IRON AND INFLAMMATION: A JOINT PROBLEM

in blood-induced arthropathy

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IJZER EN INFLAMMATIE: EEN GEZAMENLIJK PROBLEEM

in bloed-geïnduceerde gewrichtsschade

(met een samenvatting in het Nederlands)

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CHAPTER 1

GENERAL INTRODUCTION

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Introduction

Spontaneous joint bleeding is the characteristic manifestation of the inherited coagulation disorder hemophilia,[1] but can also occur in other bleeding disorders such as Von Willebrand disease,[2] as a complication of anticoagulant treatment,[3] or upon trauma[4] or 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). It has an estimated frequency of 1 in 5 000 - 10 000 male births,[6,7] approximately 80-85% representing hemophilia A. The diminished or lack of clotting factor activity leads to inadequate thrombin generation and a tendency for bleeding. The clinical phenotype is strongly correlated with the amount of clotting factor present, expressed as percentage compared to normal clotting factor activity in blood. Severe hemophilia patients have less than 1% clotting factor activity, while patients with moderate and mild hemophilia have a clotting factor activity of 1-5% and 5-40%, respectively.[8]

Spontaneous bleeding affects mainly patients with severe hemophilia, is less frequent in moderate hemophilia, and patients with mild hemophilia only experience bleeding after major trauma or surgery. The phenotype is also impacted by several other mechanisms, including genetic factors, age at first joint bleed, and concomitant thrombotic factors.[9,10] In severe hemophilia 70-80% of all bleeding events occur in the joints, spontaneously as well as in response to stress or trauma.[11,12]

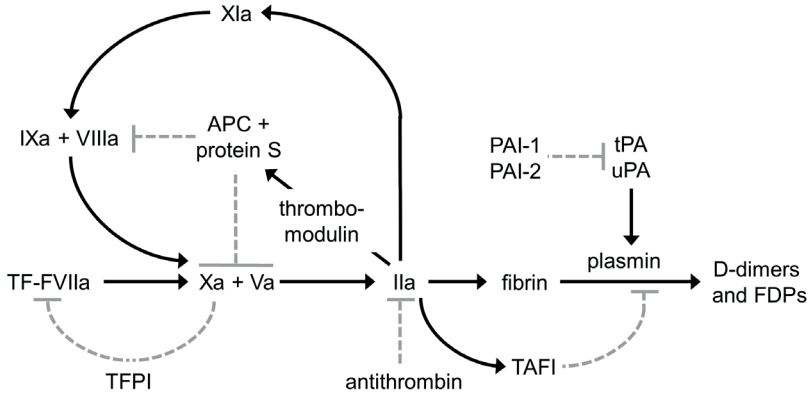

Figure 1 – Schematic representation of the coagulation cascade and fibrinolytic system
The coagulation cascade is initiated by tissue factor (TF) and results via several coagulation proteases in the formation of thrombin (factor IIa) which converts fibrinogen into fibrin. The small initial amount of thrombin generated then activates factor XI, leading to amplification of thrombin generation. Natural anticoagulant factors tissue factor pathway inhibitor (TFPI), antithrombin, protein C (APC) and protein S control the coagulation cascade. The fibrinolytic system results in plasmin formation via tissue-type and urokinase-type plasminogen activator (tPA and uPA), clot lysis, and therewith production of fibrinogen degradation products (FDPs). Plasminogen activator inhibitor 1 and 2 (PAI-1, PAI-2) inhibit tPA and uPA. Thrombin-activatable fibrinolysis inhibitor (TAFI) acts as a fibrinolysis inhibitor. Black arrows indicate activation, whereas grey striped lines indicate inhibition.

The predilection for bleeding in the joint compared with other tissue sites might be caused by the relatively low expression of tissue factor, the initiator of the coagulation cascade (Figure 1),[13,14] together with a high level of tissue factor pathway inhibitor (TFPI) in the normal joint.[15] Also increased local fibrinolysis in specifically the joints of patients with hemophilia may be causative.[16] Additionally, mechanical factors damaging the richly vascularized synovial tissue, specifically after being triggered after a first joint bleed, might be causative in this respect as well.[17]

Musculoskeletal bleeding and treatment-related complications are the most important complications in hemophilia. Development of alloantibodies directed against administered clotting factor (so called inhibitors) is common. The transmission of blood-borne virus infections due to 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 2.

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.[12] The hips and shoulders are affected to a lesser extent and bleedings in smaller joints are rare.

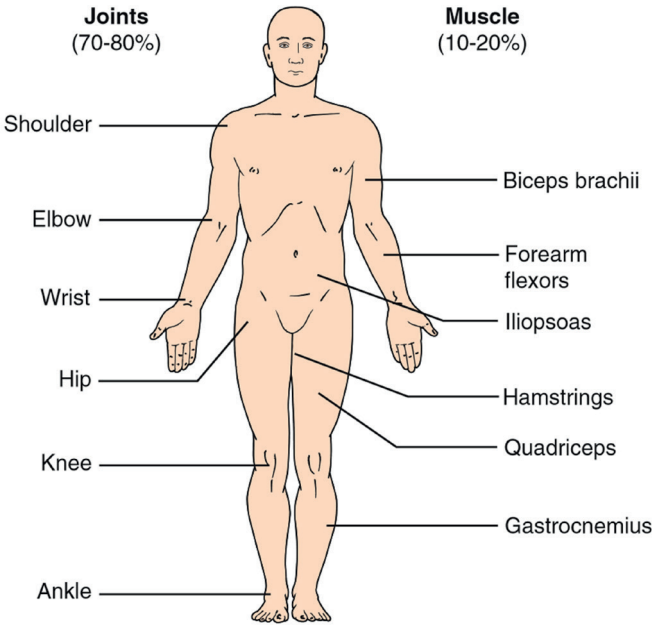

Figure 2 – Joints and muscles commonly affected by bleeding in hemophilia

Besides hemarthrosis and muscle bleeding, other major bleeds (mucous membranes, gastro-intestinal) account for 5-10% of bleeds and <5% of the bleeds occurs in the central nervous system.

In severe hemophilia the first joint bleed often occurs when children begin walking or running, at a median age of 1.8 years.[10] At this age, the 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] Flexion is the most comfortable position and disuse (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.
Joint bleeds often have a migratory pattern, moving from one joint to the other.[12] About 25% of the patients with severe hemophilia develop a so-called target joint, a joint that is more susceptible to subsequent bleedings compared to other joints. It is defined as a joint in which three or more hemarthroses have occurred in the prior six months.[17,18] Nowadays, in youngsters with hemophilia, this mostly involves the ankle joint, rather than the elbow or the knee, the latter most commonly affected before 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.[19,20] It also induces the development of a rich network of new, fragile blood vessels underneath the synovial lining making the joints vulnerable to subsequent (repeated) bleeding.[21] In this condition, the joint appears swollen, but usually not tense, is often painless and 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 3). The number of hemarthroses required to cause irreversible damage is unknown and this is likely to differ between patients. Progressive degeneration of cartilage, synovial inflammation, and bone changes cause chronic pain, joint stiffness, and a severely limited ROM. Without proper physiotherapy it is often accompanied by muscle weakness and contractures. In the most severe cases joint deformity, subluxation, joint laxity, malalignment, and even spontaneous arthrodesis can develop.
Physical activity and quality of life are severely impacted by hemophilic arthropathy.[22] Progressive synovial fibrosis decreases the frequency of hemarthroses in end-stage arthropathy.

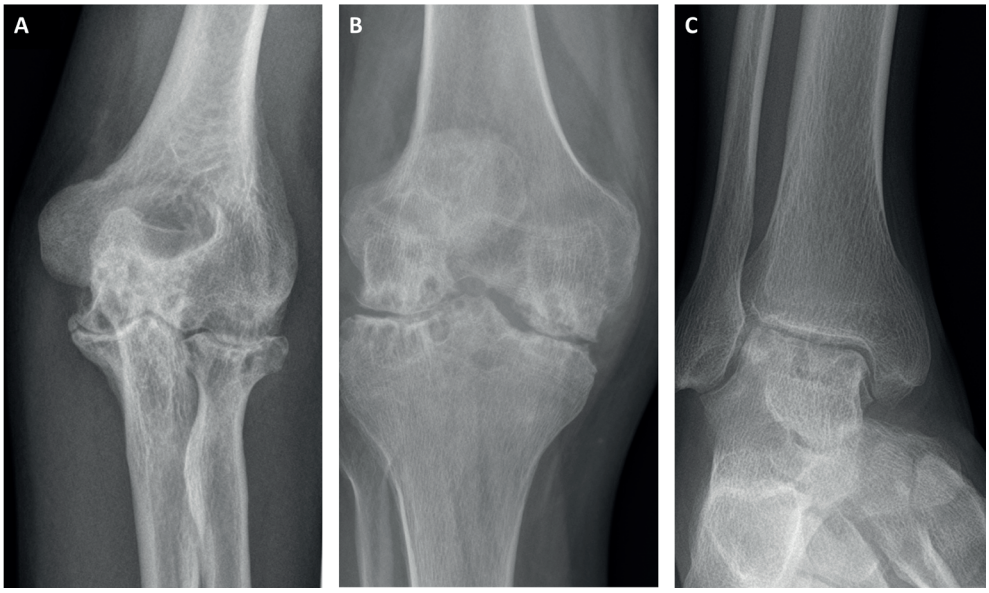

Figure 3 – Radiographic changes of hemophilic arthropathy in a 30-year old patient with severe hemophilia
(A) left elbow, (B) right knee, (C) right ankle. All showing joint space narrowing and subchondral cysts, most pronounced in the elbow and knee.

Muscle and soft tissue hemorrhage

The muscle is the second most common site of bleeding in hemophilia, accounting for approximately 10-20% of bleeding.[1] It usually results from a direct blow or sudden stretch and can occur in any muscle of the body.

The clinical features depend on the muscle involved, but overall muscle bleeding emerges more insidiously than hemarthroses and prodromal symptoms are rare. A tender swelling in the muscle with severe pain on 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, as 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.[23]

A life-threatening complication of ongoing bleeding is 'compartment syndrome' with femoral neuropathy and possible muscle necrosis. Other sites of muscle bleeding associated with neuropathy, are the posterior compartments of the lower leg and flexor group of the forearm. Muscle hemorrhage can result in permanent contracture, re-bleeding, and formation of pseudotumors.[24]

Pseudotumor

Formation of pseudotumors is a rare but serious complication unique to hemophilia, mainly occurring after inadequate replacement therapy or after development of an inhibitor.

Repeated and unresolved muscle bleeding or a progressive subperiostal hemorrhage causes an expanding encapsulated and calcified hematoma with radiographic features mimicking tumor-like conditions, hence called pseudotumors (Figure 4).[1,25]
Progressive enlargement of the pseudotumor can lead to erosion of the adjacent bone, compression of neurovascular structures, spontaneous rupture, fistula formation, and pathologic fractures.

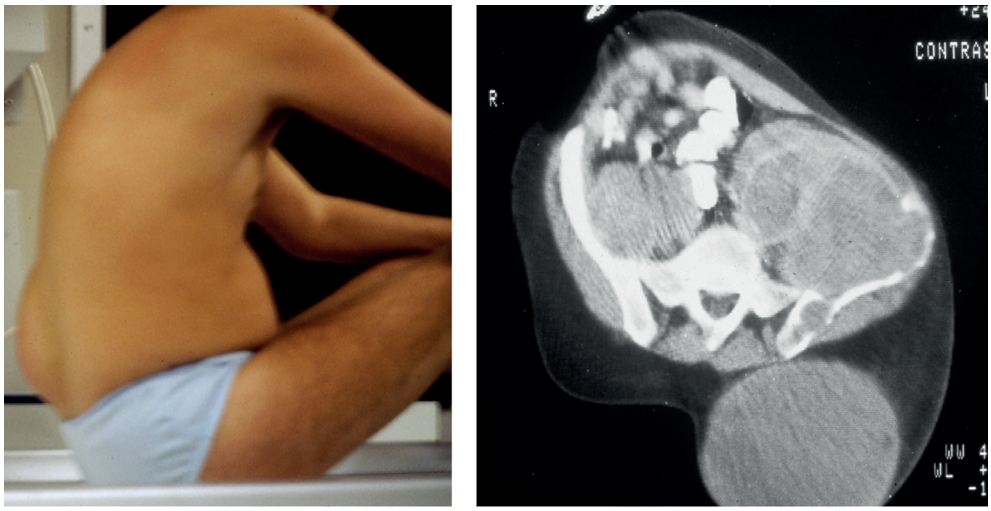

Figure 4 – Pseudotumor invading the left wing of the ilium in a patient with severe hemophilia and inhibitor

There are 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 are often amenable to conservative treatment with immobilization and clotting factor replacement.[26]

Osteoporosis
Hemophilia patients have a significantly lower bone mineral density compared to age-matched controls.[27] The pathogenesis is multifactorial, several predisposing factors are frequent in the hemophilic population: 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 its treatment.[28,29] 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 are the result of a *de novo* germline mutation, but also somatic mosaicism in a (grant)parent has been described.[30,31] 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 following trauma or surgery. Screening tests in patients with a bleeding diathesis of unknown etiology include platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT). Characteristic for hemophilia is a normal platelet count, normal PT, and a prolonged aPTT, although the aPTT might be (near) normal in mild hemophilia. Specific clotting factor assays are performed to determine the type and severity of hemophilia and for the distinction from Von Willebrand disease. The diagnosis can be confirmed by deoxyribonucleic acid (DNA)-based techniques. Given the rarity and complexity of the disease, a doctor specialized in hemostasis should be consulted.

Frequent monitoring of musculoskeletal status is indicated in hemophilia patients to diagnose complications in an early stage as prevention is the cornerstone in treatment. Use of physical examination assessment tools can help to pick up subtle early signs of joint damage, to monitor joint health over time and to evaluate treatment efficacy. Several hemophilia-specific instruments have been developed and validated.[32-34]
Clinical assessment of the musculoskeletal system at time of an acute bleed should include evaluation of warmth, bruising, swelling, tenderness, muscle tone, pain, ROM, gait, and function.[1,35]

Conventional radiography
Conventional radiology is most frequently used for imaging in hemophilia and mainly suitable for late osteochondral changes.
Radiologic classification of hemophilic arthropathy is usually performed by the Pettersson score (Table).[36] It is an additive scoring system based on assessment of the knees, ankles, and elbows in which eight criteria are 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,[37] but is only able to diagnose late arthropathic changes. It is typically used to evaluate progression of arthropathy and for planning of arthrodesis or joint replacement. It has been discussed whether digital scoring systems are more objective and have a higher inter-observer variability than the Pettersson method. Digital analysis of hemophilic arthropathy of the knee seems feasible,[38] 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.

Table – Pettersson score[36]

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 >1mm	1
	Joint space <1mm	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 (max 13 points per joint)		

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

Magnetic resonance imaging (MRI)

As prophylactic treatment improves and joint damage diminishes, MRI has obvious advantages over radiography in evaluating treatment efficacy and may be more sensitive to detect early changes. It enables visualization of small connective tissue alterations, such as hemosiderin depositions, synovial hypertrophy, and minor cartilage damage without joint space narrowing.[39] The clinical implications of small changes, especially in the absence of reported joint bleeds, remains to be established,[40] but as in osteoarthritis (OA) it may be predictive of subsequent (fast) 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. abdomen, iliopsoas).

Widespread use of MRI is restrained by its costs, availability, and requirement of sedation in young children.

Ultrasonography

With ultrasonography becoming increasingly standard in rheumatology practices, it is possible to diagnose joint effusion, synovial hypertrophy, abnormalities involving osteochondral surfaces, pseudotumors, and acute hemarthroses.[39,41] It is less costly than MRI, readily available, does not require sedation in young children, and enables dynamic investigation such as assessment of vascularity of a pseudotumor. Disadvantages are the inter-observer variability, complexity of image analyses, difficulty to identify changes in deeper structures, and a lack of validated scoring systems for hemophilic arthropathy.

Biochemical markers

In the field of OA and rheumatoid arthritis (RA) a lot of effort is put in the identification of biochemical markers in blood and/or urine that can assist in diagnosis, prognosis, and response to treatment.[42] Such markers are as yet sparsely used in clinical trials and still not used in clinical practice. Also in the field of hemophilic arthropathy we see that such markers may become of help in identifying the tissue destructive activity of a joint bleed.[43] Because of the relatively fast progression rate of tissue damage as compared to RA and OA, research in hemophilic arthropathy may become a field where marker development may become important.

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 hemophilia patients, together with *in vitro* experiments and animal models, indicated two major process to be involved: synovial inflammation and cartilage degeneration.[20,44] In this respect hemophilic arthropathy has characteristics of the RA as well as the OA joint.[45]

Synovial inflammation and proliferation

Synovial tissue is highly vascularized and in hemophilia even minimal forces can lead to hemarthroses. Blood filling the joint space leads to an influx of inflammatory cells into the synovial tissue. Synovium type A macrophage like cells and invading macrophages evacuate blood from the joint cavity completely over a period of 3-4 weeks.[17] Already four hours after induction of hemarthrosis erythrophagocytosis can be observed.[46] Adaptive changes in the expression of iron regulation proteins in the synovium of hemophilia patients after repeated joint bleedings are demonstrated.[47] With successive hemarthroses the synovial capacity is overloaded and blood remnants, especially iron, accumulate as hemosiderin depositions in synovial tissue and even cartilage.[19]

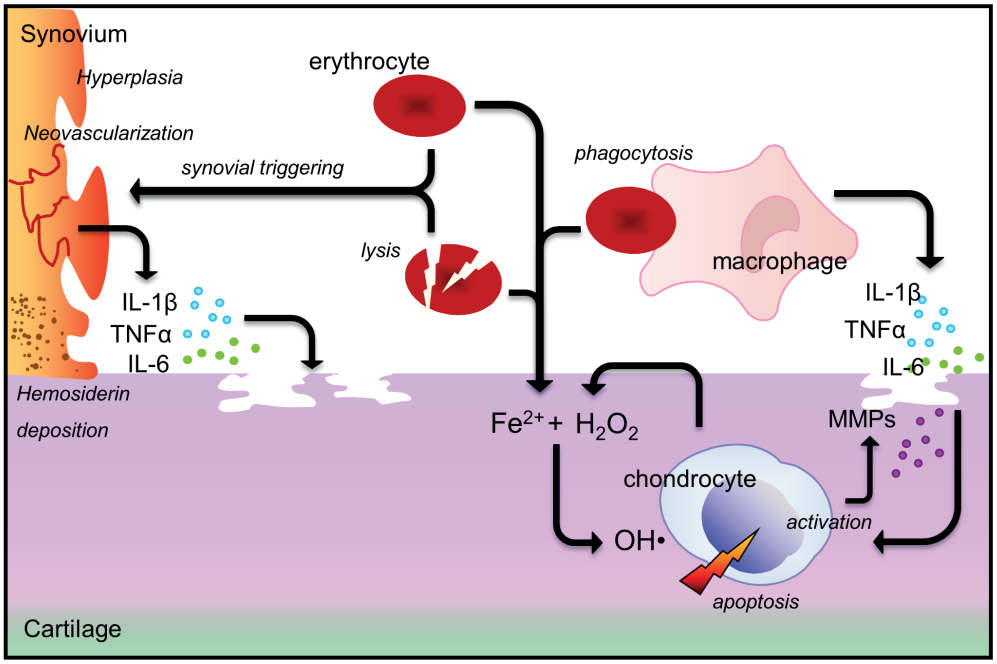

Figure 5 – Proposed mechanism of blood-induced joint damage
See text for more details.

