have a chondroprotective effect when applied *in vitro*,[31] locally in non-hemophilic animals,[32] and prophylactically in hemophilic mice.[19] The optimum dose in this study was 0.1 μg/μl, while lower concentrations were ineffective and cytotoxicity was observed in higher concentrations. The mechanism of intracellular iron deprivation resulting in apoptosis induced by iron chelators is utilized in treatment of solid[33-35] and hematologic malignancies.[36-38] In short, DFX has a chondroprotective effect, though with a small therapeutic window.
This study indicates that blood-induced cartilage damage is mediated via NF-κB-signaling. Blood-induced cartilage degeneration is mainly driven by iron overload and ROS formation in combination with inflammation, all well-known inducers of NF-κB activity.[39-41] NF-κB is a transcription factor with a central role in inflammation, immune responses, cellular differentiation and survival.[42] Dysregulation of the NF-κB pathway is a disease contributing factor in various disorders, including joint diseases such as OA,[43] rheumatoid arthritis,[39] and appears to be important in HA as well. An upregulation of the NF-κB-pathway has been observed in hemophilic mice upon joint bleeding on a molecular level[8] and in synovial tissue of patients with HA.[44] The current study is the first to demonstrate upregulation of the NF-κB expression in human cartilage tissue upon blood-exposure. While NF-κB is considered an acute phase modulator of inflammation following hemarthrosis in hemophilic mice,[8] the cartilaginous expression persisted after a recovery period in the present study. This discrepancy may be due to the lack of synovial reabsorption of residual blood in the *in vitro* culture.
This is the first study describing the inhibition of NF-κB expression by DFX in cartilage, but this effect has been observed in preclinical studies involving other tissue or cell lines than cartilage.[25,26,35,45-47] Gene expression analysis in low risk MDS patients treated with DFX revealed that 14 weeks therapy induced downregulation in several genes associated with the NF-κB pathway as well.[48] DFX is capable to interfere with the initiating factors of the NF-κB pathway, by reducing radical formation by binding catalytic iron,[24] and scavenging the already formed ROS,[25] thereby reducing H_2O_2-induced apoptosis.[49] The decrease in pro-inflammatory cytokines as demonstrated by this and previously published *in vitro* studies,[26] suggests that DFX can suppress inflammation, but literature on this effect *in vivo* is indecisive.[50-52] In addition, there is evidence that DFX inhibits the NF-κB-pathway directly without affecting its proximal activation.[26]
The main limitations of this exploratory study are the low n-value and the use of an *in vitro* model lacking synovial tissue. In this *in vitro* study a 9% damage reduction is noted, while a complete chondroprotective effect observed in hemophilic mice receiving DFX prophylaxis suggests an additional synovial mediated effect.[19] Nevertheless, the direct and protective effect on cartilage in the presence of blood highlights the importance of local efficacy. Administration of DFX remains a challenge in a clinical setting since oral on-demand treatment with DFX was ineffective in hemophilic mice,[20] and persisting concerns about intra-articular treatment, despite available adequate clotting factor substitution. Moreover,

hemarthroses and flares are sometimes difficult to distinguish for hemophilia patients,[53] which is relevant since previous[31] and the current study demonstrate a cytotoxic effect of DFX in the absence of blood.

Conclusion

This exploratory study provides insight in the potential therapeutic role of the iron chelator DFX in blood-induced cartilage damage. DFX is able to diminish blood-induced cartilage degeneration *in vitro*, with a role for the inhibition of NF-κB upregulation. However, the narrow therapeutic range due to the observed cytotoxicity in the absence of blood and the need for intra-articular treatment limit its translational value. A more comprehensive understanding of the functions and regulation of NF-κB in blood-induced cartilage may provide additional therapeutic options.

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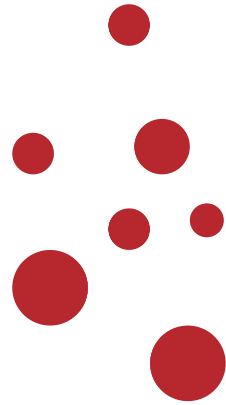

Summary and general discussion

Summary

Spontaneous joint bleeding is a characteristic manifestation of the inherited coagulation disorder hemophilia.[1] Prophylactic clotting factor replacement therapy drastically reduces bleeding events, although it is not possible to completely prevent hemarthroses.[2,3] Recurrent hemarthroses can result in hemophilic arthropathy (HA), characterized by progressive degeneration of cartilage, synovial inflammation, and bone changes with severe impact on mobility and quality of life.[4] Clinical parameters to detect patients at risk for progressive arthropathy are lacking, since the effects of joint bleeds are heterogeneous. The onset and severity of joint damage in patients with similar bleeding history varies. Especially in those patients at risk disease modifying therapy is urgently warranted to interrupt the process of blood-induced joint damage, preferably early in the disease.

This thesis aims to contribute to the knowledge of early pathophysiology of HA and to investigate a potential new treatment modality. To identify patients at risk for the development of HA, a retrospective cohort study on biomarkers was performed. Subsequently, the value of animal models in translation to the human situation was reviewed and two established animal models for blood-induced joint damage were advanced by adding new parameters. Next, a review of literature was conducted to identify possible targets for therapy based on the current knowledge of pathophysiology. Finally, the potential of the iron chelator and NF-κB inhibitor deferasirox (DFX) was explored in hemophilic mice and in a human *in vitro* study.

Early identification

The severity of damage upon a joint bleeding differs between patients, as does the development of HA. Identifying patients susceptible to fast progressive joint damage may have therapeutic implications, especially early in the disease. Imaging techniques lack the ability to detect early changes and the information provided is static. Biochemical markers, which are defined as molecules or fragments measured in blood or urine, reflect dynamic changes in joint tissue turnover at a certain point in time. The biochemical markers urinary C-terminal telopeptide of type II collagen (uCTX-II), serum cartilage cleavage product C1,2C (sC1,2C), and serum chondroitin sulphate 846 (sCS-846) are well correlated with radiographic joint damage. The combination of uCTX-II, serum cartilage oligomeric matrix protein (sCOMP), and sCS-846 correlated best with the degree of arthropathy.[5] Additionally, uCTX-II and sCS-846 increased shortly after a joint bleed in hemophilia patients, supporting their dynamic value.[6] In rheumatoid arthritis (RA) and osteoarthritis (OA), joint diseases sharing characteristics with HA, these two biomarkers were associated with radiographic progression.[7,8]
Considering the responsiveness of uCTX-II and sCS-846 to a joint bleed and the results in RA and OA, the potential prognostic value of these biochemical markers was explored in chapter 2. We assessed radiographic progression of HA over time in an original cohort of hemophilia patients used to establish the cross-sectional value of biomarkers.[5] X-rays as part of standard care were conducted with a mean follow-up of 6.5 years. None of the individual biomarkers measured at baseline predicted the presence of progression. However,

the combined index of uCTX-II and sCS-846 measured in the absence of a joint bleed could discriminate between patients with fast and slow progression of HA. This suggests that these biochemical markers are a reflection of the complexity of different chronic ongoing processes in the joint, and as such might be useful in monitoring HA and guiding treatment.

Early pathophysiological changes

In medical research, genetically engineered laboratory animals are utilized to mimic characteristics of human genetic disease. By use of knockout technology hemophilic rodents were created,[9,10] which can be used to study HA and may generate leads for research of other joint diseases, given the similarities in pathophysiology of OA and RA. The defined onset and progression rate of HA may be seen as an advantage over the other two arthropathies when it comes to identifying relevant markers (imaging and biochemical) of disease onset, progression, and treatment efficacy. In chapter 3 the translational value of animal models to study arthropathy is discussed. An artificially induced joint bleed in hemophilic rodents results in joint tissue damage resembling human disease,[11-13] although in hemophilic mice cartilage damage is relatively mild and early and extensive bone remodeling is observed in hemophilic rats.[13] Rest as a part of the treatment strategy in patients with hemophilia is another difference from animal models. These factors, in combination with differences in bone metabolism, different cartilage composition and as a result a higher turnover of cartilage, thicker layer of calcified cartilage and differences in biomechanics in small animals underline the importance of caution in translating findings from small animal models to the human situation. Nevertheless, animal models have proven indispensable for the evaluation of pathophysiological mechanisms and preclinical assessment of possible novel therapeutics.[14-15]

Optimizing preclinical hemophilia models is critical for their relevance to patient care. Chapter 4 aimed to advance the knowledge of the well-established hemarthrosis model in hemophilic mice. Firstly, induced hemarthrosis resulted in inflammation comparable for the patellar and tibiofemoral compartment. In contrast, minor cartilage damage was demonstrated in the tibiofemoral compartment, whereas patellar cartilage damage was substantial. Secondly, the additional value of a second induced joint bleed was limited, because no clear aggravation of joint damage was observed upon the second bleed, while the survival rate significantly decreased. Thirdly, the severity of cartilage damage in the injured joint was associated with development of cartilage damage in the weight-bearing area of the contralateral knee, a finding that must be taken into account when using this joint as an internal control.

Recently, a hemophilic rat model was developed in which joint bleed induction resulted in histologically assessed joint damage resembling human HA within days.[13,16] Histological evaluation is time consuming, less sensitive for subtle and early changes, and results are subject to interpretation despite initiatives to harmonize semi-quantitative scores. To improve the evaluation of very early blood-induced cartilage changes in the hemophilic rat model, the 35sulphate ($^{35}SO_4^{2-}$) incorporation assay as quantitative and sensitive outcome parameter for proteoglycan synthesis was studied in chapter 5. First, an *in vitro* study confirmed that it was possible to obtain sufficient amounts of tibial and patellar cartilage from rats to conduct the $^{35}SO_4^{2-}$-assay, and that blood exposure led to a decrease in the

proteoglycan synthesis rate as evaluated by this assay. Next, we conducted an *in vivo* study in which proteoglycan synthesis rate was assessed 4 or 16 days after joint bleed induction. An inhibition of proteoglycan synthesis in the injured knee was observed on day 4, which was irreversible after the recovery period at 16 days. Remarkably, the proteoglycan synthesis was also decreased to the same degree in the contralateral knee after 16 days. For the first time, early changes in cartilage matrix synthesis rate upon blood exposure were quantified with the $^{35}SO_4^{2-}$-assay in a hemophilic rat model, establishing this assay as a novel method to study blood-induced cartilage damage.

Early intervention

In the last part of this thesis potential new targets for treatment of HA were investigated. At present, no disease modifying therapy is available to fill the gap between preventing joint bleeding by clotting factor replacement therapy on the one hand and orthopedic surgery as a last resort for end-stage arthropathy on the other. Chapter 6 provides a literature review of the pathogenesis of HA in order to identify potential targets for therapy. Based on the pathophysiology, potential targets for therapy are iron, inflammation, vascular remodeling, hyperfibrinolysis, bone remodeling and cartilage regeneration. So far, iron chelators, anti-inflammatory therapy, anti-fibrinolytics and bone remodeling agents have demonstrated beneficial effects. Irrespectively, these interventions are predominantly tested in a preclinical setting, so there is still a long way to go for translation into clinical practice.