The presence of iron triggers an inflammatory response and stimulates proliferation of synoviocytes. Pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNFα), in addition to tissue destructive proteases like matrix-metalloproteinases (MMPs), are produced by the iron-laden synoviocytes.[19,45] The normally thin synovial membrane becomes irregular, grossly hypertrophied, villous, friable, and highly vascular.[48,49] Synovial proliferation is postulated to result from an aberrant gene expression induced by the overload of iron.[50] It induces an overexpression of the proto-oncogene *c-myc*, associated with synovial cell proliferation, and of the p53 tumour suppressor binding protein *mdm2*, leading to the abrogation of apoptosis of synovial cells.[51,52] An increased oxygen demand of the hypertrophied synovium causes hypoxia and therewith potentially induces the release of growth factors such as vascular-derived endothelial growth factor (VEGF) and the formation of a rich network of brittle capillaries underneath the hypertrophied synovium.[21,53] This sets up a vicious circle as these fragile vessels are more susceptible to recurrent bleeds. Over time chronic synovitis develops with pannus invading and eroding marginal cartilage,[54] and ultimately the synovial tissue becomes fibrotic. Clearly there are similarities to the rheumatoid joint.

Cartilage degeneration

Cartilage destruction results from both synovial-dependent and -independent processes. Synovial pannus invades cartilage at the periphery of inflamed joints. The inflamed

hemosideritic synovium produces cartilage-destructive pro-inflammatory cytokines and cartilage matrix degrading proteases.[19,49] These destructive properties of synovial tissue on cartilage are already present within 24-48 hours after a joint bleed and cause long-lasting damage.[55,56]

Hemarthroses also exert harmful effects directly on cartilage. Exposure of cartilage explants to whole blood or the combination of erythrocytes and mononuclear cells severely impacts cartilage matrix turnover and results in chondrocyte apoptosis.[57,58] The pro-inflammatory mediators IL-1β and TNFα produced directly after blood-exposure transiently decrease proteoglycan turnover. These cytokines also activate chondrocytes to increase hydrogen peroxide (H_2O_2) production, which in combination with iron derived from erythrocytes, leads to oxidative stress.[59,60] Iron in combination with H_2O_2 reacts via the Fenton reaction to form hydroxyl radicals resulting in chondrocyte apoptosis.[61] Since chondrocytes hardly proliferate and have the responsibility to produce and maintain the extracellular matrix, apoptosis of chondrocytes will result in long-lasting impaired matrix turnover. These effects are more pronounced on immature articular cartilage compared to mature cartilage,[62] and impaired cartilage is at least as susceptible to blood-induced joint damage as healthy cartilage.[63] This implicates that it is not only important to prevent joint bleeds in children, but also in patients with already affected joints. These direct effects on cartilage with chondrocyte death represent characteristics of OA.

Treatment of hemophilia

Treatment in hemophilia consists of substitution of the deficient clotting factor. This was first performed by whole blood transfusion and fractions from plasma. The introduction of plasma-derived factor concentrates in the 1960s dramatically improved treatment and quality of life in hemophilia patients. It enabled prophylactic clotting factor replacement therapy, therewith converting the disease form a severe to a milder form.[64] 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 risk of viral transmissions.

Traditionally, treatment is given at time of clinically evident bleeding (episodic treatment, on-demand therapy). Nowadays, in developed countries most patients with severe hemophilia are treated prophylactically, i.e. by regular intravenous injection to prevent anticipated bleeding.[1] This treatment is called primary prophylaxis as it is initiated before the second clinically evident large joint bleed and before the age of three years. In secondary prophylaxis regular treatment is started after two or more bleeds into large joints, but before the onset of '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, optimum dosage, frequency of injection, and whether or not prophylaxis should be given indefinitely

remain subject of discussion.[65-67] These are important questions to address as prophylaxis is a highly expensive treatment.

Prophylactic factor VIII replacement typically requires infusions 2-3 times a week, whereas factor IX can be given less frequently due to a longer half-life. The burden of regular venipunctures in young children is high and central venous access devices, such as Port-A-Cath and Hickman lines, are often inserted for reliable long-term venous access.[68]

In patients with mild hemophilia A or hemophilia A carriers, desmopressin (DDAVP), 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.[69] On average a three-fold increase in factor VIII levels can be expected, and therefore a residual factor VIII activity of >10% is required. Responsiveness to desmopressin should be tested prior to therapeutic treatment as it varies largely between patients.

Antifibrinolytic therapy is useful to treat or prevent bleedings in areas of increased fibrinolysis, such as skin and mucosal surfaces (epistaxis, menorrhagia, or oral bleeding).[70] It promotes clot stability by inhibiting plasminogen activation in the fibrin clot. Tranexamic acid is the most commonly used antifibrinolytic, acting 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 as it has a shorter half-life, is less potent, and more toxic.[71]

To improve hemophilia treatment, current research focusses on factor modifications to extend the half-life, and development of gene-based therapy.[72,73] Improved bioavailability could be 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 1-2 weeks. Development of gene therapy is also most promising in hemophilia B as the factor IX gene is considerably smaller than the factor VIII gene and anti-factor VIII immunity is far more frequent. In a trial for hemophilia B, long-term expression of factor IX in levels sufficient to improve the bleeding phenotype has been observed following adeno-associated viral (AAV) vector-mediated gene transfer to the liver.[74] The main concern with this approach is immune-mediated clearance of AAV-transduced hepatocytes. Moreover, about 40% of the men with severe hemophilia B will be excluded for this therapy based on the presence of pre-existing neutralizing antibodies to AAV.[75] Although the initial studies seem promising, there are still a number of significant hurdles to overcome.

Complications of treatment

Inhibitors

Inhibitors are alloantibodies that neutralize clotting factor activity following infusion of factor VIII or factor IX, primarily immunoglobulin G4 (IgG_4). Risk of inhibitor development is highest in the first period of treatment, especially in the first 50-75 exposure days. Patients with

severe hemophilia A have a lifetime risk of inhibitor development of 20-30%, compared to a risk of 5-10% in moderate or mild disease.[76,77] Inhibitors are much less frequently encountered in hemophilia B, occurring in less than 5% of affected individuals.[78] 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.[77] Development of factor IX inhibitors is associated with severe allergic or anaphylactic reactions to factor administration, whereas this is almost never encountered in hemophilia A patients.[79] Inhibitor development could be suspected in patients failing to respond to clotting factor infusion, especially if previously responsive. In patients with mild or moderate hemophilia, the presence of an inhibitor can convert the bleeding phenotype in a more severe form as the inhibitor may neutralize endogenously synthesized clotting factor.

Patients with inhibitors are classified according to the inhibitor titer, expressed in Bethesda units. Patients developing 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 hemophilia B.[80,81] ITI in hemophilia B patients carries the risk of anaphylactic reactions and the development of nephrotic syndrome secondary to complex formation and deposition in the kidney. In refractory patients with hemophilia A rituximab may be useful.[82,83] Other immunosuppressive treatments are high dose prednisolone, cyclophosphamide, or azathioprine.[84] 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.[85,86] These agents act by boosting thrombin generation independently of the presence of factor VIII or IX.

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 due to transmission of blood-borne virus infections. More than 90% of hemophilia patients treated with these products before 1985-1987, became infected with hepatitis C (HCV).[87-89] A chronic infection developed in the majority of them and in 20-30% it progressed to liver cirrhosis. The cumulative incidence of human immunodeficiency virus (HIV) infection in the United States in the peak years was 78% of the factor VIII recipients and 37% of the factor IX recipients.[90] Other viruses transmitted are hepatitis A, B, or G, and cytomegalovirus.

Regarding safety of plasma-derived factor concentrates different measures are taken, including selection of healthy donors, screening donations for the absence of relevant infectious blood-borne viruses and screening for viral markers by serology and nucleic acid testing. Additionally, various viral inactivation procedures are required, e.g. pasteurization, exposure to solvent-detergent, dry heat, chemical disruption with sodium thiocyanate, or ultrafiltration.[91]
The introduction of recombinant products and the implementation of these measures, almost completely eliminated HIV and HCV transmission through factor concentrate.[92,93] Hepatitis A and B immunization is still recommended to reduce infection risk, although the risk of infection nowadays is low.[94]

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. For optimal management of musculoskeletal complications multidisciplinary care is required. As prevention is the cornerstone, the hemophilia specialist is important for prevention and adequate treatment of bleedings, and early recognition of musculoskeletal issues. Physiotherapy is required for recovery after musculoskeletal bleeds and to maintain muscle strength in established hemophilic arthropathy. A physiatrist can assist in patient education to prevent disabling injuries, care during bleeding episodes, in long-term rehabilitation, and for orthotics and shoe adaptations.[95] In end-stage arthropathy, orthopedic surgery is often inevitable.

Acute hemarthrosis
The primary aim in therapy for acute hemarthrosis is to stop the bleeding as quickly as possible by administration of clotting factor concentrates. The efficacy of joint aspiration (under adequate factor replacement) is still unclear. Shortening the exposure of cartilage to blood theoretically might limit blood-induced damage.[96] Aspiration, although common in rheumatology practice, bears the risk of infection or re-bleeds and even when the joint is washed, blood remains will always be left in the joint. No data are available on long-term benefit of aspiration. The most recent *in vitro* experimental data suggest that limitation of blood-exposure to cartilage may prevent tissue damage later on.[96] However, this has to be confirmed by clinical studies that will remain difficult to perform. Clearly, if the suspicion of infection is high, arthrocentesis is mandatory for diagnosis, but in general it should be avoided. According to the WFH guidelines it may be considered in a bleeding, tense, and painful joint showing no improvement 24 hours after conservative treatment and after exclusion of inhibitor development.[1] It should be performed only after appropriate clotting factor replacement and under strictly aseptic conditions. In recurrent or massive bleedings unresponsive to appropriate clotting factor replacement,

therapeutic embolization could be considered.
Pain relief could be achieved by analgesics, but R.I.C.E. (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.[97] 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 might be achieved by (more intensive) prophylactic clotting factor concentrate replacement during 6-8 weeks combined with active physiotherapy and cooling. Cyclo-oxygenase-2 (COX-2) inhibitors may reduce inflammation.[98] Other non-steroidal anti-inflammatory drugs (NSAIDs) should be avoided concerning the possible impairment of platelet function. Moreover, they have been suggested to bear cartilage damaging effects.[99] 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 radiopharmaceuticals or chemicals. Radioisotopic synovectomy using a pure beta emitter (yttrium-90 or phosphorus-32) is preferred, as it is minimally invasive, requires little factor replacement, and less intense rehabilitation afterwards. It is very successful in reducing hemarthroses with 60-100% in the majority of patients.[100] If necessary, the procedure can be repeated after six months. However, potential direct harmful effects to cartilage in the long term should be taken into consideration.[101] 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.[102] Surgical options are preferred in cases with severe synovial hypertrophy on MRI or in case of bone cysts as they carry the risk of dispersing radioactive material outside the intra-articular space. It requires a large supply of clotting factor and intensive rehabilitation.[103]

Hemophilic arthropathy
In established hemophilic arthropathy, the goal of treatment is to improve joint function, relieve pain and assist the continuation or resumption of normal activities of daily living. With conservative procedures remarkable benefit can be provided and surgical interventions postponed. For pain relief, adequate analgesic treatment, manual traction of the joint, and in some cases transcutaneous electrical nerve stimulation (TENS) are indicated.[104] 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.[105] In advanced ankle arthropathy, immobilization with a cast can provide insight whether arthrodesis would be helpful.

In end-stage arthropathy orthopedic surgery is often indicated. Different surgical options could be considered depending on the joint or specific condition needing correction. Joint replacement therapy (knee, hip and less commonly elbow and shoulder) and ankle arthrodesis are the most performed procedures. Joint replacement therapy is successful in pain relief and regaining functional activities and participation. Hip replacement usually improves ROM, whereas after knee replacement therapy a restricted ROM may limit functional recovery.[106] Ankle arthrodesis is 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 adjacent joints of e.g. the lower limb/foot necessitating surgery of other joints.[107,108] Total ankle replacement has the advantage of preservation of motion but information on long-term follow-up is scarce. There is the concern of higher rates of aseptic loosening and deep infections due to poor bone quality and increased risk of micro-bleedings at the prosthesis-bone interface.[109,110] In this respect, new surgical approach for the ankle could be the use of joint distraction. Joint distraction in treatment of OA has gained more and more attention over the past years.[111] This technique seems also very helpful for treatment of hemophilic ankle arthropathy,[112] although larger and prospective studies have to be awaited.

Other procedures performed in end-stage arthropathy or in case of severe contractures include soft tissue contracture release, arthroscopic nettoyage 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.[113] Surgical interventions should always be performed at or in consultation with a comprehensive hemophilia treatment center.[114] 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 suffering from 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.[115] Herewith, reduced quantities of clotting factor concentrate are required and a quicker regain of participation is expected. Careful assessment by a multidisciplinary team is a prerequisite to ascertain that recovery will not be compromised for any of the procedures.

Aims and outline of this thesis

Despite the increase in the use of prophylactic infusions of clotting factor starting at an early age, it is not possible to prevent hemarthroses and subsequent joint damage completely. The aim of this thesis is to gain more insight in the pathophysiology of blood-induced joint damage, to detect joint damage early to prevent further damage, and to investigate potential new therapeutic modalities. For this purpose, a literature study, several human *in vitro* and *ex vivo* studies, animal *in vivo* studies, an observational cohort study, and an interventional pilot study in hemophilia patients were undertaken.

Iron in the pathophysiology of hemophilic arthropathy

The first part of this thesis focuses on the role of iron in the pathophysiology of blood-induced joint damage as a pivotal role is suggested and differences in iron control hypothetically explain the clinical variability in severity of joint damage observed. In the genetic disorder hemochromatosis iron control is disturbed causing iron overload, leading to organ damage and arthropathy. A previous pilot study suggested that carriership of an *HFE* mutation, which can cause hemochromatosis, aggravates hemophilic arthropathy.[116] In **chapter 2** the currently available literature is reviewed to compare the clinical and histological characteristics of arthropathy in hemophilia to those in the arthropathy of hemochromatosis. In **chapter 3** a large cohort study is described investigating whether carriership of an *HFE* mutation affects hemophilic arthropathy. Moreover, in this cohort the effect of polymorphisms in heme-oxygenase on the severity of hemophilic arthropathy is explored. This enzyme is the rate limiting step in heme degradation, has a role in inflammation, and is associated with joint damage progression in RA.[117] Its role in hemophilic arthropathy was never investigated.

Translation

At present, joint damage in hemophilia is evaluated via imaging lacking the ability to detect early changes as in general they need time to become sufficiently discriminative. MRI has the capacity to detect small changes, but is unsuitable in young children and for regular evaluation of joint damage progression. Moreover, new diagnostic strategies are needed to prospectively evaluate the effect of new treatments on joint damage in clinical trials. Biochemical markers of joint tissue damage have the potential to provide dynamic information on tissue turnover and to detect changes in an early phase. **Chapter 4** describes a study in which the effect of a joint bleed on four of these markers is investigated by measuring them prospectively in urine and serum after an induced joint bleed in a canine *in vivo* study and in hemophilia patients after an observed joint bleed.

Treatment

Targeted therapy for hemophilic arthropathy is still lacking. Treatment consists of the prevention of joint bleeding by clotting factor substitution 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. In **chapter 5**, it is investigated whether antagonizing the pro-inflammatory activity of IL-1β or TNFα can prevent blood-induced cartilage damage *in vitro*, and whether this effect is dose- and time-dependent.

Previous studies have shown a protective effect of the anti-inflammatory cytokines IL-4 and IL-10 *in vitro*,[118,119] but with limited efficacy *in vivo*.[120] In **chapter 6** a fusion protein of IL-4 and IL-10, combining the function of both cytokines and increasing their bioavailability, is tested *in vitro* in human blood-cartilage co-cultures, and *in vivo* in a murine model of hemophilic arthropathy.

For patients with established hemophilic arthropathy, limiting inflammation will not be appropriate to improve joint health. Joint distraction is a surgical alternative for ankle arthrodesis or ankle replacement, successfully used in OA.[121] In hemophilia, a retrospective study in three patients with severe ankle arthropathy has shown beneficial effects.[112] **Chapter 7** describes the preliminary one-year results of a prospective, interventional study investigating the efficacy of joint distraction in severe hemophilic ankle arthropathy.

Chapter 8 summarizes and integrates the main findings of this thesis and places them in a broader perspective. Additionally this chapter discusses the implications of these findings for patient care, elaborating on several open issues and challenges for future research.

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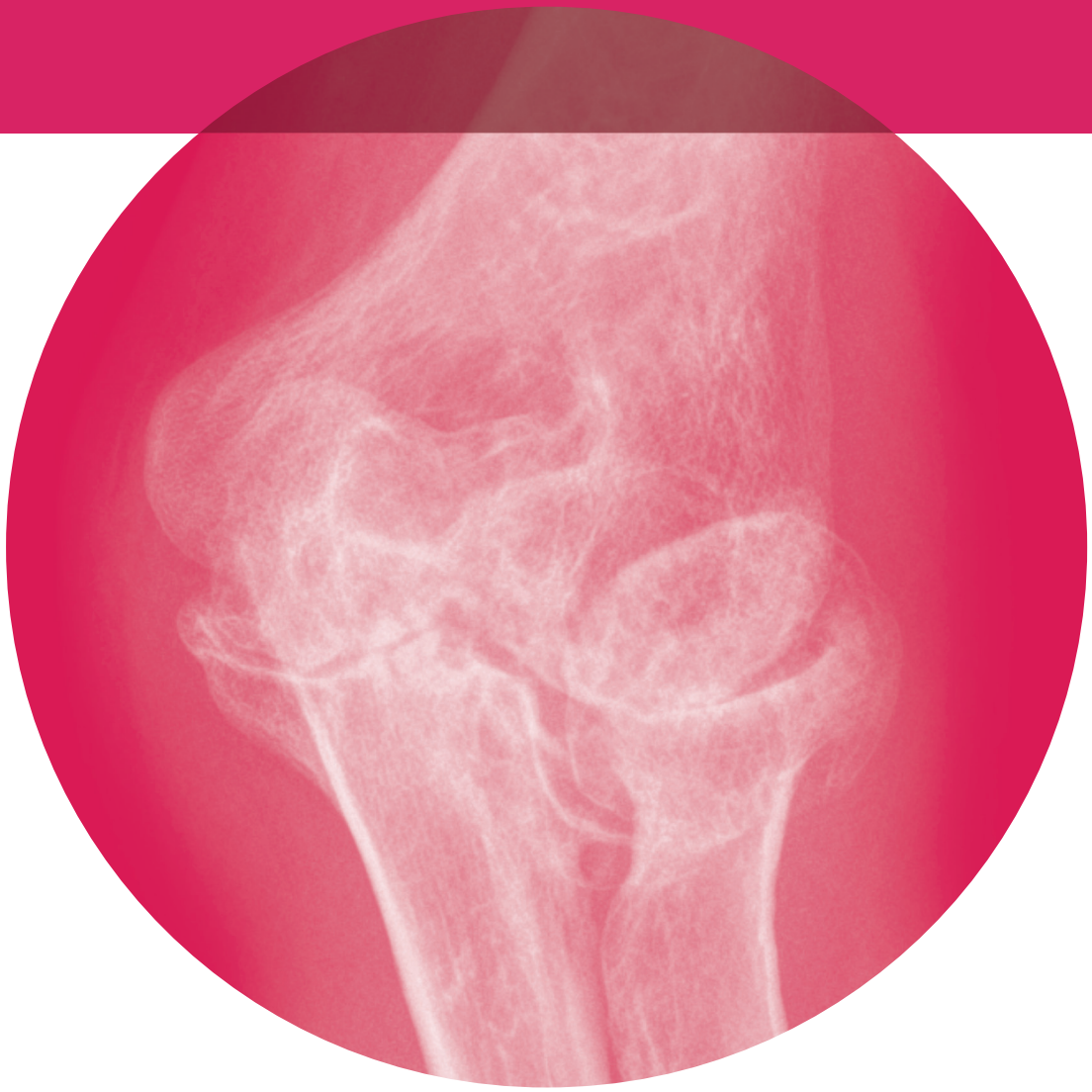

CHAPTER 2

THE DETRIMENTAL EFFECTS OF IRON ON THE JOINT

a comparison between hemochromatosis and hemophilia

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Abstract

Joint damage due to (recurrent) joint bleeding in hemophilia causes major morbidity. Although the exact pathogenesis has not been fully elucidated, a central role for iron is hypothesized. Likewise, in hereditary hemochromatosis joint destruction is caused by iron overload. A comparison between these types of arthropathy could provide more insight in the influence of iron in inducing joint damage. A literature review was performed to compare both disorders on their clinical and histological characteristics, and preclinical studies were reviewed on the influence of iron on different joint components. Similarities in the features of arthropathy in hemochromatosis and hemophilia are cartilage degeneration, subchondral bone changes with osteophyte and cyst formation, and osteoporosis. In both disorders synovial inflammation and proliferation are seen, although this is much more explicit in hemophilia. Other substantial differences are the age at onset, the occurrence of chondrocalcinosis radiographically and calcium pyrophosphate dihydrate deposition disease in hemochromatosis, and a rapid progression with joint deformity and neovascularization in hemophilia. Preclinical studies demonstrate detrimental effects of iron to all components of the joint resulting in synovial inflammation and hyperplasia, chondrocyte death, and impaired osteoblast function. These effects, particularly the synovial changes, are aggravated in the presence of a pro-inflammatory signal, which is prominent in hemophilic arthropathy and minimal in hemochromatosis. Additional research is needed to further specify the role of iron as specific target in treating these types of arthropathy.

Introduction

Iron is an essential trace element, involved in many biological reactions mainly through its ability to accept or donate electrons and thus catalyze redox reactions. Because iron is so critical, the body recycles and conserves it carefully.[1] When iron loss is increased, for instance in case of an external bleed, intestinal iron absorption is upregulated. Remarkably, no significant physiologic mechanisms exist for regulating iron loss to compensate for excessive iron uptake, as is the case in iron storage diseases. Free Fe^{2+} can contribute as catalyst to oxidant-mediated cellular injury. In this process Fe^{2+} donates an electron to form Fe^{3+}, and oxidant species accepting this electron are converted to super-oxide (O_2^-), hydrogen peroxide (H_2O_2), and the most powerful electron acceptor hydroxyl radical (OH·). Considering this electron accepting function of O_2 and its high redox potential, it is not surprising that iron in an aerobic environment can be enormously toxic.

Iron overload in hereditary hemochromatosis (HH) is caused by a dysregulation in hepcidin production. This hepatic peptide is considered the principal regulator of iron absorption. In a high iron state, hepcidin is released, binds to the iron exporter ferroportin, and induces its degradation to decrease iron uptake by the gut. This tight control of iron uptake is necessary as excessive amounts of free iron generate cellular toxicity, and no natural mechanism exists to enhance iron excretion.[1] HFE (*H*igh Iron *Fe*) is a major histocompatibility complex (MHC) class I–like molecule involved in the regulation of hepcidin expression. Cys282Tyr *HFE* gene mutations, transmitted autosomal recessively, account for most of the mutations causing the classical variant of HH[1] (Table 1), although other mutations have been described.[2]

In hemophilia, an X-linked inherited disorder involving deficiency of clotting factor VIII (hemophilia A) or IX (hemophilia B), thrombin generation is diminished causing a bleeding tendency (Table 1). Although bleeding may occur anywhere, approximately 70-80% of hemorrhages occurs in the joints, spontaneously as well as in response to mechanical stress or trauma,[3,4] causing joint degeneration by inflammation and direct tissue damage, called hemophilic arthropathy (HA).[5,6] Iron is hypothesized to be important in inducing arthropathy in HH and HA, although in both diseases the exact pathogenesis has not been fully elucidated.

The aim of this review is to collect the available data on the role of iron in inducing joint damage. We conducted a literature search to compare the clinical and histopathologic consequences of local iron overload (hemophilic arthropathy) and systemic iron overload (hereditary hemochromatosis). Moreover, we reviewed the effects of iron on different joint components as investigated *in vitro* or in animal studies. A better understanding of the role of iron in developing joint damage will translate to improved targeted treatment in both hemophilia as well as hemochromatosis.

Table 1 – Clinical characteristics of joint complaints in hereditary hemochromatosis and hemophilia

	Hemochromatosis	Hemophilia
Genetics	*HFE* gene	*FVIII* or *FIX* gene
	Autosomal*	X-linked
Origin	Iron accumulation	Joint bleed(s)
	Fe^{3+} from systemic overload	Fe^{2+} in the presence of other (inflammatory) blood components
Gender	Men > women	Men
Age	> 50 years	Starting at a young age
Prevalence of joint complaints	24-81%	Up to 100% in the elderly patients with severe haemophilia
Uni/polyarticular	Polyarticular	Polyarticular with usually a target joint
Predominant site	Hand (classic: MCP2-3), wrist, hip, knee, ankle	Large synovial joints (elbow, knee, ankle)
Articular complications	Degenerative cartilage damage	Acute joint bleed
	CPPD	Chronic synovitis
	Bone changes – osteoporosis, subchondral sclerosis	Degenerative cartilage damage
		Bone changes – cysts, subchondral sclerosis, osteoporosis
Symptoms	Stiffness, pain on movement, slight joint swelling	Acute joint bleed, chronic swelling, pain on movement
Treatment	Systemic iron depletion – phlebotomy or iron chelation	Clotting factor replacement
	Analgesics	Analgesics
	Physical therapy	Physical therapy and orthotics
	Surgery – joint replacement	Synovectomy – chemical, radioactive or surgical
		Surgery – joint replacement, arthrodesis, joint distraction

* The classical variant of hereditary hemochromatosis and juvenile hemochromatosis are autosomal recessive. In some families, autosomal dominant mutations have been described.
CPPD, calcium pyrophosphate dehydrate; *HFE*, *H*igh Iron *Fe*; MCP, metacarpophalangeal; FVIII/FIX, factor VIII/IX.

Methods

For this review, we searched literature on clinical and histopathological joint changes in hemochromatosis and hemophilia. Embase and PubMed (August 2014) were accessed for relevant articles written in the English language using the following terms: “arthropathy OR joint OR arthritis” AND “haemophilia OR hemophilia OR haemochromatosis OR hemochromatosis”. Articles were selected based on title and abstract. Articles discussing radiologic joint changes only and case reports were discarded. Assessing the references of all relevant articles retrieved additional articles.

Furthermore, separate searches were performed using terms on iron, heme and blood components, combined with terms defining different components of the joint to retrieve articles investigating the effect of iron on joint tissue in *in vitro* and animal studies.

Clinical characteristics of arthropathy in hemochromatosis and hemophilia

Symptoms

Arthropathy in HH is a progressive polyarticular disease with a preference for the second and third metacarpophalangeal (MCP) joint.[7,8] Also other joints in the hands, and large joints such as the hip, knee, ankle, shoulder, and elbow can be affected. The reason for the MCPs as predilection site is not known. Joint complaints are the presenting symptom in nearly 30% of the patients,[9] but usually occur after diagnosis and are more frequent in patients over 50 years of age.[10,11] The frequency of arthropathy reported ranges from 24-81% depending on the population studied and definition of arthropathy used.[12] Factors associated with susceptibility to arthropathy are age, a serum ferritin level >1000 µg/mL at diagnosis, and homozygosity for Cys282Tyr.[8,13]

Symptoms resemble osteoarthritis (OA) with stiffness, pain on movement, and only slight joint swelling. Joint inflammation is typically minimal, but fluctuating inflammatory symptoms might be present, especially in case of calcium pyrophosphate dihydrate crystal deposition (CPPD) disease.[12,14] In addition, osteoporosis is frequently observed with incidences varying from 25 to 50 percent.[15-17]

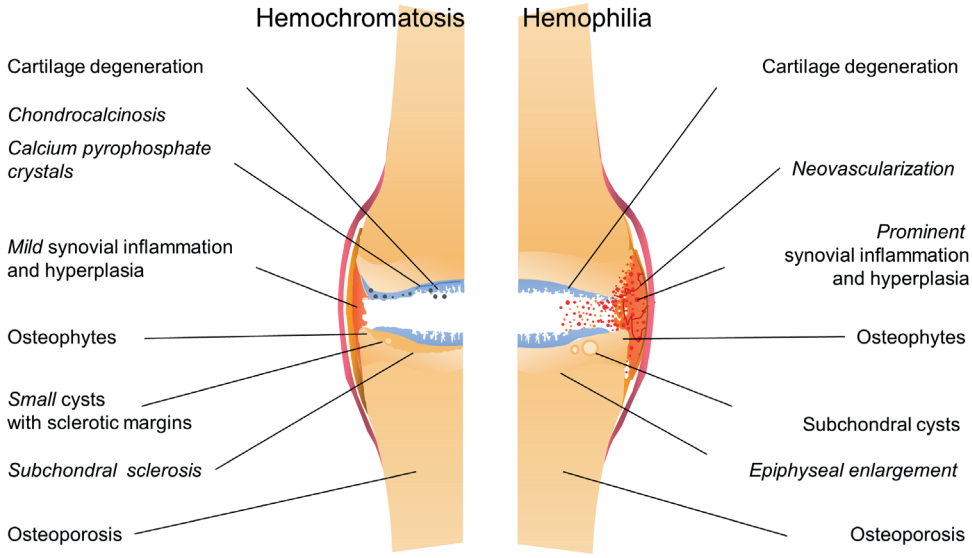

Figure 1 – Schematic representation of arthropathy in hemochromatosis versus hemophilia
The structural changes in arthropathy in hemochromatosis are shown on the left, and those in hemophilia on the right. Note the differences indicated in italics.

On radiological examination the arthropathy of HH resembles idiopathic OA and CPPD with joint space narrowing, subchondral sclerosis, and osteophyte formation (Figure 1).[7,14,18-20] Cartilage loss is often accompanied by subchondral cyst formation. Calcification within the cartilage (chondrocalcinosis) is present frequently,[18] and can be observed in articular and non-articular cartilage.[21]

Nearly all patients with severe hemophilia (clotting factor activity <1%) experience hemarthroses (joint bleeds), mainly in the elbows, knees or ankles.[22] Also for HA the preference for these joints is not fully elucidated yet. A joint bleed causes pain, a tense effusion and limitation of motion, responding rapidly to replacement of the deficient clotting factor. Once bleeding has occurred, the joint is more susceptible to recurrent bleeding and this might subsequently lead to chronic synovitis and cartilage degeneration. In chronic synovitis, the joint is swollen but usually not tense nor particularly painful.[23] Ultimately HA may develop, characterized by stiffness, chronic pain, deformities, and a severely limited range of motion. Cartilage is destroyed, bony erosions occur, and synovial inflammation is replaced by progressive fibrosis. Secondary soft tissue contractures, muscle atrophy, and angular deformities with loss of motion cause invalidity. Factors known to influence the development of HA are the severity of hemophilia and age at first joint bleed.[22] A genetic factor suggested to be associated with the severity of arthropathy is the *HFE* mutation,[24] although these data require confirmation.
Many features of HA on radiological assessment are similar to arthropathy in HH including joint space narrowing and subchondral bone changes, such as sclerosis, cysts and osteophyte formation (Figure 1).[25,26] Hemarthrosis before epiphyseal plate closure may cause epiphyseal enlargement. HA is often accompanied by osteopenia and even osteoporosis. In end-stage HA severe malalignment of the joint and ankylosis occur.

Treatment
Treatment for HH consists of systemic iron depletion by phlebotomies (venesection) or use of iron chelation therapy. In contrast with its effect on visceral iron overload, this is seldom helpful in relief of joint pain. Some patients even report an increase in symptoms after phlebotomy.[27]
Low-dose colchicine, non-steroidal anti-inflammatory drugs, intra-articular glucocorticoids or other analgesics alleviate pain but have no influence on the progression of arthropathy. Physiotherapy and conservative measures might be useful, but in end-stage arthropathy joint replacement is often necessary.

In hemophilia, treatment aims at preventing intra-articular hemorrhages and the consequent development of HA. Although less, hemarthroses and the consequent joint damage still occur.[28,29] Once arthropathy has developed, conservative management, including analgesia, physiotherapy and orthotics, is needed to alleviate symptoms, maintain mobility and muscle

strength, and prevent contractures, disability and invalidity.[30,31] In chronic synovitis with frequent recurrent bleeding which could not be controlled otherwise, synovectomy can be performed, either with chemical or radio-isotopic substances, or surgically. In advanced stages of HA, surgical procedures are the last resource, e.g. prosthetic joint replacement and ankle arthrodesis.[23]

Histopathology

Knowledge on histologic changes occurring in the joint in both HA and HH has mainly been obtained from surgical specimens derived at the time of joint replacement surgery, and is therefore only representative of advanced end-stage disease. These findings are discussed in this paragraph and summarized in table 2. *In vitro* and animal models used to study early(-er) changes and the role of iron on isolated joint components are discussed in the next paragraph.

The presence of iron is demonstrated in synovial tissue of both hemophilia and hemochromatosis patients. In HH hemosiderin, an intracellular iron-storage complex, is found primarily within superficial synovial lining cells, but also in macrophages due to phagocytosis of iron loaded synoviocytes.[32,33] In the deeper layers, very little iron can be detected, only occasionally in macrophages.[33]
In HA iron is also most abundantly present in the superficial layer, but is also demonstrated in the deeper layers.[34-36] Remarkably, in hemophilia macroscopically brownish tissue can be found adjacent to normal appearing white synovial tissue.[36] Deeper iron deposits are mainly seen in the brownish tissue and consist both of extracellular aggregates and intracellular deposits in subsynovial fibroblasts.[36,37] As in HH, iron-laden macrophages might be found in the deeper portions of the synovium.[35,38] Also the normal appearing tissue shows iron staining in the lining, but much less iron in the deeper layers.
A recent study suggested an adaptation of synovial tissue to an increased iron processing in HA compared to healthy controls.[39] A clear upregulation is demonstrated for the iron regulating proteins ferroportin, feline leukemia virus subgroup C receptor (FLVCR), and heme carrier protein 1 (HCP-1). The overall expression of hepcidin is not altered nor is the total expression of CD163, a scavenger receptor for the hemoglobin-haptoglobin (Hb-Hp) complex. CD163 mediates the internalization of Hb-Hp complexes by macrophages but is also a marker of alternative activation of macrophages. In contrast to its overall expression, CD163 in the lining is increased in HA compared to healthy controls, the same enhanced expression is seen in the lining of rheumatoid arthritis (RA) patients. In contrast, CD163 expression in the lining of HH is significantly lower compared with RA synovium, and more comparable to OA synovium.[40] This low expression in HH compared to HA is probably due to a difference in inflammation and the absence of Hb-Hp complexes intra-articularly in HH. None of the other iron regulating proteins has been studied in HH.

Table 2 – Histopathologic changes in arthropathy in hemochromatosis and hemophilia

	Hemochromatosis	Ref.	Hemophilia	Ref.
Synovium				
Hyperplasia	Mild	32	Prominent	36,37
Villus formation	Microvilli	32	Macrovilli	36,37
Inflammation	Low-grade	40	Prominent	37,41,42
Predominant type of immune cells	Macrophages and neutrophils	40	Monocytes/ macrophages	43
Iron deposits				
Lining				
Intracellular	In synovial cells and macrophages	32,33	Predominantly in synovial cells	34,36
Extracellular	No		Siderosomes	36
Sublining	Very little, in macrophages	33	In fibroblasts	36,37
Neovascularization	No	40	Prominent	43,44
Cartilage				
Iron deposits	Occasionally in superficial chondrocytes	45,46	Occasionally in superficial chondrocytes	42
Chondrocytes	Clustering	46	Clustering (in the surface layer)	42,47
Matrix	Proteoglycan loss	46	Proteoglycan loss	
Chondrocalcinosis	Frequent	21	Rarely	48
Tidemark	Sometimes a split	45,49	Not reported	

Ref – reference

Arthropathy in HH is generally considered to be non-inflammatory and a chronic inflammatory cell infiltrate is rarely seen.[32] The amount of lymphocyte infiltration is similar to OA, but a more prominent macrophage infiltration in the sublining is demonstrated.[40] In most cases there is only low-grade synovitis,[40] mild intimal cell hyperplasia, and formation of microvilli.[32] Neutrophil infiltration is confined to hemosiderin positive samples suggesting an association with iron deposition.[40]

In hemophilia synovial inflammation, which is more notable than in HH, is also seen particularly around hemosiderin depositions.[37,41,42] Pro-inflammatory cytokines like interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNFα), are produced significantly more by hemosideritic synovial tissue compared to normal appearing tissue of the same patient.[36] Hyperplasia and marked villous hypertrophy develop, comparable to other types of proliferative synovitis.[36,37] Moreover, pro-angiogenic mediators like vascular endothelial growth factor (VEGF) are upregulated and synovial vascularization is increased,[43,44] potentially induced by hypoxia as

a result of an increased oxygen demand due to inflammation[50] and reduced blood flow upon increased intra-articular pressure.[51] In contrast, synovium of HH patients shows no signs of neovascularization.[40] The production of pro-inflammatory cytokines by synovial tissue of HH patients is never reported on.

In summary, although iron plays an important role in synovial changes in hemophilia, data from hemochromatosis indicate that the presence of iron on itself is not the sole element responsible for inducing synovitis.

Cartilage

Whereas the presence of synovial iron is ubiquitously for both disorders, in cartilage this is less clear. Some studies report no iron depositions in the cartilage at all,[32,38,47,49] while others report some limited deposition within chondrocytes in both HA[42] and HH.[45,46] Depositions in the extracellular matrix are not described.

Clustering of chondrocytes, accompanied by a decrease in proteoglycan content comparable to OA, is seen in both HA and HH.[42,46,47] Clefts within the cartilage might be observed,[47] as well as fibrillation.[42] Although synovial inflammation is a distinct feature in HA, cartilage resembles more the degenerative changes as seen in OA and HH than those seen in RA.[42] Damage is most prominent in the weight-bearing areas whereas peripheral cartilage appears relatively normal in HA, in contrast to cartilage damage in RA showing a centripetal pattern.[52,53]

The impact of blood exposure on extracellular matrix turnover is demonstrated in HA patients, showing an increase in biomarkers of joint tissue turnover upon a joint bleed.[54] Interestingly, also iron removal seems to impact extracellular matrix turnover. An increase in biomarkers of type II collagen turnover is detected after phlebotomy,[55] explaining at least in part the increase in arthralgia during phlebotomy in some HH patients.

Cartilage calcification due to CPPD or apatite deposition is a frequent finding in the arthropathy of HH.[46] There is no close spatial relationship between iron and CPPD crystals and crystals can be found even in the absence of iron deposits.[46] The assumption that factors other than local iron deposition may be relevant is underlined by the fact that RA, although associated with synovial hemosiderosis, is negatively associated with chondrocalcinosis and CPPD.[56,57] In HA, although rarely, chondrocalcinosis is demonstrated.[48] The lack of demonstrable cartilage calcification might result from the rapid progression of cartilage damage compared to HH.