Iron is one of the promising targets to intervene early in the process of blood-induced joint damage, because of its central role in the pathophysiology by inducing both inflammation and oxidative stress. Prophylactic treatment with the iron chelator DFX, starting 8 weeks prior to joint bleed induction in hemophilic mice, attenuated cartilage damage upon blood exposure.[17] However, in hemophilia patients this approach is not opportune given the unpredictable occurrence of hemarthroses. In chapter 7 the effectiveness of on-demand DFX treatment, initiated immediately after joint bleed induction, was evaluated in hemophilic mice. This study demonstrated that on-demand treatment with DFX does not prevent synovitis nor cartilage damage following blood exposure in this experimental setup. These harmful processes were irreversibly initiated before DFX could exert its effect. As a consequence, the application of systemic on-demand treatment with DFX as a therapeutic solution for HA is deemed as not feasible.

A possible explanation between prophylactic and on-demand treatment results is a difference in DFX concentration in the joint at the moment of the bleed, suggesting that local availability is essential to prevent blood-induced cartilage damage. The way prophylactic DFX exerted its chondroprotective effect is unknown, but suggested mechanisms are iron chelation,[18-20] reduction of oxidative stress,[21] and inhibition of the nuclear factor (NF)-κB pathway independently from ROS reduction.[22,23] Though little is known about the exact role of the NF-κB-pathway in HA, an upregulation is demonstrated in hemophilic mice upon joint bleeding.[24]

Chapter 8 describes the results of an *in vitro* study investigating the possible immunomodulatory effect of DFX with a focus on the NF-κB-pathway in more detail. We demonstrated that blood-induced cartilage damage is mediated via NF-κB-signaling in human explants and that DFX can partially inhibit this effect *in vitro*. Moreover, a decrease

in the pro-inflammatory cytokines TNFα and IL-6, as representative inflammatory mediators for blood-induced cartilage damage, confirms that DFX can suppress inflammation. However, the observed chondrocytotoxicity when DFX was applied in the absence of blood and the need for intra-articular treatment hampers the translation to clinical use. Nevertheless, a more comprehensive understanding of the functions and regulation of NF-κB in blood-induced cartilage may provide additional therapeutic options.

General discussion

The last decades, clotting replacement therapy has been the cornerstone in the management of hemophilia, successfully reducing mortality and morbidity. The number of joint bleedings experienced in the past is leading in the treatment strategy to prevent future bleedings and development of HA. The bleeding pattern and the severity of subsequent joint damage varies among hemophilia patients.[3] In addition, dissimilar synovial and osteochondral changes are observed between hemophilia patients with late-stage joint changes.[25,26]
The first part of this discussion focusses on identifying those patients at risk of progressive arthropathy. The study of potential targets for therapy to intervene early in the process of blood-induced is topic of the second part. In the last part the relevance of this thesis is related to the emergence of innovative non-replacement therapy like emicizumab and advances in gene therapy.

Prediction of progressive arthropathy

Patients with inherited hemophilia usually exhibit a bleeding tendency of a severity proportional to the degree of plasmatic deficiency of the coagulation factor. However, 10-15% of patients with severe hemophilia experience a milder bleeding tendency.[27,28] The clinical bleeding pattern and differences in phenotypic expression of joint pathology is multifactorial and the result of the complex interaction between genetic[29-38] and environmental factors.[3,28,39-43] The question is which parameters can be used to predict a progressive course of the disease. Described parameters to predict the bleeding pattern are the age at which the first bleeding occurs,[27] synovial hypertrophy on MRI,[44] the biomarker type XVIII collagen,[45] and thrombin generation tests such as endogenous thrombin potential (ETP),[46-48] and rotational thromboelastometry (ROTEM).[49] However, clinical parameters reflecting early dynamical changes in the joint correlating to structural joint damage are lacking. Promising tools in this respect are three-dimension gait analysis to detect early walking disturbance before irreversible joint damage,[50] joint bleed volume,[51] and biochemical markers. Biochemical markers reflect dynamic changes in joint tissue turnover and are considered potential tools in monitoring joint disease, but are not useful in daily practice yet.[52-54] Several studies report on the potential of biochemical markers as a diagnostic tool for detecting a joint bleed[6,55] or the presence of HA.[5,56-58] Most studies describe an association between biochemical markers and well-established and readily available imaging techniques, which has at present no added value except for scientific interest. On the other hand, data from these cross-sectional studies provided the basis of the hypothesis-generating study of the prognostic value of the biomarkers uCTX-II and sCS-846 in chapter 2.[5,6] Evaluating

biochemical markers is still promising, although the findings in chapter 2 obviously need further validation in a prospective study. The combination of different biochemical markers is expectated to have added value, because previously neither of the individual biomarkers by itself had a prognostic value, reflecting the complexity of different processes contributing to joint damage after blood exposure.

Early intervention: potential targets for therapy

The presence of iron and inflammation are key factors in blood-induced synovitis, cartilage degeneration and bone remodeling. Processes resulting in blood-induced joint damage are complex and targeting only one element seems insufficient to interrupt the vicious circle (chapter 6). Preclinical data show effect either on cartilage (iron chelation,[17,59] IL-4 plus IL-10[60]) or synovium (anti-TNFα,[61] anti-IL-6 in combination with clotting factor[62]), but not both. From this perspective, a combined approach or targeting a joint pathway would be interesting.