A specific finding in HH arthropathy is a split at the level of the tidemark or at the cartilage-bone interface.[45,49] This is described especially in hip arthropathy, even in the absence of radiographic chondrocalcinosis or CPPD crystals. It is accompanied by marked narrowing or total absence of the calcified zone, and hypothesized to result from an increased susceptibility to shearing forces.[45] Although unusual, horizontal splitting of the cartilage is also found in primary OA,[58,59] but not described in hemophilia.

Altogether, cartilage degeneration is seen both in HH and HA, and is comparable to the changes seen in OA. The presence of CPPD and cartilage calcification, as seen in other metabolic disorders, is observed frequently in HH, but not in HA.

Bone

The most prominent bone changes observed in HA are cyst formation, subchondral sclerosis, osteophyte formation, epiphyseal enlargement, and osteoporosis.[26] In HH also cysts, subchondral sclerosis, and osteoporosis develop.[60] The subchondral cysts in HH are remarkably smaller than the ones seen in HA. Probably, as many of these changes are comparable to those seen in other degenerative diseases like OA, they are secondary to cartilage degeneration and independent of iron. However, this would contrast the recent ideas on a causative role of subchondral bone changes in cartilage degrading processes in OA.[61]

Little is known about the role of iron in inducing bone changes, except for osteoporosis. A major pathway regulating bone turnover consists of the receptor activator of nuclear factor-κB (RANK), RANK ligand, and osteoprotegerin (OPG). The expression of this pathway in synovial tissue from HA patients shows a shift towards more osteoclastic activity.[62] Also in HH an increased osteoclastic activity is shown.[63] An increase in concentrations of midfragments of the parathyroid hormone has been shown, and these levels correlate with both the number of joints with subchondral arthropathy and with ferritin levels,[18] still suggesting a direct relation with iron.

However, the development of osteoporosis is a multifactorial process. Contributing factors in hemophilia are 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 its treatment.[64,65] In hemochromatosis, potential contributing factors are hypogonadism and the consequent low levels of testosterone, as well as cirrhosis; although osteoporosis also occurs in HH patients without these factors.[15-17]

Thus, the bone changes observed in hemochromatosis and hemophilia, consisting of osteoporosis and subchondral changes resembling OA, are rather similar, but the role of iron in inducing these changes remains speculative.

Preclinical studies on the effects of iron versus blood on the joint

Although arthropathy in hemophilia and hemochromatosis share many characteristics, as described in the previous paragraph, striking differences might be explained by the exposure to either iron in case of HH compared to heme-derived iron together with other blood components in case of HA. In this paragraph preclinical studies are discussed investigating the effect of iron and heme with/without other blood components *in vitro* or in animal models. The results are summarized in Figure 2.

Iron and synovial cell proliferation

In the induction of synovial hyperplasia and villus formation, a central role for iron is hypothesized. However, the effect of iron alone is limited, iron administration either intra-articularly or systemically in rabbits induces at most a moderate synovial thickening and

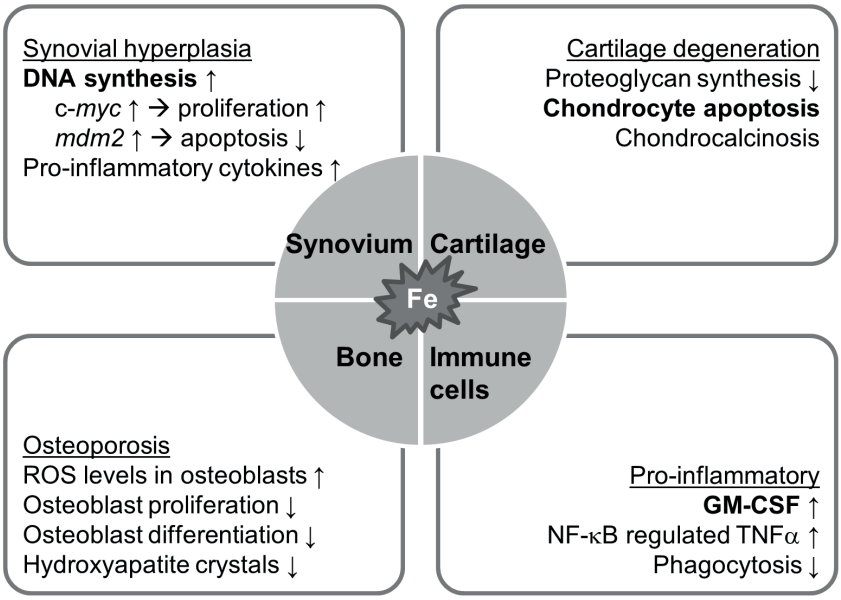

Figure 2 – Influence of iron on different components of the joint
In bold are effects of iron enhanced in the presence of pro-inflammatory cytokines.
DNA, deoxyribonucleic acid; GM-CSF, granulocyte macrophage colony-stimulating factor; NF-κB, nuclear factor-κB; ROS, reactive oxygen species.

increase in cellularity.[66,67]

Changes upon a single intra-articular blood injection are reversible in rabbits,[68,69] and it causes only minor changes in dogs.[70] In contrast, in hemophilic mice a single joint bleed leads to extensive and persistent synovial hyperplasia and villus formation.[71-73] This discrepancy is probably due to the duration of blood exposure, as a single bleed is removed quickly by the synovium of a healthy joint, but its capacity is overwhelmed in case of persistent or recurrent joint bleeds (i.e. in hemophilia).

An augmented effect by whole blood compared to iron alone is shown *in vitro*. Hemoglobin causes an increase in synovial cells to 164% of control condition, compared to 223% by whole blood.[74] It remains to be identified which components are responsible, but an augmenting effect by pro-inflammatory cytokines is likely. *In vitro,* pro-inflammatory cytokines enhance the synovial deoxyribonucleic acid (DNA) synthesis induced by iron.[75]

Specifically an upregulation in genes involved in synovial cell proliferation and apoptosis is demonstrated *in vitro*. Increased expression of the proto-oncogene c-*myc* accompanies the enhanced proliferation of synovial fibroblasts by iron.[76] C-*myc* is a key regulating gene in controlling cell growth, differentiation, and apoptosis. Also *mdm2* gene expression is upregulated both *in vitro* in synovial cells by iron and *in vivo* upon a joint bleed in hemophilic mice.[77] *Mdm2* is a negative regulator of the p53 tumor suppressor protein and with its upregulation, synovial proliferation is increased and apoptosis abrogated.

As such, the induction of synovial proliferation by iron alone in case of HH is increased in

the presence of inflammatory cells in case of a joint bleed, accounting for the more explicit synovial proliferation in HA.

Immunological consequences of iron

The influence of iron on immunity has long been appreciated. An iron-rich environment is favorable for bacterial growth, affects the gut microbiota,[78] and impairs the function of different immune cells. The phagocytic function of neutrophils is inhibited,[79] T-cell surface molecule expression is modulated impairing T-cell recognition and T-cell activation,[80] and a decrease in circulating lymphocytes is suggested, but not observed consistently.[81,82] In HH the mutation in *HFE*, an MHC class I-like molecule, is also thought to impair antigen presentation.[83] These impairments of immunity are not demonstrated locally in the joint.

Indeed, in the joint iron induces inflammation potentially via activating the NF-κB pathway. NF-κB is a transcription factor required for the expression of a number of genes involved in innate immunity and inflammation. NF-κB mediated TNFα production is increased in hepatic macrophages cultured in the presence of iron,[84] probably by increased production of reactive oxygen species (ROS) as a result of elevated intracellular levels of iron. Upregulation of NF-κB-associated signaling pathways is shown also upon a joint bleed in hemophilic mice.[85] In HH these pathways may plausibly be similarly upregulated as the expression of vascular adhesion molecule 1 (VCAM-1), produced upon NF-κB activation, is increased.[86]

In vitro the induction of IL-1β production by macrophages is significantly enhanced in the presence of heme, whereas neither Fe^{2+} nor Fe^{3+} induces IL-1β production.[87] The iron within the heme-molecule appears critical as other porphyrins with substitutions in place of the iron atom cannot induce IL-1β production, and the production induced by heme is inhibited by an iron chelator. This advocates for a stronger induction of inflammation upon a joint bleed due to the exposure to heme-iron, further enhanced by simultaneous exposure to inflammatory cells, compared to non-heme iron overload in case of HH.

This is supported by *in vivo* data demonstrating only a mild pro-inflammatory response by iron solely, compared to the persistent and explicit synovial inflammatory reaction after a joint bleed. A single intra-articular injection of iron dextran, though containing iron in its less toxic form Fe^{3+}, causes a mild transient inflammatory reaction in rabbits,[66] whereas repeated systemic administration of iron dextran lack the induction of any notable inflammatory cell reaction.[67] In dogs, intra-articular iron injection leads to iron deposition in the synovial intimal cells, but an inflammatory response remains absent.[88] In contrast, clear synovial inflammation is present after repeated intra-articular autologous blood injections in non-hemophilic rabbits or dogs, or after inducing a single joint bleed in hemophilic mice.[68,72,88-90]

The discrepancy between a mild or absent inflammatory response induced by iron alone compared to an obvious response by whole blood, might be ascribed to the capacity of iron to enhance the inflammatory response induced by other pro-inflammatory stimuli, a phenomenon already mentioned in the previous paragraph. *In vitro* IL-1 stimulates granulocyte macrophage colony-stimulating factor (GM-CSF) production by synovial

fibroblasts in the presence of iron.[91] GM-CSF stimulates the production of cells of macrophage lineage, and has an important role in experimental murine arthritis.[92]
In HH, the mild inflammatory response can be also be induced by calcium phosphate crystals enhancing IL-1β production.[93] A genetic association between IL1RN and joint pain is suggested,[94] and therapeutic efficacy of an IL-1β receptor antagonist is described in two patients with HH-related hand arthritis.[95]
Altogether, the combination of heme-iron together with inflammatory cells and signals from whole blood mutually stimulate each other in inducing synovial inflammation, whereas the gradual accumulation of iron in HH only mildly induces a pro-inflammatory response.

The influence of iron on chondrocytes
Iron can be detected in isolated chondrocytes after a short culture period in the presence of either iron or hemoglobin,[96] which negatively influences DNA content and proteoglycan synthesis rate.[97] This inhibiting effect is reversible in the absence of a pro-inflammatory signal.[98] To exert long-lasting harmful effects on cartilage, the combination of heme-iron with IL-1β, or mononuclear cells to produce IL-1β, is necessary. Neither red blood cells nor mononuclear cells separately induce persistent damage. The permanent effect results from chondrocyte apoptosis via the formation of hydroxyl radicals.[98,99] IL-1β stimulates chondrocytes to produce hydrogen peroxide which is converted to hydroxyl radicals under catalytical conditions created by Fe^{2+}.
The combined effect of iron with an inflammatory signal is also demonstrated in hemophilic mice as cartilage destruction upon a joint bleed can be attenuated either by treatment with deferasirox, an iron chelator,[100] or by an anti-inflammatory treatment with IL-4 and IL-10.[101]

Iron and osteoporosis
Recent studies demonstrate a direct influence of iron in triggering bone loss or limiting calcification. Iron inhibits growth of hydroxyapatite crystals *in vitro* and has a negative influence on its quality.[102,103] In a mouse model of hemochromatosis, bone shows an increased iron content accompanied by an increased number of osteoclasts.[104] These adverse effects of iron on bone mineral density are related to oxidative stress and can be prevented by administration of an antioxidant.[105] *In vitro* a concentration-dependent increase in intracellular ROS production is observed in osteoblasts due to iron,[106] causing inhibition of osteoblast proliferation and differentiation,[103] and alterations in gene expression associated with the osteoblast phenotype.[107]

Iron and calcium pyrophosphate deposition
CPPD deposition including chondrocalcinosis, as frequently seen in HH and infrequently in HA, is the result of precipitation of inorganic pyrophosphate. In rabbits intra-articular injection with autologous blood significantly decreases the clearance rate of calcium pyrophosphate crystals from knee joints.[108] *In vitro* iron, as Fe^{2+} but not as Fe^{3+} ions, inhibits

pyrophosphatase[109] leading to a diminished hydrolyzation of inorganic pyrophosphate[110] and as such contributes to the precipitation of inorganic pyrophosphate with calcium.
Factors inhibiting crystal formation are less well investigated and understood. Hypothetically, the presence of inflammatory cells and the absence of a gradually increasing iron concentration in the synovial fluid of hemophilia patients may explain that CPPD is not prevalent in HA.

Conclusion

A pivotal role for iron in arthropathy in HH and HA is demonstrated by clinical, histopathological, animal, and *in vitro* findings. Iron deposition within the joint triggers a number of pathologic events, and these effects are amplified in the presence of an inflammatory signal. Iron deposition in HH derives from gradual accumulation of the less catalytic Fe^{3+} compared to recurrent bursts of exposure to Fe^{2+} derived from heme together with inflammatory cells in HA (Figure 3). Therefore, the production of ROS and therewith the induction of chondrocyte apoptosis, synovial inflammation and proliferation is much more explicit in HA compared to HH. Moreover, the development of arthropathy in hemophilia starts with hemarthroses already in early childhood compared to iron accumulation over time revealing clinical symptoms only later in life in HH. Also, in HH the alterations caused by iron deposition are superimposed on joint changes resulting from CPPD, which is not seen in HA.

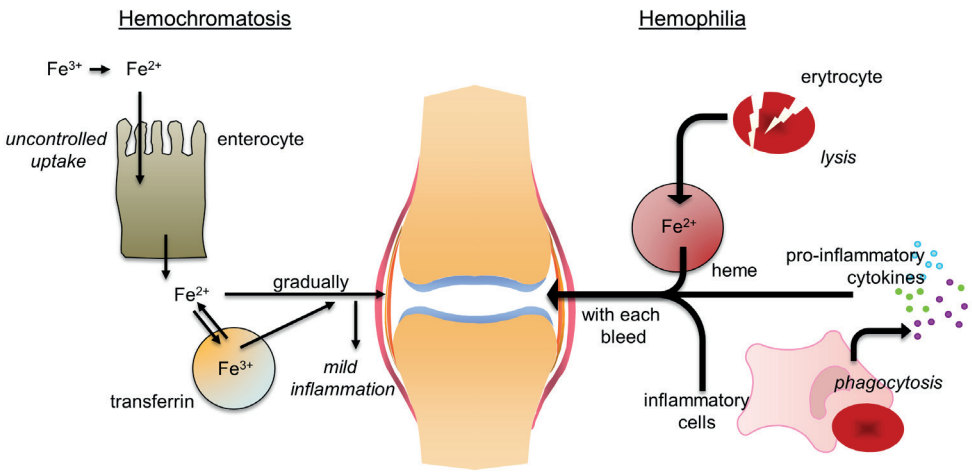

Figure 3 – Iron deposition in the joint in hemochromatosis versus hemophilia
In hemochromatosis, iron derived from increased absorption accumulates gradually over time, therewith evoking a mild inflammatory response. The iron accumulating is non-heme derived, which is less toxic compared to Fe^{2+} incorporated within heme and released together with inflammatory cells in hemophilia during a joint bleed.

In both diseases, the cornerstone of treatment is prevention; either preventing joint bleeds in case of hemophilia, or iron overload in HH. Once structural joint damage is present, treatment options are limited. Based on the present review, the rationale for iron chelation therapy to treat arthropathy remains appealing. Nonetheless, only limited data are available. In HH contradictory results after venesection are observed, as improvement (not prevention) and deterioration of symptoms are both described.[7,27,111] For HA only data from a hemophilia mouse model are available showing a protective effect on cartilage damage.[100] Clearly, more experimental and clinical data are needed to further elucidate the role of iron as target in the treatment of arthropathy in hemophilia and hemochromatosis. As the detrimental effects of iron are enhanced in the presence of an inflammatory signal, also treatment aiming at reducing inflammation specifically in the case of HA needs investigation.

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CHAPTER 3

GENETIC POLYMORPHISMS IN IRON AND HEME CONTROL ARE NOT ASSOCIATED WITH THE SEVERITY OF HEMOPHILIC ARTHROPATHY

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Abstract

Marked heterogeneity in joint damage exists among hemophilia patients. It was suggested previously that genetic differences in iron and heme control impact the development of hemophilic arthropathy. Dietary iron uptake is strictly regulated by the *HFE* gene, whereas heme degradation is dependent on heme-oxygenase encoded by the *HMOX1* gene. In this study we evaluate whether the severity of hemophilic arthropathy is associated with genetic differences in *HFE* and *HMOX1*. In a large cross-sectional cohort study of 252 patients with severe and moderate hemophilia A and B (median age of 44y (range 18-79y)) the presence of an *HFE* gene mutation (Cys282Tyr and His63Asp) and the $(GT)_n$-repeat length in the *HMOX1* promoter region were analyzed. In 95 patients an *HFE* mutation was detected, in these patients levels of iron and transferrin saturation were significantly increased, and their clotting factor consumption was higher compared to wild type patients. Patients with a short $(GT)_n$-repeat length had a lower annualized bleeding rate. Neither presence of an *HFE* mutation, nor a long $(GT)_n$-repeat length was associated with radiographic joint damage after adjustment for hemophilia severity, annualized joint bleeding rate, clotting factor consumption, age at evaluation, age at entry at the Van Creveldkliniek, and year of birth. This unique large monocenter cohort study cannot support the hypothesis that carriership of an *HFE* mutation or polymorphisms in *HMOX1* are associated with the severity of hemophilic arthropathy.

Introduction

In hemophilia a genetic deficiency of clotting factor VIII (hemophilia A) or factor IX (hemophilia B) leads to a life-long bleeding tendency with spontaneous bleeding occurring mostly within the musculoskeletal system and ultimately leading to hemophilic arthropathy. The bleeding tendency is primarily determined by the hemophilia severity expressed as percentage of the normal clotting factor activity.[1] However, a considerable heterogeneity in radiological joint damage is observed even in patients with a similar bleeding pattern (clinical observations,[2,3]). The distinction of patients at risk of more severe arthropathy is important for individualization of treatment. At present we are unable to predict which patients will develop a rapidly progressive arthropathy and who will not. A genetic predisposition is suggested,[4] but the impact of genetic differences in iron and heme homeostasis in this context is unknown.

Iron has a pivotal role in the pathogenesis of hemophilic arthropathy.[5,6] Upon a joint bleed heme-derived iron accumulates as hemosiderin within the synovium where it induces inflammation and cell proliferation, causing chronic synovitis and synovial hyperplasia.[7-9] Moreover, iron catalyzes the conversion of hydrogen peroxide, produced by activated chondrocytes, into hydroxyl radicals leading to chondrocyte apoptosis.[10] These processes ultimately result in the development of hemophilic arthropathy.

The body has several protective mechanisms against the damaging effects of iron and heme. Hepcidin, which expression is regulated by *HFE* (*H*igh Iron *Fe*), inhibits oral iron absorption.[11] Genetic mutations in the *HFE* gene are common among the Caucasian population, can lead to iron overload, and are associated with osteoarthritis.[12,13] In a small cohort, a relationship between carriership of *HFE* mutations and the severity of hemophilic arthropathy is hypothesized.[14]

Heme itself is also tissue destructive by inducing oxidative and inflammatory stress and by providing a source of iron.[15] Heme is broken down via a cascade of different enzymatic steps, with heme-oxygenase 1 (HO-1) being the rate-determining factor. The level of HO-1 is influenced by chemical and physical stimuli, and the intensity of this response is affected by the length of a guanine-thymidine $(GT)_n$-repeat polymorphism in the promoter region of the *HMOX1* gene, the gene encoding HO-1. A higher response in HO-1 levels is observed in people with homozygosity for a short $(GT)_n$-repeat (n-repeat < 25),[16] and these people are less susceptible to develop rheumatoid arthritis (RA).[17] Moreover, a short $(GT)_n$-repeat is shown to protect against progression of joint damage in RA patients.[18] Considering the importance of heme-derived iron in the pathogenesis of hemophilic arthropathy, we hypothesized that an impaired iron or heme handling might impact the severity of blood-induced joint damage. To test this hypothesis, we conducted a large cross-sectional cohort study to investigate the influence of *HFE* mutations and *HMOX1* polymorphisms on the severity of hemophilic arthropathy.