Targeting a joint pathway: the NF-κB-pathway in hemophilic arthropathy

Repeated or ongoing joint bleeding result in accumulation of erythrocyte derived iron in synovial hemosiderin deposits,[63] which induces NF-κB mediated expression of pro-inflammatory cytokines IL-1β, IL-6, interferon-gamma and TNFα.[24,64] This is supported by high expression of synovial levels of receptor activator of NF-κB in patients with HA.[65] In the presence of iron, in particular in combination with pro-inflammatory cytokines, activated NF-κB stimulates synovial hypertrophy[66] by promoting proliferation via c-Myc[67,68] and inhibiting synovial cell apoptosis via mdm2.[69,70] NF-κB is also pivotal in controlling hypoxia regulating factors (HIF-1α), growth factors (VEGF) inducing neoangiogenesis, and cartilage degrading enzymes.[24]
While activation of NF-κB is considered the major pathway responsible for synovial inflammation and neo-angiogenesis upon bleeding, its role in blood-induced cartilage damage is relatively unknown (chapter 8). Upon blood-exposure, macrophages are attracted to the joint,[71] and activate NF-κB via the rapid canonical pathway[26] after being triggered by several pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). As a result, IL-1β is produced, the key cytokine initiating the inflammatory response leading to cartilage damage.[72] Additional cartilage damage occurs by catabolic gene expression in chondrocytes, promoting the expression of MMP's, pro-inflammatory cytokines and other destructive mediators.[24,73-75] Further research is needed to clarify the role of NF-κB in chondrocytes, especially in conjunction with the synovium.
Because NF-κB is a ubiquitously expressed transcription factor that regulates the processes leading to blood-induced joint damage, inhibition of the NF-κB signaling pathway has become a potential target for pharmacological intervention. Several clinically important anti-inflammatory (such as glucocorticoids) and non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the NF-κB pathway in preclinical models of joint disease.[76,77] However, so far no studies with structural joint changes or long-term outcome in HA as an endpoint have been performed.

The idea of using the iron chelator DFX as a disease modifying agent is very appealing, based

on the pathophysiological concept, (chapter 6) the chondroprotective effect upon joint bleeding observed in hemophilic mice,[17] and its ability to inhibit the nuclear factor (NF)-κB pathway independently from ROS reduction.[22,23] DFX may act as a NF-κB inhibitor and was able to reduce NFkB expression in cartilage *in vitro* (chapter 8). Unfortunately, in chapter 7 was demonstrated that systemic on-demand treatment with DFX did not result in joint status improvement upon joint bleed induction in hemophilic mice. Does this result mark the end of DFX as a treatment option in blood-induced joint damage? Or is the value of the hemophilic mouse model in this study design more limited than expected?

Animal models as research tool in hemophilic arthropathy

Animal models have proven indispensable in pathophysiological and pharmacological studies. The discovery of a hemophilic dog and successful breeding of a colony emerged the earliest animal studies on hemophilia and HA.[78-81] Since then, other animals with spontaneous clotting factor deficiency have been described, such as the sheep and pig.[15] With the development of techniques allowing for genetic modification, hemophilic rodent models have emerged.[9,10] Rodents are easy to handle and breed and are suitable for clinical imaging. These models enable studying blood-induced joint damage in a homogenous population, in which the initiation of blood-exposure can be controlled and subsequent mechanisms can be studied directly at joint tissue level. An additional advantage is that the joint destructive process is accelerated in comparison to the human situation and already visible after a few days to weeks.[11,13] The lack of spontaneous bleeding in mice allows for studying the disease without the interference of non-induced joint bleeds and need for clotting factor administration, though spontaneous hemorrhages in soft tissue including the joint are reported.[82]

However, the hemophilic mouse model also has limitations, which makes the results of DFX on demand study difficult to interpret (chapter 7). First, the amount of intra-articular blood cannot be controlled nor quantified, while bleed volume correlates with the subsequent degree of HA.[51] The induced joint damage could have been too severe for DFX to improve, especially in the absence of coagulation correction. In addition, the mice had a mediocre intake 1-2 days after joint bleed induction. As a result, the administration of DFX, dissolved in drinking water, was at least suboptimal in those crucial first days. Repeating this experiment in the hemophilia rat model would overcome these disadvantages. In contrast to mice, rats have a larger blood volume allowing multiple blood sampling and bleed spontaneously, which is considered an advantage from a translational perspective.[16] Moreover, bleed volume can be quantified with imaging and the therapeutical agent can be administered by oral gavage.

Yet, I would not argue for a new animal study since differences in biomechanics, composition of cartilage tissue and potential for intrinsic healing raise questions about the translational value of rodent models.[83] Although animal testing is still required by law in development of new drugs, this limited translational value is the main reason for increasing ethical and public concerns in the Netherlands and Europe about the use and welfare of laboratory animals.[84] It is considered important to reflect societal values within scientific practice and emerging technology, especially in case of publicly funded research. Therefore animal

models are constantly improved by application of the 3 R's: reduction, refinement and replacement.[85] A contribution to the improvement of the hemophilic mouse model was part of this thesis as well. The observation in chapter 4 that a second joint bleeding has limited value in studying blood-induced joint damage can result in a reduction in laboratory animals and their suffering in future studies. Adding a sensitive outcome parameter for cartilage damage contributes to refinement of the model, as described in chapter 5. Replacement by animal-free innovations is a challenge for the research community. Interesting alternatives are the development of *in vitro* co-cultures of cartilage and synovium[86,87] and biofabrication to generate biologically responsive articular joints which pave the way for an organ-on-a-chip technology.[88] This concept is rapidly advancing in medicine and aims to simulate specific organ functions and pathologies.[89] A personalized joint-on-a-chip will probably be possible in the future,[88] providing valuable insights into pathophysiology and patient tailored drug efficacy. The important role of mechanical loading in the development of joint disease is one of the issues that must be addressed in the development of these new techniques. Further, computational modeling ranging from biomechanical to gene regulatory network models may be used to study whole joint disease, predict disease progression and identify potential targets for treatment.[90] In silico models are not embedded in research focusing on HA yet.