Methods

Patients and data collection

Patients with severe or moderate hemophilia A or B (factor VIII/IX activity ≤ 5%) regularly visiting the outpatient clinic of the Van Creveldkliniek, University Medical Center Utrecht (UMCU), the Netherlands, were included in this cross-sectional study after written informed consent. The study was approved by the Institutional Review Board of the UMCU and performed in accordance with the Declaration of Helsinki.

Outcome was the severity of hemophilic arthropathy in knees, elbows, and ankles on X-rays as measured by the Pettersson score.[19] Blood was drawn during regular visits. From the patients' medical files demographic data were collected as well as information on clotting factor activity, history of clinically relevant inhibitors (defined as a current or historic high-titer inhibitor (≥ 5 Bethesda Unit (BU)) or a long-term inhibitor (≥ 1 year and ≥ 1 BU)), bleeding pattern, current treatment, clotting factor consumption, and the age at entry at the Van Creveldkliniek. Clotting factor consumption was calculated as the mean annual FVIII/FIX consumption per kilogram bodyweight over all treatment years registered since 2006. Clotting factor consumption because of surgical interventions was excluded. The annualized joint bleeding rate (AJBR) was calculated as the mean number of joint bleeds per year in the wrists, elbows, shoulders, hips, knees, and ankles, using all data available, independent of treatment received.

Pettersson score

All radiographs were obtained in standard care according to standard procedures and scored according to the Pettersson method[19] with use of a reference atlas[20] by a single rater with experience using the Pettersson score. The most recent set of radiographs made before inclusion was used, with a mean interval of 5.8 years. The age at which the radiographs were taken was called 'age at evaluation'.

On posterior-anterior and lateral X-rays the following eight criteria were scored in each joint: osteoporosis, epiphyseal enlargement, irregularity of subchondral surface, narrowing of joint space, subchondral cysts, erosions at joint margins, incongruence between joint surfaces, and the angulation and/or displacement of articulating bone ends, with a maximum score per joint of 13, and a maximum total score per patient of 78. Joints with a prosthesis or after arthrodesis were assigned 13 points.

DNA analysis

Genomic deoxyribonucleic acid (DNA) was extracted from ethylenediaminetetraacetic acid (EDTA)-preserved blood according to a standard protocol using the Chemagic Magnetic Separation Module 1' (MSM1) DNA extraction robot.

The presence or absence of an *HFE* mutation was analyzed at the DNA diagnostics laboratory of the UMCU. Exon 2 and 4 were amplified in a final volume of 25 µl containing

12.5 µl AmpliTaq Gold 360, 5 µl DNA at a concentration of 20 ng/µl, and a primer mixture as outlined in Supplemental table 1. Amplification was performed on a Gene Amp 9700 thermal cycler (Applied Biosystems) using an initial denaturation step at 95°C for 10 min, followed by 33 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step of 10 min at 72°C. The amplified fragments were sequenced to detect the presence of c.845G>A (Cys282Tyr) or c.187C>G (His63Asp) on an ABI Prism 3700 DNA analyzer (Applied Biosystems).

As indicator of HO-1 expression, the $(GT)_n$-repeat in the promoter region of the *HMOX1* gene was established at the DNA diagnostics laboratory of the Radboud University, Nijmegen, the Netherlands. The 5'-flanking region containing $(GT)_n$-repeats of the HO-1 gene was amplified by polymerase chain reaction (PCR) using a FAM-labeled sense primer, 5'-TCACAGGTCAGTTGTAGGGATG-3', and an unlabeled antisense primer, 5'-ACAGCTGATGCCCATTTCT-3'. PCR products were generated in a final volume of 10 µl containing 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), AmpliTaq PCR buffer II, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5 pmoles of each primer, and 50 ng genomic DNA. After a 10 min denaturation period at 94°C, PCR products were amplified in 35 cycles of 94°C for 1 min, 62.4°C for 1 min, and 72°C for 1 min. A final extension step of 10 min at 72°C completed the reaction. One µl of each amplified product (10x diluted) was combined with size standard (LIZ-600; Applied Biosystems) and formamide, and analyzed on an ABI3730 Genetic Analyzer (Life Technologies).

Iron status

Serum was obtained from blood collected into Vacutainer tubes (10 mL, Becton Dickinson, Oxford UK). A total of eight units of recombinant clotting factor concentrate (factor VIII or factor IX, as appropriate) was added. Blood was kept at room temperature for at least one hour to ensure proper coagulation. Thereafter, samples were centrifuged at 1,300 *g* for 10 min. Serum aliquots were obtained and stored at -80°C until use. Serum ferritin was determined using an automated chemiluminescence immunoassay on a Unicel Dxl 800 (Beckman Coulter, Brea, CA, USA). Serum iron was estimated using a colorimetric assay on a DxAU 5811 (Beckman Coulter). Serum transferrin values were obtained by immunochemical turbidimetry on a DxAU 5811. Total iron binding capacity (TIBC) (µmol/L) was calculated as serum transferrin (g/L) × 25.14, and transferrin saturation as the ratio of serum iron to TIBC. As a marker for inflammation, C-reactive protein (CRP) was measured by immunochemical turbidimetry on a DxAU 5811.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 21.0.0 for Windows (IBM, Chicago, Ill, USA). Comparison between two groups was performed by independent *t*-test, after ln-transformation if necessary to achieve normal distribution. Comparisons between three groups were performed by the oneway ANOVA with post-hoc Bonferroni to correct for

multiple testing. Nominal data were analyzed by the Pearson Chi-square test. *P*-values <0.05 were considered statistically significant.

According to the *HFE* mutation patients were allocated into two groups:

- Group 1, referred to as "wild type"(WT), included patients without a Cys282Tyr or His63Asp mutation;
- Group 2, referred to as "mutated", included patients heterozygous for Cys282Tyr and/or His63Asp, or homozygous for His63Asp or Cys282Tyr.

For the *HMOX1* polymorphism, patients were allocated into three groups:

- Group 1, referred to as SS, included patients homozygous for a $(GT)_n$-repeat < 25;
- Group 2, referred to as LL, included patients homozygous for a $(GT)_n$-repeat ≥ 25;
- Group 3, referred to as SL, included patients heterozygous for a $(GT)_n$-repeat ≥ 25.

Linear regression analysis was performed to assess the association of the *HFE* mutation and *HMOX1* polymorphism with the Pettersson score. We performed a stepwise linear regression to include all potential confounders of the relationship between the genetic mutations and the Pettersson score. Covariates investigated were hemophilia type and severity, AJBR, current treatment (prophylaxis versus on demand), clotting factor consumption, presence of clinically relevant inhibitors, age at evaluation, age at entry at the Van Creveldkliniek, and year of birth. Patients were grouped in three cohorts according to their year of birth (≤1965; 1966-1980; ≥1981) as access to treatment significantly improved over the years. For the age at entry at the Van Creveldkliniek, patients were allocated in two groups with a cut off of four years of age, as this was also assumed to impact access to treatment.
Factors with a *P*-value < 0.10 were considered significant confounders and included in the model. Model assumptions were checked using a Q-Q plot and normality of the residuals was checked with a normal P-P plot.

Results

Patients
Patient and treatment characteristics are presented in Table 1. In total 252 patients were included at a mean age of 44 years (range 18-79), the majority suffering from severe hemophilia A (223 of 252; 88%). As expected, the AJBR and clotting factor consumption were higher for patients with severe hemophilia as compared to moderate hemophilia (median AJBR 2.3 (IQR 1.0-4.6) versus 0.5 (0.1-2.2), p=0.00; median clotting factor consumption 1851 IU/kg/y (IQR 1143-2341) versus 236 (69-1793), p=0.00), as was the Pettersson score (median 22 (IQR 5-44) for severe hemophilia; median 4 (1-13) for moderate hemophilia, p=0.00, unadjusted for age at evaluation, Figure 1).

Table 1 – Baseline characteristics according to hemophilia severity

	Severe n=211	**Moderate** n=41	***P*-value**
Age at inclusion, mean (range)	44 (18-79)	44 (18-75)	0.95
Age at evaluation, mean (range)	37 (11-79)	42 (15-75)	0.05
Hemophilia A, n (%)	185 (88%)	38 (93%)	0.44
Inhibitor history, n (%)	26 (12%)	1 (2%)	0.09
Currently on prophylaxis, n (%)	165 (78%)	3 (7%)	**0.00**
Clotting factor consumption (IU/kg/y), median (IQR)	1851* (1143-2341)	236 (69-1793)	**0.00**
AJBR, median (IQR)	2.3 (1.0-4.6)	0.5 (0.1-2.2)	**0.00**
Pettersson score, median (IQR)	22 (5-44)	4 (1-13)	**0.00**

AJBR, annualized joint bleeding rate; IQR, interquartile range; SD, standard deviation.
* n=208; three patients excluded because of NovoSeven usage.

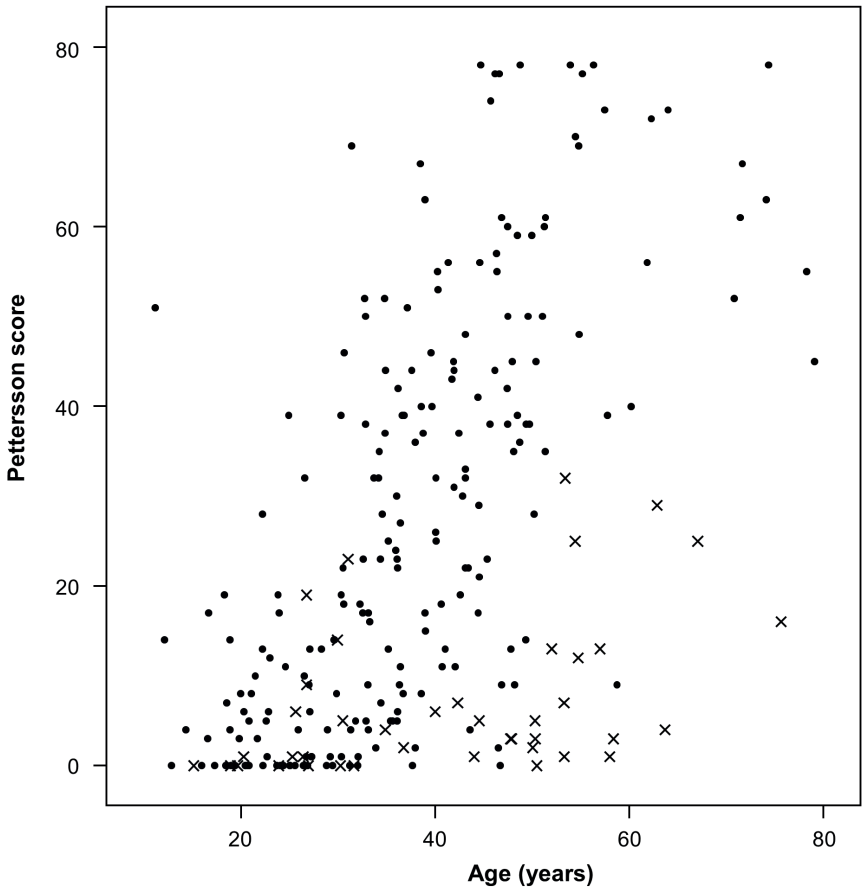

Figure 1 – Pettersson score according to age and severity of clotting factor deficiency
Dots represent patients with severe hemophilia, crosses represent patients with moderate hemophilia.

Patient characteristics according to HFE mutation status

Carriership of an *HFE* mutation was detected in 38% of the patients, one patient was found to be homozygous for the Cys282Tyr mutation and six patients were compound heterozygous for Cys282Tyr and His63Asp. This frequency is similar to the frequency observed in the general Dutch population (Supplementary table 2).

WT patients did not differ from patients carrying a mutation in terms of age at time of inclusion and at time of evaluation, hemophilia type or treatment, AJBR or Pettersson score. Clotting factor consumption was slightly but significantly higher in patients carrying a mutation (1549 vs 1827 IU/kg/y; p=0.03, Table 2).

In patients with an *HFE* mutation transferrin saturation and iron levels were significantly increased (transferrin saturation 27% vs 32%; p=0.01, iron 17 vs 19 µmol/L; p=0.04). A transferrin saturation exceeding 45% (cut-off used for the suspicion of hemochromatosis) was detected in six (4%) WT patients compared to 13 (14%) patients carrying a mutation (p=0.01). In addition, ferritin showed a trend towards increased levels in the mutated group (p=0.06), whereas CRP levels were similar (p=0.16). Ferritin levels of >250 µg/L were observed in five (3%) WT patients versus 10 (11%) in the mutated group (p=0.02).

Table 2 – Patient characteristics according to *HFE* mutation status

HFE	**Wild type** (n=157)	**Mutated** (n=95)	***P*-value**
Age at inclusion, mean (range)	43 (18-79)	45 (18-76)	0.27
Age at evaluation, mean (range)	37 (11-79)	39 (13-76)	0.59
Hemophilia A, n (%)	142 (90%)	81 (85%)	0.21
Severe hemophilia, n (%)	132 (84%)	79 (83%)	0.85
Inhibitor history, n (%)	18 (11%)	9 (9%)	0.68
Currently on prophylaxis, n (%)	107 (68%)	61 (64%)	0.52
Clotting factor consumption (IU/kg/y), median (IQR)	1549* (600-2160)	1827# (986-2244)	**0.03**
AJBR, median (IQR)	2.0 (0.6-4.3)	2.2 (0.9-4.6)	0.51
CRP (mg/L), median (IQR)	1.5 (0.5-3.3)	2.1 (0.6-4.9)	0.16
Ferritin (µg/L), median (IQR)	86 (54-133)	98 (58-157)	0.06
Iron (µmol/L), median (IQR)	17 (13-21)	19 (16-24)	**0.04**
Transferrin (g/L), mean (SD)	2.66 (0.38)	2.59 (0.41)	0.20
Transferrin saturation, mean (SD)	0.27 (0.10)	0.32 (0.13)	**0.01**
Pettersson score, median (IQR)	14 (4-40)	18 (5-39)	0.32

AJBR, annualized joint bleeding rate; CRP, C-reactive protein; IQR, interquartile range; SD, standard deviation.
* n=156; # n=93

Patient characteristics according to HMOX1 polymorphism

In one patient the $(GT)_n$-repeat could not be determined due to technical issues. Only 22 patients were homozygous for the short $(GT)_n$-repeat (SS), whereas 116 patients were homozygous for the long $(GT)_n$-repeat (LL) and 113 patients were heterozygous (SL).

Patients in the SL cohort more frequently suffered from hemophilia A compared to SS and LL patients (p=0.012; Table 3), this did not differ between SS and LL patients. SS patients had a lower AJBR compared to SL patients (p=0.02), whereas there was no difference between SS and LL patients (p=0.08). Other baseline characteristics were similar between the three cohorts.

Table 3 – Patient characteristics according to *HMOX1* $(GT)_n$ microsatellite polymorphism

HMOX1	SS (n=22)	SL (n=113)	LL (n=116)	*P*-value
Age at inclusion, mean (SD)	44 (14)	41 (14)	46 (15)	**0.02**
Age at evaluation, mean (SD)	39 (11)	36 (13)	39 (14)	0.17
Hemophilia A, n (%)	17 (77%)	107 (95%)	98 (84%)	**0.01**
Severe hemophilia, n (%)	16 (73%)	99 (88%)	96 (83%)	0.19
Inhibitor history, n (%)	0 (0%)	12 (11%)	15 (13%)	0.20
Currently on prophylaxis, n (%)	12 (55%)	78 (69%)	78 (67%)	0.42
Clotting factor consumption (IU/kg/y), median (IQR)	1644 (433-1928)	1639* (865-2341)	1619# (779-2152)	0.71
AJBR, median (IQR)	1.3 (0-3.4)	2.3 (1.0-4.2)	2.0 (0.5-4.6)	**0.02**
Pettersson score, median (IQR)	5 (1-46)	14 (5-38)	20 (3-41)	0.16

AJBR, annualized joint bleeding rate; IQR, interquartile range; SD, standard deviation.
* n=112; # n=114

No association of HFE mutation or HMOX1 polymorphism with Pettersson score

The association between genetic polymorphisms and radiographic joint damage was investigated via linear regression analysis. In this analysis 248 patients were included, three patients were excluded because their clotting factor consumption could not be assessed due to NovoSeven usage, one patient was excluded because his *HMOX1* polymorphism was unknown. There was no association between *HFE* and *HMOX1* and the Pettersson score without adjustment for other factors (unstandardized beta 2.8; p=0.34 and 1.9; p=0.40 respectively). Factors associated with the Pettersson score were the severity of hemophilia, age at time of evaluation (Figure 1), access to clotting factor replacement (defined as birth cohort and age of entry at the Van Creveldkliniek), clotting factor consumption, and the AJBR. All these factors were significantly associated with the Pettersson score in our cohort (p<0.05; Table 4). Data were similar according to hemophilia type (A versus B), current prophylactic treatment (yes or no), and inhibitor presence (data not shown). R-square of

the linear regression model including all significantly associated factors was 0.61, addition of the *HFE* and *HMOX1* data to this model did not improve this (unstandardized beta 0.4; p=0.81 for *HFE* and -0.5; p=0.74 for *HMOX1*).

Table 4 – Linear regression analysis for determinants of Pettersson score

Predictor	Beta	95% CI	*P*-value
Age at evaluation	0.42	0.20 – 0.64	**0.000**
Clotting factor activity	-15.0	-20.6 – -9.4	**0.000**
Clotting factor consumption	0.003	0.002 – 0.005	**0.001**
AJBR	1.1	0.5 – 1.6	**0.000**
Year of birth (≤1965; 1966-1980; ≥1981)	-8.8	-12.6 - -5.0	**0.000**
Entry at the VCK (≤ or > 4 years of age)	6.3	2.0 – 10.6	**0.004**
***HFE* mutation**	0.4	-3.3 – 4.2	0.814
***HMOX1* polymorphism**	-0.5	-3.3 – 2.3	0.739

AJBR, annualized joint bleeding rate; CI, confidence interval; VCK, Van Creveldkliniek.

Discussion

This is the first study specifically designed to investigate the association between the severity of hemophilic arthropathy and genetic polymorphisms influencing iron and heme homeostasis. In this cohort, severity of hemophilic arthropathy was not associated with *HFE* mutations or the $(GT)_n$-repeat in the promotor region of the *HMOX1* gene, even after adjustment for well-known factors impacting the development of hemophilic arthropathy. Although carrying an *HFE* mutation significantly increased serum iron levels and transferrin saturation, this did not result in increased joint damage reflected on X-rays.

Understanding factors affecting the observed difference in joint damage progression might result in more individualized treatment regimens or targeted therapy for joint damage. If we are able to identify patients at highest risk of damage beforehand, a more stringent prophylactic regimen could be feasible. Investigating the impact of a single factor on joint damage progression is difficult as the hemophilia population is heterogenic and many factors affect outcome. Hemophilic arthropathy is a cumulative result of bleeds, treatment, joint loading, exercise, etc. developing over many years, with many of these factors being difficult to measure.

Our study is a unique large single-center cohort study with a complete set of detailed data on treatment and bleeding without interference of center-specific treatment guidelines. By including all data in a linear regression model, we were able to explain 61% of the observed variation in the Pettersson score. Addition of the *HFE* mutation and *HMOX1* polymorphism did not improve the model. The genes investigated in our study were selected because iron

has a pivotal role in the pathogenesis of blood-induced joint damage.[6,21] Long-lasting cartilage damage upon blood-exposure results from iron-induced radical formation and subsequent chondrocyte apoptosis.[22] In hemophilia, iron is responsible for the upregulation of genes involved in synovitis,[8,9] iron deposits stimulate synovial production of pro-inflammatory cytokines,[7] and blocking the damaging effects of iron limits cartilage degeneration upon joint bleeding in hemophilic mice.[5]

A genetic predisposition for the development of hemophilic arthropathy was recently suggested in a study investigating the impact of over 10,000 genetic markers on joint range of motion in children and adolescents with hemophilia A.[4] A group of 25 genetic markers mainly involved in inflammation and immune modulation, was associated with range of motion at joint level. Range of motion is mainly influenced by synovial changes, especially in children, whereas we principally investigated osteochondral tissue damage. Their study population was significantly younger, patients were treated on demand only, and data on the AJBR were lacking. It would be interesting to validate their findings in our cohort.

A role for *HFE* in the pathobiology of hemophilic arthropathy was suggested previously.[14] Our findings contradict this study by Cruz *et al*, that has several limitations; the population size was small, the AJBR in patients with severe hemophilia was high, joint damage was more subjectively defined as the number of affected joints and the number of hemarthroses, and treatment data were not included. In our cohort clotting factor consumption was higher in patients with an *HFE* mutation compared to WT patients, but the absolute the difference is not of clinical relevance. In addition, serum levels of iron and transferrin saturation were increased in patients with an *HFE* mutation. This however did not result in a difference in Pettersson score, AJBR, or number of affected joints (data not shown), demonstrating the complexity of investigating factors affecting joint damage in hemophilia.

HMOX1 is the gene encoding HO-1, the rate-limiting step in the degradation of heme into biliverdin, iron, and carbon monoxide (CO). HO-1 can be induced by multiple stimuli like oxidative stress, hypoxia, cytokines etc. It acts anti-oxidative and anti-inflammatory and is present in cartilage of osteoartrhitis[23] and even more abundantly in synovial tissue of RA.[24] In osteoarthritic chondrocytes and articular explants, HO-1 counteracts the catabolic effects of IL-1.[25] In murine arthritis models, pharmacologic upregulation of HO-1 as well as administration of CO and biliverdin attenuate inflammation and cartilage degradation.[26,27] *HMOX1* polymorphisms are related to the susceptibility to RA and subsequent inflammation.[17,18] In our study, no relation with hemophilic arthropathy was found. Theoretically, increased inflammation will result in osteochondral damage over time, causing an increase in the Pettersson score. Nevertheless, it is possible that an effect on synovial inflammation is missed.

To objectively assess arthropathy, we used plain radiographs of the ankles, knees and elbows, and scored them according to the Pettersson scoring method as this is a widely used method to quantify osteochondral changes with a good reliability and reproducibility.[20,28] A major limitation is its insensitivity to detect early small changes and its low sensitivity to

detect soft-tissue changes. As such, it is possible that an association of *HFE* mutations or *HMOX1* polymorphisms with soft-tissue changes including inflammation is missed.
Another limitation of our cohort is the wide age range including differences in treatment over time. To address this, we grouped patients according to their date of birth with cut-offs at landmark years in the history of hemophilia treatment. Ideally, studying the impact of genetic variations is performed in a homogenous population with similar treatment, bleeding frequency, and assessment of joint damage at specific time points.
An interesting observation is the absence of inhibitors in patients with an SS genotype of *HMOX1*, although there was no significant difference compared to the frequency in SL and LL patients. The AJBR was lower in SS patients, but there was no difference in clotting factor consumption. The SS cohort consisted of only 22 patients, and the number of inhibitor positive patients in our cohort was low. Nonetheless, it is in line with a case-control study showing a higher prevalence of a long $(GT)_n$-repeat among hemophilia patients developing inhibitory antibodies to factor VIII.[29] An increased HO-1 induction is shown to protect from developing inhibitory antibodies via immunosuppressive and anti-inflammatory properties ascribed to HO-1.
In conclusion, this cross-sectional cohort study does not support the hypothesis that hemophilic arthropathy is associated with *HFE* mutations or *HMOX1* polymorphisms.

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Supplementary tables

Supplementary table 1 – Primers used for polymerase chain reaction to detect *HFE* mutations

Forward	5'-*GTTTTCCCAGTCACGAC*CTGCAACTCACCCTTCACAA-3'
Reverse	5'-CAACAGTGAACATGTGATCCC-3'
Forward	5'-GGTCTTTCCTTGTTTGAAGCTT-3'
Reverse	5'-*GTTTTCCCAGTCACGAC*AAATTCCTTCCCTCTTCCCTG-3'
Forward	5'-ATAGAAGGAAGTGAAAGTTCCAGTC-3'
Reverse	5'-*GTTTTCCCAGTCACGAC*CAAGGTTATCCAGCCCTGGTA-3'
Forward	5'-*GTTTTCCCAGTCACGAC*GTGTCGGGCCTTGAACTAC-3'
Reverse	5'-CATAATTACCTCCTCAGGCACTC-3'

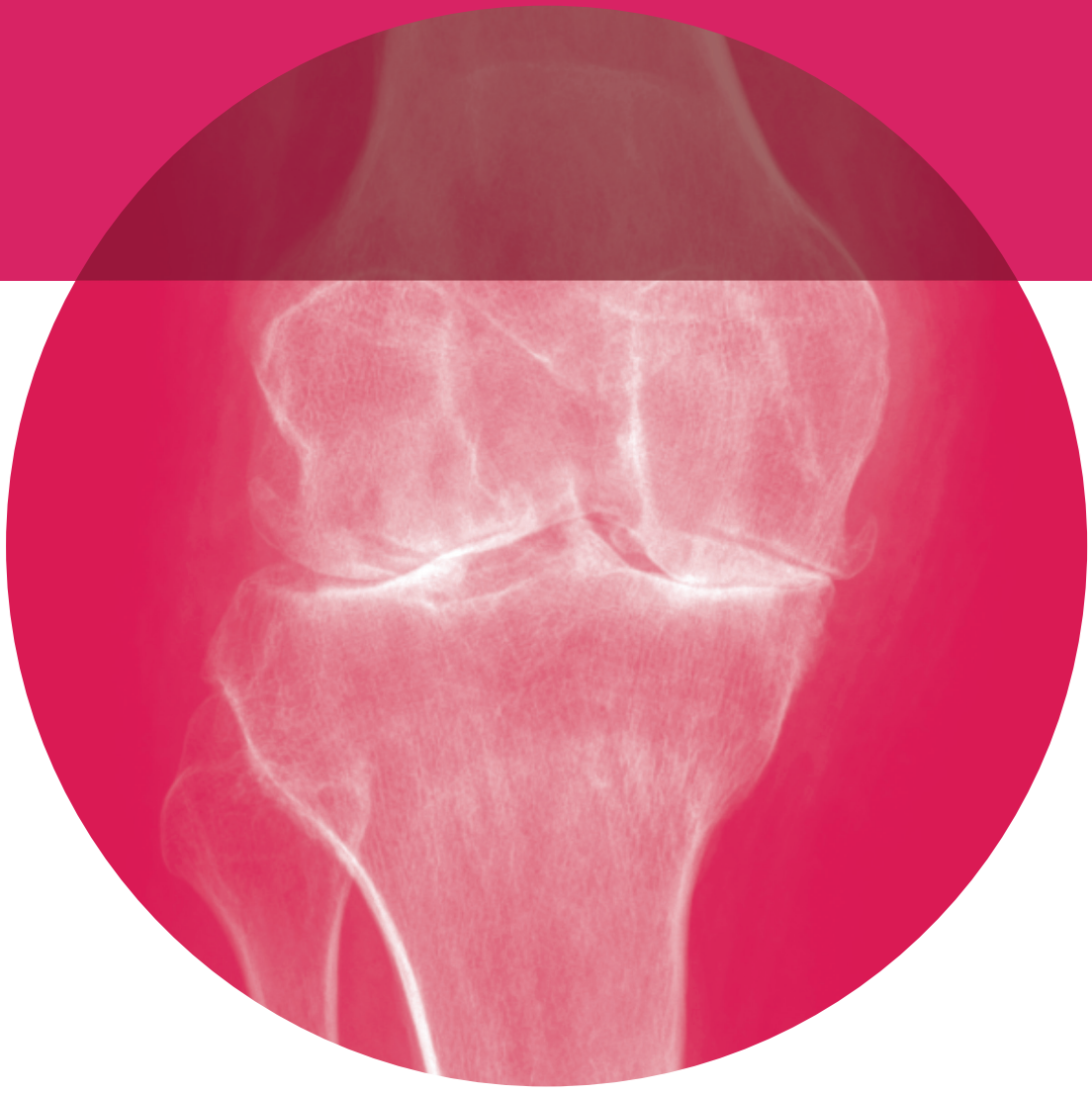
In *italic* the M13 sequence used as universal-tail for sequencing.

Supplementary table 2 – *HFE* mutation in our cohort of hemophilia patients and in a control population*

Genotype	Hemophilia patients (n=252)	Controls*
Wild type	157 (62%)	
Cys282Tyr/Cys282Tyr	1 (0.4%)	0.5-1.5%
Cys282Tyr/His63Asp	6 (2.4%)	1-3%
Cys282Tyr (heterozygous)	24 (9.5%)	3.5-15%
His63Asp (heterozygous)	61 (24%)	20%
His63Asp/His63Asp	3 (1.2%)	

* Dutch Hemochromatosis Guideline, numbers based on North-European people.

TRANSLATION



CHAPTER 4

BIOCHEMICAL MARKERS OF JOINT TISSUE DAMAGE INCREASE SHORTLY AFTER A JOINT BLEED

an explorative human and canine *in vivo* study

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Abstract

Objective: Evaluation whether biomarkers of joint damage are sensitive to change shortly after a joint bleed in hemophilia patients and in a canine model of blood-induced joint damage.

Methods: Blood and urine samples were collected from 10 hemophilia patients after they reported a joint bleed: within two days, after 3-5 days, and 12-14 days. Additionally, 90 days after the bleed a blood and urine sample was taken and considered to represent baseline condition. Commercial serum and urine biomarker assays were performed: urinary C-terminal telopeptide of type II collagen (uCTX-II), serum cartilage oligomeric matrix protein (sCOMP), serum cartilage cleavage product C1,2C, and serum chondroitin sulfate 846 (sCS846).
The same panel of biomarkers was explored in dogs (n=7) after induction of a first joint bleed by intra-articular blood injections. Biosamples were collected at baseline, day two, one and two weeks later.

Results: In hemophilia patients, levels of uCTX-II and sCS846 increased five days after joint bleeding when compared to baseline (+52%; p=0.021 and +14%; p=0.011, respectively). In dogs, uCTX-II increased statistically significant from day two to day seven (from 75% to 155% of baseline; p=0.018), and sCOMP from baseline to day two (+46%; p=0.028).

Conclusions: This study demonstrates that biochemical markers of joint tissue damage increase shortly after a single joint bleed, both in humans with established hemophilic arthropathy and in an animal model of joint damage upon a first joint bleed. Biomarkers might be useful in monitoring the impact of a joint bleed and in evaluation of treatment of such bleeds.

Introduction

Progressive damage of cartilage and bone, and synovial inflammation are the main characteristics of different joint diseases, either primarily degeneration-mediated such as osteoarthritis (OA), primarily inflammation-mediated such as rheumatoid arthritis (RA), or a combination of both processes as seen in hemophilic arthropathy (HA).[1] Developing diagnostic tools to identify patients at high risk of developing or progression of joint damage early in the disease remains an important, yet challenging aspect. Assessments are mainly based on imaging techniques, involving plain radiography, ultrasonography (US), and magnetic resonance imaging (MRI).[2-4] Each technique has its own advantages and limitations, regarding availability, accuracy, costs, examination time, and scoring systems.

An alternative approach could be offered by the use of serum or urine biochemical markers of joint tissue turnover.[5] They have the potential to reflect dynamic changes in joint tissue turnover, whereas imaging techniques provide information as a result of these dynamic processes over time. As such these biochemical markers might be of use as diagnostic tool to treat patients more adequately and to monitor disease progression and treatment efficacy more closely. Unfortunately, despite extensive research, at present none of the biochemical markers of joint damage is sufficiently valuable for diagnosis or prognosis of joint diseases at the level of an individual patient.[6]

Investigating biochemical markers in diseases like OA and RA is challenging as associations might be obscured by the presence of presumed but not yet fully characterized phenotypic differences within the OA[7] and RA populations.[8] Patients being at different stages of the disease, the involvement of single to multiple joints, aggressive treatment, and a systemic inflammatory component in case of RA, may influence biomarker metabolism significantly.[9] Hence, it is attractive to investigate biochemical markers in a disease model with a clear trigger for damage progression as is the case in hemophilia at the moment of a joint bleed. In hemophilia blood-induced joint damage is still the most important cause of morbidity, despite the increased use of prophylactic infusions of clotting factor concentrates.[10] It has been demonstrated clinically and experimentally, that recurrent bleeds but also a limited number or even a single joint bleed cause damage.[1,11-14] A joint bleed initiates a combined cascade of inflammatory and degenerative processes causing a relatively fast progressive arthropathy. Inflammation is restricted to the joint as opposed to the chronic systemic inflammation seen in RA, and joint damage is mainly restricted to the elbows, knees, and ankles, which facilitates the quantification of the extent of joint damage as compared to diseases like OA and RA affecting also multiple smaller joints like hands and spine.

The Pettersson scoring method, based on conventional radiography of the main involved joints (elbows, knees, and ankles), is a well-validated scoring method to assess joint damage

in hemophilia.[15,16] A good cross-sectional correlation is demonstrated between biochemical markers of joint damage and radiographic joint damage for urinary C-terminal telopeptide of type II collagen (uCTX-II), serum cartilage oligomeric matrix protein (sCOMP), serum cartilage cleavage product C1,2C (sC1,2C), and serum chondroitin sulfate 846 (sCS846).[17]

To date, no data are available on the response of biochemical markers to a joint bleed. The purpose of this explorative study was to investigate whether these biochemical markers are sensitive to change upon blood-induced joint tissue damage in patients with hemophilia with established HA and in a canine model of blood-induced joint damage without pre-existing joint damage.

Materials and methods

Patients

Patients with moderate or severe hemophilia A or B (≥18 years, n=10) reporting a joint bleed in their elbow, knee or ankle were included. Blood and urine samples were prospectively collected at three successive time-points: within two days, between day three and five, and between 12-14 days after the bleed. Additionally at about 90 days after the reported joint bleed a blood and urine sample was taken, considered to represent baseline. Exclusion criteria were the presence of human immunodeficiency virus infection, the presence of antibodies that neutralize clotting factors, or a body mass index > 30 kg/m^2.

The study was undertaken in accordance with the Declaration of Helsinki, and was approved by the Medical Ethical Review Board. Written informed consent was obtained from all study participants.

Human serum and urine sampling

Ten milliliters of blood was collected into Vacutainer tubes (Becton Dickinson, Oxford UK). A total of eight IU of recombinant clotting factor concentrate (factor VIII or factor IX, as appropriate) was added. Blood was kept at room temperature for at least one hour to ensure proper coagulation. Thereafter, samples were centrifuged at 1,500 *g* for 10 minutes. Serum aliquots were obtained and stored at -80°C until use. Urine samples were kept at 4°C for a maximum of eight hours, and then aliquoted and stored at -80°C. Non-fasted samples were collected between 8:00 A.M. and 12:00 A.M.

Canine in vivo experiments

Seven skeletally mature Beagle dogs (3/4 male/female; mean age 2.1±0.1 years; weight 12.2±0.4 kg) were obtained from the animal laboratory of the Utrecht University, The Netherlands, and housed in groups of 2-3 dogs. They were let out at least two hours daily, and fed a standard commercial diet with water ad libitum. The Utrecht University Animal Experiments Committee approved the study protocol.

In all dogs a joint bleed was mimicked by injecting 1.8±0.1 mL freshly collected (vena puncture) autologous blood intra-articularly in the left knee. These procedures were performed under short-term anesthesia (Dormitor/Antisedan). Accuracy of the injection was checked randomly by placing a second needle at the other side of the joint. A blood flow from the injected joint was demonstrated in all cases, indicating complete filling of the joint with blood. After removal of the needles, pressure was put on the puncture holes until the bleed stopped, preventing leakage of blood outside the joint cavity.
Four blood injections in the left knee were given in four successive days, because of the rapid clearance of blood from the canine joint in contrast to humans.[14] As previously shown, this results in a period of at least four days continuous blood exposure with at least 20% v/v blood and leads to mild progressive cartilage damage and synovitis over time mimicking HA.[13,14]

Canine serum and urine sampling
Serum and urine samples were collected from each dog before the intra-articular blood injections (baseline), halfway the injection period (day 1-2), one (day 7) and two weeks (day 14) later. To validate the surrogate baseline values used in the human study (obtained at day 90 post bleeding), biosamples were also obtained at week 13 and compared to baseline. All samples were obtained in the morning, but were non-fasted, similar to the human clinical situation. Ten mL of blood was collected into Vacutainer tubes (Becton Dickinson) and allowed to coagulate for at least one hour at room temperature. Subsequently samples were centrifuged at 1,500 *g* for 10 minutes, aliquoted and stored at -80°C until the biomarker analysis was performed. Urine was obtained by catheterization and kept at 4°C for a maximum of eight hours, and then aliquoted and stored at -80°C.

Biomarker assays
All biomarkers were measured in duplicate using standard enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. All samples for any single patient or dog were tested on the same plate to avoid inter-plate variance, with all plates purchased from the same batches. Urine samples were assessed for the level of uCTX-II (human samples CartiLaps; canine samples Pre-Clinical Cartilaps; both from IDS Ltd, Boldon, UK) and these levels were corrected for creatinine levels (Cayman Chemicals, Ann Arbor, MI). In serum samples sCOMP (specific kit for human or canine COMP, both from Novatein Biosciences, Woburn MA), sC1,2C, and sCS846 (kits with broad cross-reactivity including human and dog, both from IBEX, Montréal, Québec, Canada) were determined.

Statistical analysis
Data distribution was assessed using the Shapiro-Wilk tests for normality. None of the biomarkers was normally distributed, and this could not be achieved by natural log transformation for all biomarkers. Accordingly, non-parametric tests for related samples (Friedman's test followed by a post hoc Wilcoxon signed rank test) were used. All statistical

analyses were performed using absolute data. Values are expressed as percentage compared to baseline and depicted as individual values together with the median.
Statistical calculations were performed with SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 (two-sided) were considered statistically significant.