The future role of the iron chelator DFX in blood-induced joint damage

Due to the translational limitations of the hemophilic mice study, the effect of DFX on blood-induced joint damage in the human situation is uncertain. Still, the concept of reducing the effect of iron is interesting considering its pivotal role in the pathophysiology. Theoretical interventions to achieve this could be the application of DFX systemically or locally, or reducing the amount of blood (iron) by joint aspiration.

To study the potential effect of DFX without the interference of a disturbed coagulation cascade, deteriorated joints or intercurrent joint bleeds, a study population of patients with trauma-related hemarthrosis can be chosen. Those patients can be randomized between six week treatment with DFX versus placebo. Biomarker studies and MRI could be performed at baseline, 6 and 12 weeks to evaluate subtle and early joint changes upon bleeding. Next, a similar study design could be applied to hemophilia patients. A scientific disadvantage could be that due to successful new treatment options such as emicizumab, a bispecific antibody mimicking factor VIII, spontaneous joint bleeding will only occur sporadically and it takes more time to include enough patients for analysis.

A second approach for a follow-up study involves local application of DFX. Local availability seems essential to prevent blood-induced cartilage damage.[59,60] The intra-articular administration of DFX would be possible with adequate coagulation correction, however, the observed chondrocytotoxicity when DFX was applied in the absence of blood raises concerns. Therefore, the presence of blood in the joint should first be checked (i.e. by ultrasound).

A third intervention to reduce the effect of iron is by arthrocentesis, considering the prolonged adverse effects observed after even a single joint bleed.[91] The estimated natural evacuation time of blood from the joint space is at least four days.[92] There is evidence that aspiration of blood within two days of hemarthrosis can reduce long-term cartilage

damage.[93] Joint aspiration remains controversial in the management of acute hemarthrosis and current guidelines emphasize correction of coagulation. The potential risk of aggravation of the joint bleed or infection are frequent arguments against aspiration. However, accelerated recovery after a joint bleed is reported when aspiration was combined with appropriate replacement therapy.[94,95] The estimated incidence of infectious complications is 0.0003% and probably lower when performed by experienced physicians.[96]

Impact of novel treatment options

At the start of this PhD program, clotting replacement therapy was the cornerstone of the management of hemophilia, successfully reducing mortality and morbidity. Challenges were the risk of inhibitor development, therapy adherence due to the burdensome frequent intravenous injections, high costs and restricted access to therapy for many patients worldwide. However, the landscape of hemophilia treatment has changed dramatically over the past years due to the emergence of non-replacement therapy like emicizumab and advances in gene therapy.

Emicizumab is a bispecific antibody mimicking factor VIII and recently approved as novel therapeutic agent. Advantages are the subcutaneous administration and the effective prophylaxis irrespective of inhibitor status. As a result, even patients with factor VIII inhibitors hardly bleed anymore, which has an enormous impact on quality of life. Another type of non-replacement therapy is targeting the natural inhibitors of coagulation, such as antithrombin (fitusiran),[97] tissue factor pathway inhibitor (concizumab),[98,99] or activated protein C (SerpinPC).[100] These agents are currently evaluated in (pre)clinical trials.

The ultimate goal of hemophilia treatment would be a phenotypical cure, which is achievable by gene therapy. This therapy aims at sustained endogenous production of FVIII and FIX proteins at concentrations that provide effective prophylaxis without the need for exogenous factor replacement therapy. The predominant strategy for gene transfer in patients with hemophilia is the liver-directed delivery of factor VIII or IX with the use of recombinant non-integrating adeno-associated viral (AAV) vectors. Data from multiple small phase 1 and 2 trials, show that the majority of patients have measurable FVIII and FIX concentrations in plasma after 8–10 years, which are sufficient to withdraw regular prophylaxis.[101] The safety and efficacy of several agents are under investigation in clinical trials,[102] but it seems a matter of time before gene therapy will find its way into clinical practice.

For patients with hemophilia, these developments in non-replacement and gene therapy will redefine the perception of their disease again. After the introduction of factor replacement concentrate, hemophilia changed from a lethal disease to a chronic disease, with the disease burden being determined by complications of the treatment (HIV, hepatitis) and comorbidity due to hemophilic arthropathy. Current factor replacement therapy is safe and prophylaxis drastically reduces bleeding events, although it is not possible to completely prevent hemarthroses.[2,3] If non-replacement therapy and / or gene therapy is as successful as expected, the next generation of hemophilia patients will grow up without or with a very limited number of joint bleeds. In addition, the price of coagulation factor concentrate will fall due to competition from new treatment options, making this treatment available to patients in developing countries who currently do not have access to it. In the light of the above, you can argue whether the research of blood-induced joint damage is still warranted.

However, there is emerging evidence for a role of FVIII in regulating processes beyond coagulation. A decreased factor VIII is associated with several non-hemostatic pathologies, such as reduced bone mineral density,[103-106] neoangiogenesis,[107,108] deficits in tissue regeneration,[109,110] impaired hematopoiesis,[111] and endothelial dysfunction. It is unknown whether these non-hemostatic functions of factor VIII are sufficiently supported by the emerging non-replacement therapy. In addition, for the next 50 years there still will be patients with hemophilia who have experienced joint bleedings in the past. In addition, the occurrence of microbleeds in patients treated with non-replacement therapy is unknown. Moreover, non-replacement therapy and / or gene therapy will not be readily available in developing countries. Therefore, focusing on the prevention of hemarthroses and subsequent joint deterioration remains of great importance.

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APPENDICES

Nederlandse samenvatting

Dankwoord

List of publications

Curriculum Vitae

Nederlandse samenvatting

Introductie

Hemofilie is een aangeboren bloedingsziekte die veroorzaakt wordt door een tekort aan stollingsfactor VIII (hemofilie A) of stollingsfactor IX (hemofilie B). De ziekte komt vrijwel uitsluitend voor bij mannen, omdat het gen voor beide stollingsfactoren op het (vrouwelijke) X-chromosoom ligt. Naar schatting wordt 1 op de 5.000-10.000 jongens geboren met hemofilie, waarvan 80-85% hemofilie A heeft.

Patiënten met hemofilie hebben last van bloedingen in voornamelijk spieren en gewrichten. De ernst van de hemofilie wordt bepaald door de resterende stollingsfactoractiviteit. Patiënten met ernstige hemofilie hebben minder dan 1% stollingsfactoractiviteit ten opzichte van de gezonde populatie, bij de matig-ernstige vorm is dit 1-5% en bij milde hemofilie 5-40%. Hemofilie wordt gekenmerkt door bovenmatig bloeden bij een trauma of operatie en, in het geval van ernstige hemofilie, ook door spontane bloedingen. Het merendeel (70-80%) van deze spontane bloedingen treedt op in de gewrichten, waarbij de ellebogen, knieën en enkels het vaakst zijn aangedaan. Bloedingen in de heupen en schouders komen minder vaak voor en bloedingen in kleinere gewrichten zijn zeldzaam. Blootstelling van een gewricht aan bloed leidt tot degeneratie van kraakbeen, synoviale inflammatie en uiteindelijk ook tot botafwijkingen. Wanneer herhaaldelijke gewrichtsbloedingen in verloop van tijd resulteren in onherstelbare schade aan het gewricht, wordt gesproken van hemofilie arthropathie. Deze aandoening heeft grote invloed op de mobiliteit en kwaliteit van leven van hemofiliepatiënten.

De behandeling richt zich op correctie van de stolling ten tijde van een bloeding én het voorkomen van gewrichtsbloedingen. Bij de start van dit promotietraject was profylactische toediening van de ontbrekende stollingsfactor (replacement therapie) de hoeksteen van de behandeling. Met deze stollingsfactorvervangende therapie wordt de dalspiegel van factor VIII/IX verhoogd. Desondanks blijkt het onmogelijk om gewrichtsbloedingen geheel te voorkomen. Daarnaast kunnen patiënten antistoffen ontwikkelen die het effect van de stollingsfactoren verminderen en is door de kosten deze zorg beperkt toegankelijk voor hemofiliepatiënten in een groot deel van de wereld. Met de komst van non-replacement therapie (bijvoorbeeld emicizumab, een bispecifiek antilichaam dat factor VIII nabootst) en de ontwikkelingen in gentherapie zal de behandeling van bloedingen bij hemofilie verder verbeteren.

In dit proefschrift staat het vroege ziekteproces van bloedgeïnduceerde gewrichtsschade centraal. Allereerst gaat het daarbij om het tijdig identificeren van hemofiliepatiënten die risico lopen op ernstige gewrichtsschade op de langere termijn. Daarnaast wordt een bijdrage geleverd aan inzicht in de vroege pathologische veranderingen in het gewricht na blootstelling aan bloed en wordt een mogelijke nieuwe behandeling onderzocht.

In een vroeg stadium opsporen van patiënten die risico lopen op hemofilie arthropathie

Een bloeding leidt niet bij elke patiënt tot dezelfde schade aan het gewricht of ontwikkeling

tot hemofilie arthropathie. Het identificeren van patiënten die gevoelig zijn voor snel progressieve gewrichtsschade kan gevolgen hebben voor de behandeling, vooral in het begin van het ziekteproces. Beeldvormende technieken, zoals röntgenfoto's, echo en MRI, kunnen vroege veranderingen in het gewricht niet goed op sporen, zijn tijdrovend en/of duur. Biomarkers zijn stoffen die vrijkomen bij de opbouw en afbraak van kraakbeen en bot en zijn daarmee een afspiegeling van de gewrichtschade op dat moment. Ze kunnen gemeten worden in bloed of urine. Voor een aantal biomarkers is aangetoond dat ze goed verband houden met de mate van gewrichtsschade op röntgenfoto's bij hemofiliepatiënten. Dat is het geval voor de biomarkers C-terminaal telopeptide van type II collageen (gemeten in urine; uCTX-II), serum cartilage cleavage product C1,2C (gemeten in serum; sC1,2C), serum cartilage oligomeric matrix protein (sCOMP) en serum chondroïtine sulfaat 846 (sCS-846). Eerdere studies hebben laten zien dat uCTX-II en sCS-846 kort na een gewrichtsbloeding bij hemofiliepatienten stijgen en bij patiënten met andere gewrichtsaandoeningen zoals reumatoïde artritis en artrose zijn deze twee biomarkers geassocieerd met radiologische progressie van de gewrichtsschade.