Results

Patient characteristics
The study population consisted of six patients with severe hemophilia A, three patients with moderate hemophilia A, and one patient with severe hemophilia B. The age at time of the joint bleed ranged from 18-71 years (mean 53 years, SD 18 years). Three patients received clotting factor replacement therapy as prophylaxis and seven received treatment on demand. Three patients reported a joint bleed in the knee, six in the elbow, and one patient had an ankle joint bleed. There was a wide variation in the amount of clotting factor consumption (300-6000 IU/kg/year), average number of joint bleeds per year (0-20), number of affected joints (1-6) and total Pettersson score (5-68). Two patients had undergone joint replacement in the past.

uCTX-II and sCS846 change upon a joint bleed in hemophilia patients
Baseline levels of the biomarkers could not be determined previous to a joint bleed, since the occurrence of a joint bleed could not be predicted. Therefore samples collected around 90 days after the joint bleed were considered baseline. Table 1 shows the median (25-75% percentile) of the absolute biomarker concentrations at each time point. Additionally, data are depicted as percentage compared to baseline (100%) in Figure 1 because biomarker baseline values varied widely between patients. A clear correlation between the Pettersson score and baseline values was lacking in our study (data not shown).
uCTX-II levels (Table 1) changed statistically significantly over time (p=0.016). Within two days after the bleed, no change in uCTX-II concentration was seen compared to baseline levels (+22%; p=0.878; Figure 1A). At day five, a significant increase was detected when compared to baseline (+52%; p=0.021). This increase was approaching statistical significance when compared to the uCTX-II concentration measured within two days after the joint bleed (p=0.086). The concentrations dropped at 14 days to almost baseline levels (+16% compared to baseline value; p=0.037), which was a statistically significant decrease compared to day five (p=0.008).
Also sCS846 showed a significant change over time (Table 1; p=0.004). Immediately after the bleed, the concentrations were still comparable to baseline (+5%; p=0.575). At day five, an increase in sCS846 concentrations was observed (Figure 1D), both when compared to baseline levels (+14%; p=0.011) as well as compared to the concentrations measured within two days (p=0.008). The concentrations returned to baseline values at 14 days after the bleed (+2% compared to baseline value; p=0.285; compared to day five p=0.021).

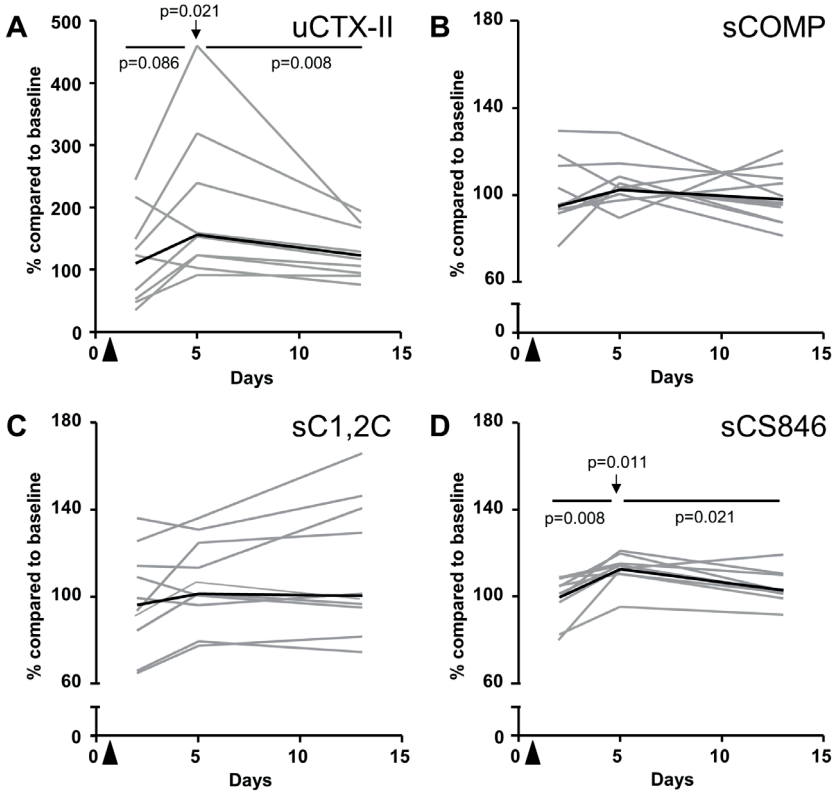

Figure 1 – Biomarkers of joint damage increase after an acute joint bleed in hemophilia patients
Blood and urine samples were collected after a joint bleed in 10 hemophilia patients. uCTX-II (A) was measured in urine samples and corrected for creatinine levels. sCOMP (B), sC1,2C (C), and sCS846 (D) were measured in serum samples. All data are presented as percentage compared to baseline. Friedman's test followed by Wilcoxon signed rank test was performed on absolute data.
Each grey line represents an individual patient, black lines represent median values.

Table 1 – Change in biomarkers after a joint bleed in hemophilia patients

Biomarker	Baseline	2 days	5 days	14 days	*P*-value[a]
uCTX-II (mg/mmol creat)	0.38 (0.20-0.60)	0.30 (0.23-1.08)	0.57 (0.38-1.10)	0.38 (0.31-0.74)	**0.016**
sCOMP (ng/mL)	89.4 (76.0-92.5)	85.5 (78.1-100.1)	91.0 (80.4-96.3)	84.4 (78.9-96.2)	0.435
sC1,2C (ng/mL)	530 (489-682)	480 (449-640)	599 (506-674)	645 (517-709)	0.316
sCS846 (ng/mL)	202 (136-252)	196 (144-268)	227 (158-283)	235 (138-261)	**0.004**

Absolute values of biomarkers are presented as median (25-75% percentile). Statistically significant changes are in bold. Creat, creatinine.
[a] Friedman's test

Neither sCOMP nor sC1,2C showed significant changes over time (p=0.435 and p=0.316 respectively), although all patients showed an increase in sCOMP at some point within the first five days. In four patients sCOMP levels increased already within two days after the bleed when compared to baseline, whereas the other six patients showed an increase from day two to day five. Such a trend was not observed for sC1,2C.

uCTX-II and sCOMP change upon an experimentally induced joint bleed in dogs

The observed changes in biochemical markers upon a joint bleed in this small population of hemophilia patients with pre-existing HA prompted us to conduct a study in a canine model of blood-induced cartilage damage to have a more controlled set-up in arthropathy naïve joints. In seven Beagle dogs changes in the same panel of biomarkers were investigated upon an experimentally induced joint bleed. For none of the biomarkers levels at week 13 were higher than at baseline suggesting normalization of the biomarkers after such a time period post bleeding.

Absolute concentrations of biomarkers are shown in Table 2 and in Figure 2 data are depicted as percentage compared to baseline. The concentrations of uCTX-II and sCOMP changed significantly over time (p=0.011 and p=0.004 respectively), whereas sC1,2C and sCS846 showed no significant changes over time (p=0.178 and p=0.100 respectively; Table 2).

The uCTX-II concentrations measured directly after the experimentally induced joint bleed (day 1-2) were not statistically significantly different compared to levels at baseline (-25%; p=0.398). One week after the bleed, concentrations increased to 155% of baseline levels (p=0.176), which was a significant increase compared to levels at day 1-2 (p=0.018). One week later, uCTX-II concentrations decreased slightly, but remained elevated compared to day 1-2 (p=0.063), and compared to baseline (+42%; p=0.237).

sCOMP increased immediately after the bleed compared to baseline (+46%; p=0.028) and was still marginally but statistically significantly increased at day seven (+8%; p=0.043). Two weeks after the bleed, the concentration of sCOMP decreased (p=0.018) to a concentration slightly below baseline (-8%; p=0.499).

Compared to baseline, sCS846 tended to decrease after the bleed and stayed decreased, whereas sC1,2C did not show a clear tendency.

Discussion

The present study demonstrates that certain biochemical markers of joint tissue turnover increase immediately upon a harmful trigger (a joint bleed) in case of already existing joint damage (hemophilic arthropathy; HA) as well as in damage naïve joints (a canine model for blood-induced joint damage). This provides potential for the further development and utilization of biochemical markers in monitoring joint diseases such as OA, RA, and HA. Moreover, it demonstrates that a canine model of blood-induced joint damage might be of use in development and validation of new biomarkers.

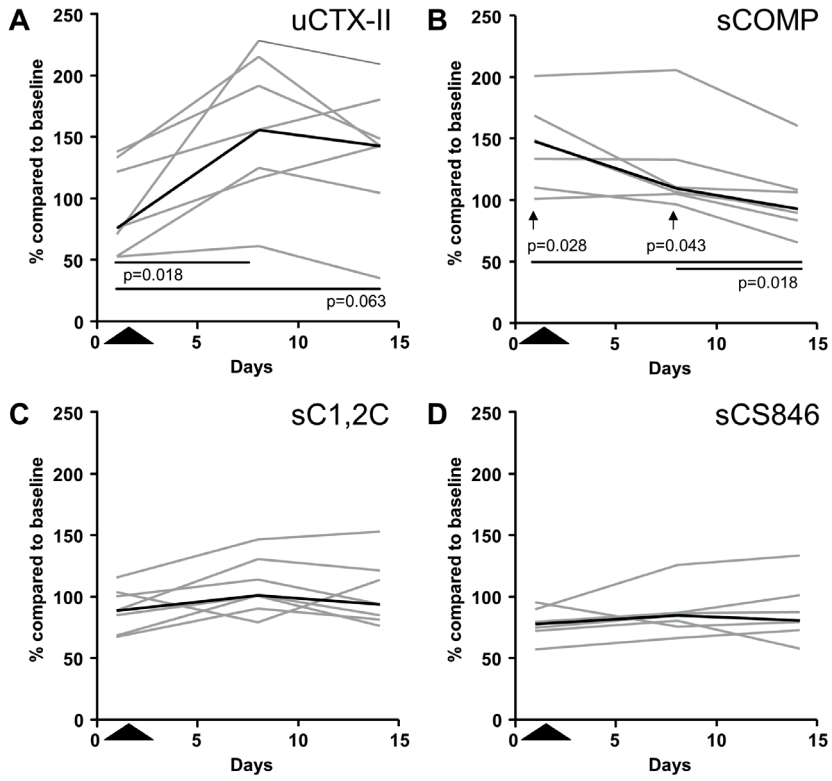

Figure 2 – Biomarkers of joint damage increase in dogs after an experimentally induced joint bleed
In 7 dogs, blood and urine samples were collected at four different time points after an experimentally induced joint bleed. uCTX-II (A) was measured in urine samples and corrected for creatinine levels. sCOMP (B), sC1,2C (C), and sCS846 (D) were measured in serum samples. All data are presented as percentage compared to baseline. Friedman's test followed by Wilcoxon signed rank test was performed on absolute data.
Each grey line represents a single dog, black lines represent median values.

Table 2 – Change in biomarkers after an experimentally induced joint bleed in dogs

Biomarker	Baseline	1-2 days	8 days	14 days	*P*-value[a]
uCTX-II (mg/mmol creat)	1.97 (1.29-4.35)	2.34 (1.10-2.86)	2.99 (2.61-4.21)	2.69 (1.90-3.47)	**0.011**
sCOMP (ng/mL)	31.5 (25.5-37.0)	46.0 (36.8-55.0)	38.2 (34.0-40.1)	31.9 (27.7-35.0)	**0.004**
sC1,2C (ng/mL)	903 (697-1159)	777 (613-998)	1039 (546-1214)	935 (763-1267)	0.178
sCS846 (ng/mL)	239 (219-317)	205 (169-288)	256 (162-312)	299 (147-312)	0.100

Absolute values of biomarkers are presented as median (25-75% percentile). Statistically significant changes are in bold. Creat, creatinine.
[a] Friedman's test

In hemophilia patients, an increase in uCTX-II and sCS846 levels was detected within a week after a joint bleed. Also a bleed in a single large joint in a dog resulted in an elevation in uCTX-II and sCOMP levels within a week after the bleed, but not in sCS846. This demonstrates that it takes a few days before breakdown products of joint tissue enter the circulation and can be measured in serum and urine. Within two weeks the elevated levels had normalized, indicating that the original tissue destructive process is temporary. As development of blood-induced joint damage is a progressive process becoming evident a long time after a bleed,[18] the first initial peak of tissue damage is anticipated to be followed over time by an intrinsic low level damage. This latter low level process can apparently not be detected by the presently used biochemical markers. This might add to the explanation why many of the biochemical markers are only discriminative at group level.[6]

It is tempting to speculate that a change in biochemical markers reflects the devastating effects of the bleed on a joint over time. However, it remains to be investigated whether this initial increase correlates with joint damage progression over time, aiding in monitoring the impact of a joint bleed and in evaluation of treatment efficacy (e.g. clotting factor on demand, or anti-inflammatory treatment[19-21]). It is important to extend the panel of biochemical markers tested in HA, to gain a more detailed insight in the changes in joint tissue turnover upon a joint bleed and to increase the usefulness of such biochemical markers in clinical application.

Screening new biomarkers in a model of blood-induced joint damage might be helpful in developing a reliable biomarker of joint damage for OA and RA. Despite all effort, at present none of the investigated biochemical markers has sufficient diagnostic or prognostic value in these diseases.[9,22] Blood-induced damage is a rather aggressive form of joint damage with features of both OA and RA.[11,23] Severely damaged joints are found at a much younger age than in case of OA or RA[24,25] and the pathologic processes are initiated by a clear trigger. Clearly, it cannot be excluded that there are pathways of joint damage (including cartilage, bone, and/or synovium) specific for a certain joint disease and not generic for all.

Of the screened biomarkers, uCTX-II showed the most evident change as an increase was demonstrated to approximately 150% of baseline in both the human and the canine study. CTX-II is seen as a marker of articular cartilage degradation,[26] but is also suggested to be involved in calcified cartilage layer changes or even bone metabolism.[27,28] The rapid elevation seen after a joint bleed is in accordance with studies demonstrating release of CTX-II into synovial fluid within one week after joint injury in humans[29] and a rise in uCTX-II levels in two different canine OA models.[30]

sCOMP is a biomarker sensitive to mechanical loading of the joint. Transient changes are demonstrated after physical exercise,[31-33] even a 30-min walk can cause elevated levels of sCOMP. The change is detected only within a few hours after exercise, but in marathon runners levels remained elevated for at least 48h.[33] In our canine study sCOMP rose almost immediately after the joint bleed, whereas in hemophilia patients effects were marginal and variable. A bleeding can be very painful, reducing joint use significantly and with that

potentially reducing a rise in sCOMP in hemophilia patients. In contrast, all animals quickly regained joint loading after an experimental joint bleed. Another possible confounder is a difference in duration of blood-exposure. Patients were included in this study within two days after they reported a joint bleed, therewith inducing already a variation of 48 hours. Duration of the bleed and response to clotting factor replacement were not taken into account. Moreover, diurnal variation in biomarker levels could be an interfering factor in the human study compared to the canine study because for logistical reasons sampling in humans was variable over the day. Also, pre-existing joint damage in hemophilia patients might have influenced biomarker levels as sCOMP was shown to correlate with the Pettersson score.[17] In general, sCOMP might be more sensitive to change upon different variables and as such less sensitive to change regarding the actual joint tissue damage. However, this needs to be verified in specifically designed studies.

Whereas sCS846[34] increased significantly in hemophilia patients, no change was detected in the canine model. This might be due to the specificity of the used ELISA kit, which is optimized for analyzing human samples and has cross-reactivity for different species including dogs. The absence of pre-existing joint damage in the canine study compared to an already increased proteoglycan turnover at time of the joint bleed in hemophilia patients might explain why a rise in CS846 was detected only in hemophilia patients. In a canine model of early OA induced by surgical transection of the anterior cruciate ligament (ACL), serum levels of CS846 were elevated already at three and 12 weeks compared to preoperative levels.[35] ACL transection causes an unstable joint and therewith a constant low level trigger compared to the temporary high impact of a single large joint bleed. In our canine study CS846 levels even tended to decrease after the joint bleed. These findings resemble the decreased levels of CS846 in serum of patients with rapid progressive RA compared to increased levels in patients with slow progressive disease,[36] and the relatively lower synovial levels after acute joint inflammation or joint injury compared to OA.[37]

The hemophilia population included in this study was relatively small and heterogeneous with respect to age, pre-existing joint damage, and annual bleeding frequency. Even despite this heterogeneity, levels of biomarkers increased in response to a joint bleed. On the other hand, it underlines the importance to relate those levels to an individual baseline level. A limitation of the human experiments is the use of surrogate baseline values measured 90 days after the joint bleed. Obtaining baseline samples prior to the bleed was impossible as patients were enrolled at the time of a joint bleed. The canine experiment demonstrated that biomarker levels 13 weeks after the joint bleed dropped to (or even below) baseline values. Nonetheless, the surrogate baseline values might have been disturbed by recurrent (sub)clinical joint bleeds experienced during follow up. The last bleed was reported 35 days before collection of biosamples for the surrogate baseline. Since biomarker levels dropped already within 14 days after the bleed, these in-between joint bleeds were considered irrelevant to the baseline values.

In conclusion, this explorative study demonstrates that a rise in biochemical markers of joint tissue turnover can be detected directly after a single trigger on the joint, in this study a joint bleed in patients with varying existing (hemophilic) arthropathy as well as in a canine model of blood-induced joint damage without pre-existing joint damage. This indicates biomarker evaluation still to be a promising tool in detecting the destructive properties of joint damage and possibly treatment-efficacy. This study also confirms the changes in joint homeostasis induced by a single bleed, stressing its joint destructive properties. Moreover, it emphasizes the usefulness of blood-induced joint damage as a model to evaluate biochemical markers of joint damage.

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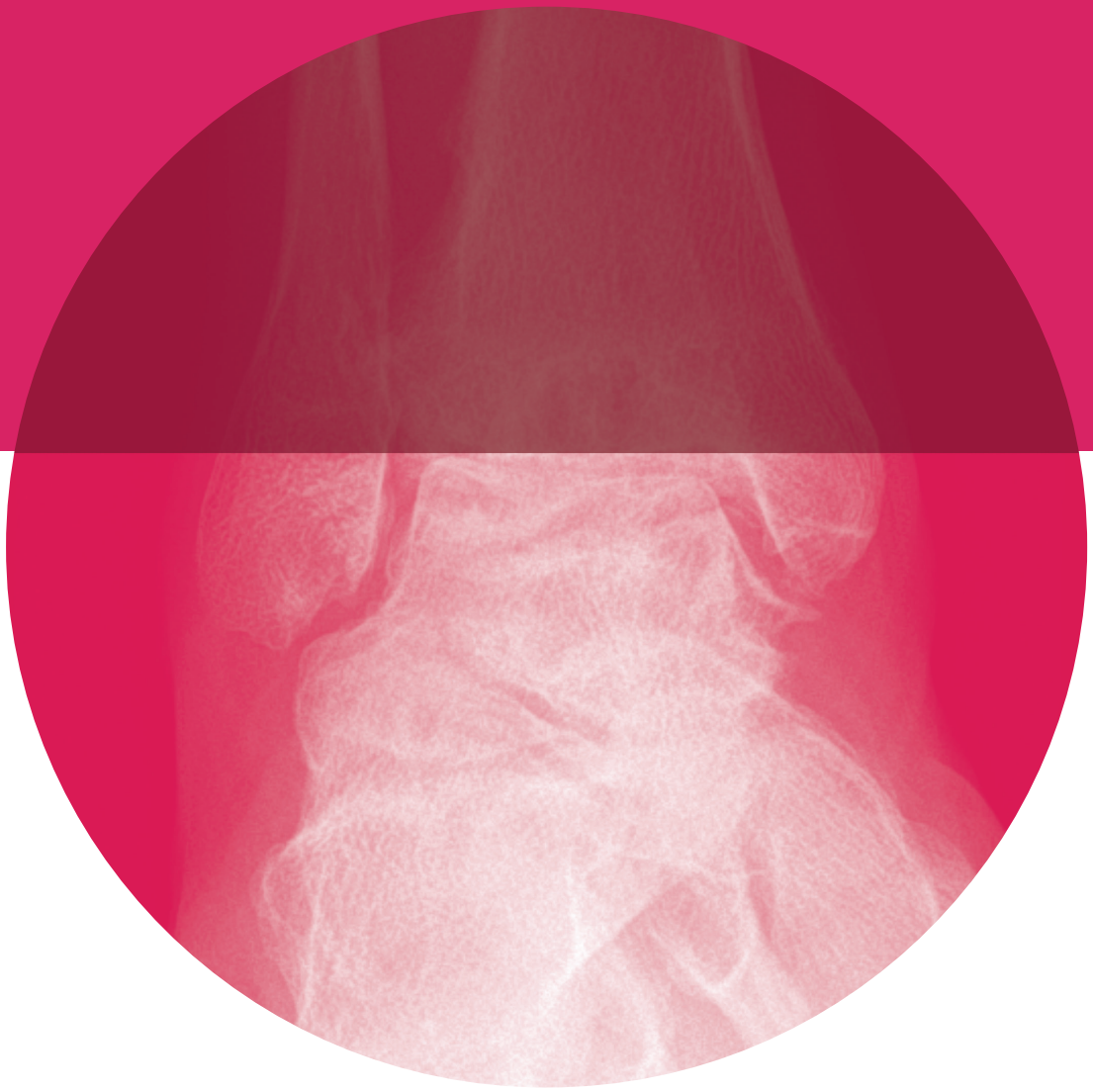
TREATMENT



CHAPTER 5

IL-1β, IN CONTRAST TO TNFα, IS PIVOTAL IN BLOOD-INDUCED CARTILAGE DAMAGE AND AS SUCH A POTENTIAL TARGET FOR THERAPY

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Abstract

Joint bleeding after (sports)trauma, major joint surgery, or as seen in hemophilia in general leads to arthropathy. Joint degeneration is considered to result from the direct effects of blood components on cartilage as well as indirectly from synovial inflammation. Blood-provided pro-inflammatory cytokines trigger chondrocytes and induce the production of cartilage-degrading proteases. In the presence of erythrocyte-derived iron, cytokines stimulate radical formation, in the vicinity of chondrocytes inducing apoptosis. To unravel the role of IL-1β and TNFα in the pathogenesis of this blood-induced cartilage damage, the effect of antagonizing these cytokines was examined in human *in vitro* cultures. Addition of recombinant human IL-1β monoclonal antibody (IL-1βmAb) or IL-1 receptor antagonist (IL-1RA) resulted in a dose- and time-dependent protection of cartilage from blood-induced damage. In higher concentrations, almost complete normalization of cartilage matrix proteoglycan turnover was achieved. This was accompanied by a reduction in IL-1β and IL-6 production in whole blood cultures, while TNFα production remained unaffected. Interestingly, addition of a TNFα monoclonal antibody, although demonstrated to inhibit the direct (transient) effects of TNFα on cartilage, exhibited no effect on blood-induced (prolonged) cartilage damage.
It is demonstrated that IL-1β is crucial in the development of blood-induced joint damage, whereas TNFα is not. This hierarchical position of IL-1β in blood induced joint damage warrants studies on targeting IL-1β to potentially prevent joint degeneration after a joint bleed.