In **hoofdstuk 2** is de prognostische waarde van de biomarkers uCTX-II en sCS-846 onderzocht in een retrospectieve cohort studie. Bij een groep hemofiliepatiënten zonder recente gewrichtsbloeding werden biomarkers gemeten. Na gemiddeld 6,5 jaar werd gekeken naar gewrichtsschade op een röntgenfoto van de knie. De individuele biomarkers konden de ernst van de gewrichtsschade niet voorspellen, maar de combinatie van uCTX-II en sCS-846 maakte onderscheid tussen hemofiliepatiënten met snel en langzaam progressieve hemofilie arthropathie. Deze biomarkers kunnen bruikbaar zijn bij het vervolgen van patienten met hemofilie arthropathie, bijvoorbeeld om richting te geven aan de behandeling.

Vroege pathofysiologische veranderingen in het gewricht na blootstelling aan bloed

In medisch-wetenschappelijk onderzoek worden proefdieren ingezet om erfelijke aandoeningen beter te begrijpen en het effect van nieuwe behandelingen te bestuderen. In het geval van hemofilie worden proefdieren ingezet die factor VIII missen, met een verhoogde bloedingsneiging tot gevolg. De gewrichtschade na een gewrichtsbloeding bij het proefdier lijkt sterk op het beeld wat gezien wordt bij de mens, dus deze factor VIII knock-out dieren worden gebruikt om het proces van hemofilie arthropathie te bestuderen. Tegelijkertijd zijn er verschillen tussen proefdieren en de mens: de mate van kraakbeen- en botschade na een bloeding, de samenstelling van het kraakbeen, de biomechanica bij kleine dieren en de rol van rust als onderdeel van de behandeling. In **hoofdstuk 3** wordt de translationele waarde van diermodellen voor het bestuderen van artropathie besproken en het belang onderstreept van een zorgvuldige vertaling van bevindingen in diermodellen naar de humane situatie.

Vooralsnog blijven diermodellen onmisbaar voor de bestudering van pathofysiologische mechanismen en de preklinische beoordeling van mogelijke nieuwe therapieën. Het optimaliseren van deze modellen is van cruciaal belang, zowel met oog op dierenwelzijn als relevantie voor de klinische praktijk. In **hoofdstuk 4** wordt het gevestigde hemartrosemodel

in hemofiliemuizen bestudeerd. Een gewrichtsbloeding resulteerde in inflammatie van zowel het tibiofemorale als het patellaire compartiment. De kraakbeenschade aan de tibia en femur was mild, terwijl de kraakbeenschade aan de patella aanzienlijk was. Daarnaast wees dit onderzoek uit dat de toegevoegde waarde van een tweede bloeding beperkt was, terwijl er meer dieren overleden. Opvallend genoeg werd bij de dieren met ernstige kraakbeenschade in het gewricht waar een bloeding werd veroorzaakt, ook schade gezien aan de contralaterale knie. Met deze bevinding moet rekening worden gehouden bij het gebruik van de niet-aangedane knie als interne controle.

Recent is ook een hemartrosemodel in ratten zonder factor VIII ontwikkeld, waarin met histologie de gewrichtsschade na een bloeding werd bestudeerd. Histologisch onderzoek kost veel tijd, vroege en subtiele veranderingen zijn lastig vast te stellen en de resultaten zijn subjectief ondanks semi-kwantitatieve scoringssystemen. Om de vroegste veranderingen in het kraakbeen te detecteren, is in **hoofdstuk 5** de ^{35}Sulphate incorporation assay met succes toegepast in het rat model. Deze analyse meet de proteoglycaan synthese en is een maat voor kraakbeenschade. Er kon voldoende kraakbeen van de tibia en patella verkregen worden om de analyse mee uit te voeren en blootstelling aan bloed kon worden gekwantificeerd. In het model werd 4 dagen na blootstelling aan bloed een remming van de proteoglycaan synthese gezien en dit effect was niet reversibel na 16 dagen. Net als in het muismodel werd 16 dagen na de gewrichtsbloeding ook kraakbeenschade gezien in de contralaterale knie. Concluderend is de ^{35}Sulphate incorporation assay een nieuwe methode om bloedgeïnduceerde kraakbeenschade in diermodellen te bestuderen.

Vroege interventie

In het laatste gedeelte van dit proefschrift worden potentiële nieuwe aangrijpingspunten voor de behandeling van hemofilie arthropathie onderzocht. De huidige strategie richt zich op het voorkomen van bloedingen en in het geval van een gewrichtsbloeding correctie van de stoling met stollingsfactorsubstitutie therapie, symptoombestrijding, behoud van functionaliteit en op indicatie orthopedisch ingrijpen. Tot op heden is er geen behandeling die direct op het ziekteproces ingrijpt. **Hoofdstuk 6** geeft een literatuuroverzicht van de pathogenese van hemofilie arthropathie om kansrijke aangrijpingspunten voor een behandeling te identificeren. Interessant zijn de rol van ijzer, inflammatie, vasculaire remodelering, hyperfibrinolyse, botremodelering en kraakbeenregeneratie. In overwegend preklinische modellen zijn gunstige effecten aangetoond van ijzerchelatoren, ontstekingsremmende therapie, antifibrinolytica en bisfosfonaten, maar er is nog een lange weg te gaan voor toepassing in de klinische praktijk.

IJzer speelt een centrale rol in de pathofysiologie van bloedgeïnduceerde gewrichtsschade door het veroorzaken van zowel inflammatie als oxidatieve stress. Het is dan ook een veelbelovend aangrijpingspunt om vroeg in het proces van bloedgeïnduceerde gewrichtsschade te interveniëren. Profylactische behandeling van hemofiliemuizen met de ijzerchelator deferasirox gedurende 8 weken vóór en 5 weken na een gewrichtsbloeding resulteerde in minder kraakbeenschade. Deze benadering met een oplaadperiode is bij hemofiliepatiënten echter niet toepasbaar in de dagelijkse praktijk gezien het onvoorspelbare

optreden van hemarthrosen. Daarom werd in **hoofdstuk 7** in hemofiliemuizen de effectiviteit van deferasirox gestart op het moment van een gewrichtsbloeding (on-demand) bestudeerd. Behandeling met deferasirox on-demand voorkomt geen synovitis of kraakbeenschade na blootstelling aan bloed in deze studie. Daarmee lijkt de toepassing van systemische ijzerchelatie als therapeutische oplossing voor hemofilie arthropathie niet haalbaar.

Lokale beschikbaarheid van deferasirox in het gewricht is een mogelijke verklaring van het verschil tussen de resultaten behaald met de profylactische en on demand behandeling. Het precieze mechanisme dat ten grondslag ligt aan het chondroprotectieve effect van deferasirox is onbekend, maar ijzerchelatie, de vermindering van oxidatieve stress en de remming van de nucleaire factor (NF)-κB-pathway kunnen een rol spelen. **Hoofdstuk 8** beschrijft de resultaten van een *in vitro* onderzoek naar het mogelijke immuunmodulerende effect van deferasirox met een focus op de NF-κB-pathway. Bloedgeïnduceerde kraakbeenschade wordt gemedieerd via NF-KB-signalering in humaan kraakbeen. Deferasirox kan deze schade *in vitro* gedeeltelijk voorkomen, door onder andere de ontstekingsmediatoren TNFα en IL-6 te remmen. Echter, deferasirox is schadelijk voor chondrocyten in afwezigheid van bloed, wat de toepassing in de klinische praktijk bemoeilijkt. Een beter inzicht in de functies en regulatie van NF-κB in bloedgeïnduceerd kraakbeen levert in de toekomst wellicht nieuwe aangrijpingspunten voor therapie op.

Conclusie

Bij het starten van dit promotietraject was het vervangen van de deficiënte stollingsfactor (replacement therapie) de enige optie om een gewrichtsbloeding te behandelen en te voorkomen. Met de komst van emicizumab (non-replacement therapie) en mogelijk gentherapie in de toekomst, zal de volgende generatie hemofiliepatiënten opgroeien met een beperkt aantal of zelfs zonder gewrichtsbloedingen. Factor VIII lijkt echter ook een rol te spelen in processen buiten de stolling om, waarbij de vraag is of non-replacement therapie deze functie voldoende ondervangt. Daarnaast is het onbekend of microbloedingen onder non-replacement therapie ontstaan en zal deze nieuwe behandeling niet direct wereldwijd beschikbaar zijn. Zolang gewrichtsbloedingen nog voorkomen, blijft de focus op het voorkomen van bloedgeïnduceerde gewrichtsschade van groot belang.

Dit proefschrift onderschrijft het belang van vroeg ingrijpen in het proces van bloedgeïnduceerde gewrichtsschade. Dat begint met het identificeren van de hemofiliepatiënten met een verhoogd risico op de ontwikkeling van hemofilie arthropathie, waarbij de biomarkers uCTX-II en sCS-846 kunnen ondersteunen. Kennis over de vroege pathofysiologische veranderingen in het gewricht na blootstelling aan bloed uit preklinische modellen levert aanknopingspunten op voor gerichte behandeling. Het verminderen van de rol van ijzer in dit proces door het toedienen van de ijzerchelator deferasirox direct na de gewrichtsbloeding geeft geen afname in schade. Kortom, dit proefschrift heeft het inzicht in de pathofysiologische veranderingen na een gewrichtsbloeding vergroot waarmee een basis is gelegd voor de ontwikkeling van nieuwe behandelstrategieën om hemofilie arthropathie te voorkomen.

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Curriculum Vitae

Astrid Pulles was born January 11th 1984 in Nijmegen, the Nederlands. She grew up in Nijmegen and Curaçao and graduated from secondary school at the Stedelijk Gymnasium Nijmegen in 2002. She started her study Bestuurs- en Organisatiewetenschappen (School of Governance) at the University of Utrecht and received her bachelor of science degree in 2006. Meanwhile she started medical school at the University of Utrecht in 2004, during which she participated in the Excellent Traject and did internships in Nicaragua (2007) and Curaçao (2008). After obtaining her medical degree in November 2010, she started her clinical career as a medical doctor (ANIOS) at the department of Internal Medicine at the Sint Antonius Hospital in Nieuwegein. In May 2011 she started her residency Internal Medicine alternately at the University Medical Center Utrecht (prof. dr. M.M.E. Schneider and prof. dr. H.A.H. Kaasjager) and the Sint Antonius Ziekenhuis in Nieuwegein (dr. A.B.M. Geers). From June 2015 till December 2018 she interrupted her residency for the PhD project described in this thesis at the Van Creveld Kliniek and department of Rheumatology & Clinical Immunology at the University Medical Center Utrecht, under direct supervision of prof. dr. R.E.G. Schutgens, prof. dr. F.P.J.G. Lafeber, dr. S.C. Mastbergen and dr. L.F.D. van Vulpen. In the beginning of 2019 she resumed her residency and started her Fellowship Hematology in July 2019 (dr. R.A.P. Raymakers, dr. A. van Rhenen and prof. dr. J.H.E. Kuball), which is expected to be completed in February 2022.

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