Introduction

A bleed inside a joint after major joint surgery, (sports)trauma or as seen in hemophilia can lead to irreversible joint damage.[1-3] Over the years evidence has been gathered that even a single exposure of blood to a joint has profound damaging effects on joint cartilage.[4-6] This devastating effect is not primarily the result of synovial inflammation induced by blood components, an important aspect upon repeated bleeding as in hemophilia. After a single bleeding these synovial effects are considered transient,[7] whereas effects on cartilage are devastating. A single bleeding is able to induce chondrocyte apoptosis,[8] and will result in long-lasting impaired matrix turnover which eventually results in progressive joint damage becoming clinically evident over years.

The pathogenesis of blood-induced cartilage damage is still not fully understood.[7] Cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α are produced by blood-derived mononuclear cells,[9,10] by the subsequently activated synoviocytes,[11,12] as well as by stimulated chondrocytes,[13,14] and induce a variety of biological activities. Importantly, IL-1 and TNFα are considered upstream in the cascade of cytokines and induce the production of several other pro-inflammatory cytokines such as IL-8 and IL-6, as well as the synthesis of proteases including matrix metalloproteinases.[15] The production of those cytokines provides a transient imbalance in joint homeostasis but is unable to induce irreversible damage.[16,17] However, the presence of erythrocyte-derived iron in addition to the pro-inflammatory cytokines provides a mechanism for more permanent damage. The catalytic heme-derived iron is involved in the Fenton reaction, resulting in the formation of reactive oxygen species in the vicinity of chondrocytes activated by pro-inflammatory cytokines. These oxygen radicals induce apoptosis of the chondrocytes, the only cell type of cartilage and responsible for maintenance of the extracellular matrix.[8] Considering the relatively low number of chondrocytes within an abundant extracellular matrix[18] and the extremely low replenishment of these chondrocytes in adult human articular cartilage,[19] the induced apoptosis is rather devastating. It leads to a permanent disturbance in cartilage matrix turnover, causing loss of proteoglycans essential for resilience of cartilage tissue. Diminished mechanical properties of the cartilage will make it more vulnerable for subsequent mechanical damage initiating a vicious circle ultimately leading to progressive joint destruction.

Despite increasing insight in the mechanisms involved in the devastating effects of blood on joint tissues, targeted therapies are lacking. The aim of the current study is to dissect the role of IL-1β and TNFα in the pathophysiology of blood-induced cartilage damage. Understanding the contribution of individual cytokines to the development of blood-induced arthropathy is important to provide leads for targeted therapy.

Material and methods

Cartilage culture technique

Healthy human articular cartilage tissue was obtained within 24 hours post mortem of donors without a known history of joint disorders (n=31; age 60.6±16.6 years; 17 males and 14 females). Procedures were according to the medical ethical regulations of the University Medical Center Utrecht. Full-thickness cartilage was cut aseptically from the humeral head excluding the underlying bone, and kept in phosphate-buffered saline (PBS, pH 7.4). Within one hour after dissection, slices were cut into full-thickness square pieces and weighted aseptically (range 5-15 mg; accuracy ±0.1 mg).

Each explant was cultured individually in a 96-wells round-bottomed microtiter plate (at 5% CO_2 in air, 37°C, 95% humidity) in 200 µl culture medium per well. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µl/mL; all Paisley, UK), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled human male AB serum (Invitrogen).

For each experiment, fresh blood was drawn from healthy human donors (n=19; age 28.3±7.1 years; 9 males and 10 females) into Vacutainer tubes (Becton Dickinson). Explants were exposed to 50% v/v whole blood for four days, supposed to be the natural evacuation time of blood from the joint cavity.[20] After blood exposure, cartilage explants were washed twice for 45 minutes under culture conditions to remove all additives and cultured for an additional 12 days without additives (recovery period, to differentiate transient from permanent effects). Medium was refreshed every four days.

Experimental design

In the first set of experiments, the effect of IL-1β blockade on blood-induced cartilage damage was investigated by addition of an IL-1β monoclonal antibody (IL-1βmAb; R&D systems, Minneapolis) or IL-1RA (IL-1 receptor antagonist; anakinra, Swedish Orphan International Ltd) in a broad concentration range during blood-exposure.

In the second set of experiments, 100 ng/mL IL-1βmAb or 1000 ng/mL IL-1RA was added directly after addition of blood, or 2, 4, 8, 24, or 48 hours later (therapeutic approach). In those two experiments, long-term effects (after 12 days of recovery) on cartilage matrix turnover were assessed.

Thirdly, to determine the direct effects of IL-1βmAb on cartilage in the absence of blood, explants were cultured for four days in the presence of 10 ng/mL IL-1βmAb. Both short-term (immediate after the four day culture) and long-term effects (after 12 days of recovery) on cartilage matrix turnover were determined.

To investigate the effect of blocking TNFα on blood-induced cartilage damage, 10 µg/mL TNFαmAb (adalimumab, AbbVie; at this concentration completely inhibiting TNFα production by LPS-stimulated monocytes[21]) was added to blood-cartilage co-cultures for four days and proteoglycan turnover was determined after 12 days of recovery. As no effect

was demonstrated, the efficacy of the TNFαmAb was investigated by culturing cartilage explants for four days in the presence of recombinant TNFα (R&D Systems) with/without 10 µg/mL TNFαmAb. As TNFα alone exhibits only reversible effects on proteoglycan turnover,[17] proteoglycan synthesis rate was determined after four days culture.

Determination of proteoglycan turnover

Each experiment was performed with cartilage from a single donor. To correct for possible biological variations between samples, the mean value of 8-10 cartilage explants (depending on the amount of cartilage tissue available) per parameter per donor, obtained randomly and handled individually, was taken as a representative value.

Proteoglycan turnover was evaluated at the end of each experiment. Proteoglycans, consisting of negatively charged glycosaminoglycans (GAGs) attached to a core protein, attract water into the cartilage, therewith exerting a swelling pressure to withstand loading.[22] So, a decreased proteoglycan synthesis, release of GAGs and overall a loss of GAG content results in a diminished resilience of cartilage and consequently in tissue degeneration. Assessment of quantitative biochemical proteoglycan turnover is more sensitive than histological Alcian Blue staining.[23]

For proteoglycan synthesis rate, sulphate incorporation was determined by addition of $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont; 74 kBq per well) for four hours to pulse label the sulphated GAGs. Subsequently, the cartilage explants were washed twice in cold PBS, digested for two hours at 65°C with 2% papain (Sigma), and stored at -20°C. Proteoglycan synthesis rate was determined by precipitation of GAGs with 0.3M hexadecylpyridinium chloride monohydrate (CPC; Sigma) in 0.2M NaCl. The precipitate was dissolved in 3M NaCl and the amount of radioactivity measured by liquid scintillation counting. Radioactivity was normalized to the specific activity of the medium, labeling time, and wet weight of the explant. Results were expressed as nanomoles of sulphate incorporated per gram wet weight of cartilage tissue (nmol/h*g).

The proteoglycan content of each cartilage explant and release of proteoglycans into culture medium were established by staining and precipitation of GAGs with Alcian Blue (Sigma) in the papain digest of cartilage samples and in culture medium, respectively. Staining was quantified by spectrophotometry at 620 nm using chondroitin sulphate (Sigma) as a reference. Results were normalized to the wet weight of cartilage tissue.

Immunohistochemistry

For immunohistochemistry, human cartilage explants were cultured for four days with/without 50% v/v whole blood, and 100 ng/mL IL-1βmAb or 10 µg/mL TNFαmAb was added. Explants were harvested, snap frozen in liquid nitrogen and stored at -80°C. Frozen sections were sliced and fixed with 4% paraformaldehyde and 80% methanol.

Apoptosis of chondrocytes was detected using an anti-single-strand deoxyribonucleic acid (ssDNA) monoclonal antibody (clone F7–26/apostain; Alexis Corporation, The Netherlands).

Tissue was permeabilized with 0.2 mg/mL saponin (Sigma) and heated to 56°C in formamid to denature unstable DNA and subsequently transferred to ice-cold PBS. The sections were incubated with anti-ssDNA antibody and the antibody complex was visualized using 3,3'-diaminobenzidine (DAB; Vector). The tissue was counterstained with hematoxylin. Representative images of a limited number of experiments are provided.

Blood cultures

To study the effect of IL-1βmAb, IL-1RA, and TNFαmAb on the production of pro-inflammatory cytokines, separate whole blood (50% v/v) cultures without cartilage were performed. It was previously demonstrated that the production of pro-inflammatory cytokines by healthy cartilage itself is negligible,[24] and that the production of these cytokines is not different between 50% blood cultures with and without cartilage.[25]

Blood of six healthy donors (age 30.7±9.1 years; four males and two females) was cultured for four days. IL-1βmAb, IL-1RA, or TNFαmAb was added in a concentration of 100 ng/mL, 1000 ng/mL, or 10 µg/mL respectively (i.e. the highest concentrations tested in blood-cartilage co-cultures). After four days samples were collected, centrifuged at 1,500 *g* for 10 minutes, and supernatants stored at -80°C. The concentrations of IL-1β, IL-6, and TNFα were determined with commercially available enzyme-linked immunosorbent assay (ELISA) (for IL-1β and IL-6 Invitrogen, for TNFα Sanquin) and analyses were performed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS statistics package for Windows (Version 21.0, IBM Inc., Chicago, IL, USA). As data were non-normally distributed, the Wilcoxon-signed rank test for related samples was performed to determine statistical significance between treatments and controls of cartilage or blood from the same donor sample.

All statistical analyses were performed using absolute data. Values are expressed as percentage of control culture conditions and depicted as median values ± interquartile range, unless stated otherwise.

P-values <0.05 were considered statistically significant.

Results

Dose-dependent protection of blood-induced cartilage damage by IL-1βmAb and IL-1RA

Culturing cartilage explants in the presence of 50% v/v whole blood for four days strongly impacted proteoglycan turnover determined 12 days later (Figure 1). Proteoglycan synthesis rate was inhibited with 75%; p=0.012 (A) and 76%; p=0.018 (B), GAG release was increased with 129%; p=0.017 (C) and 125%; p=0.018 (D), and GAG content decreased with 14%; p=0.017 (E) and 18%; p=0.028 (F), all compared to control condition (100%; dotted line).

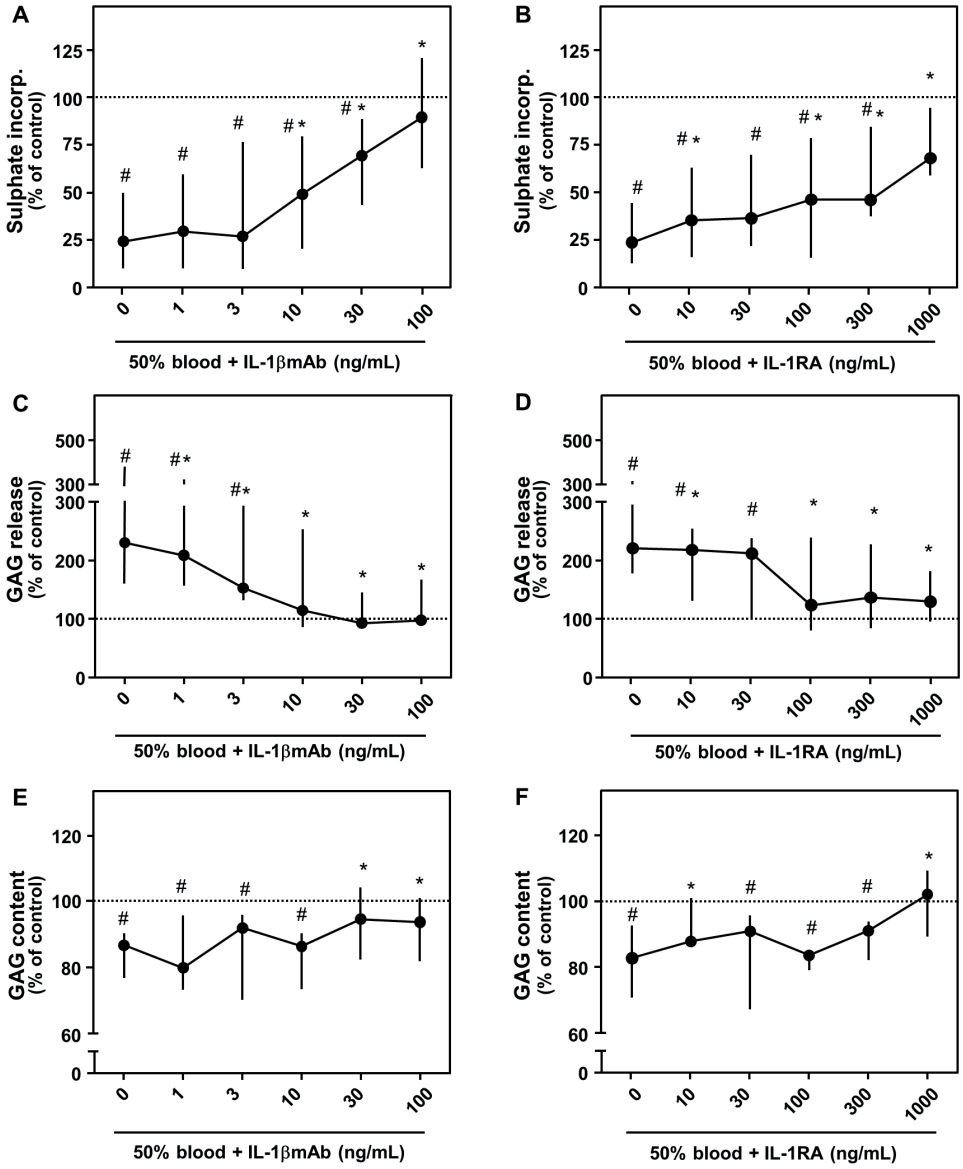

Figure 1 – Concentration dependent effect of antagonizing IL-1 activity

Healthy human cartilage was cultured for four days in the absence (control; dotted line) or presence of 50% blood. During blood-exposure, a monoclonal antibody directed against IL-1β (IL-1βmAb) (A,C,E; n=8) or an IL-1 receptor antagonist (IL-1RA) (B,D,F; n=7) was added.

Proteoglycan synthesis rate (A,B), -release (C,D) and -content (E,F) are depicted. Dotted line represents control conditions (cartilage cultures without additives).

# *P<0.05* compared with control values

* *P<0.05* compared with blood-exposed cartilage without IL-1βmAb or IL-1RA addition

Addition of IL-1βmAb during the four-day blood-exposure resulted in a dose-dependent recovery of proteoglycan synthesis rate, with a statistically significant increase from 10 ng/mL compared to blood exposure without additives (this and all higher concentrations p=0.012). At a concentration of 100 ng/mL, proteoglycan synthesis rate was not different anymore compared to control (91%; p=0.401). GAG release decreased already with addition of 1 ng/mL IL-1βmAb (all concentrations p<0.03 vs blood alone). From a concentration of 10 ng/mL on, GAG release was not different anymore compared to control (p>0.441). Proteoglycan content improved significantly in the presence of 30 ng/mL IL-1βmAb or higher (both p=0.025 compared to blood alone).

To confirm that the protective effects of IL-1βmAb were IL-1 specific, we repeated the experiments with addition of IL-1RA, a natural inhibitor of both IL-1α and IL-1β. Similar effects with the antagonist as with the neutralizing antibody were found. Addition of IL-1RA prevented blood-induced cartilage damage in a concentration-dependent way. In nearly all concentrations tested, proteoglycan synthesis rate improved (Figure 1B; asterisks). GAG release improved already at the lowest concentration of IL-1RA added, and was not significantly different to control condition anymore from a concentration of 100 ng/mL and higher (Figure 1D). Addition of 1000 ng/mL significantly improved GAG content compared to blood-exposure only (+2% compared to control; p=0.018; Figure 1F; asterisk). Pre-incubation of cartilage explants with IL-1RA before blood exposure, did not further improve the antagonizing efficacy (data not shown).

Time-dependent protection of blood-induced cartilage damage by IL-1βmAb and IL-1RA

In a separate set of experiments it was shown again that addition of a high concentration of IL-1βmAb (100 ng/mL) or IL-1RA (1000 ng/mL) immediately after blood-exposure resulted in a complete restoration of proteoglycan turnover, matching control values again (Figure 2; all parameters p>0.05 compared to control).

Blocking IL-1 within eight hours after blood-exposure was most effective, thereafter the effect diminished. Proteoglycan synthesis improved in a time-dependent manner (Figure 2A&B). A significant decrease in GAG release by addition of IL-1βmAb or IL-1RA was seen at all timepoints (Figure 2C&D), but when the antibody was added after 24 hours the effects was clearly less pronounced (panel C).

GAG content significantly improved when IL-1βmAb was added within eight hours after blood-exposure (Figure 2E; p≤0.043) but for IL-1RA only when added shortly after blood-exposure (Figure 2F; p=0.018). Compared to control cartilage GAG content was significantly lower (viz. additives were not effective anymore) when IL-1RA was added after eight hours or later (all p=0.018), and for IL-1βmAb when added after 24 or later (p≤0.028).

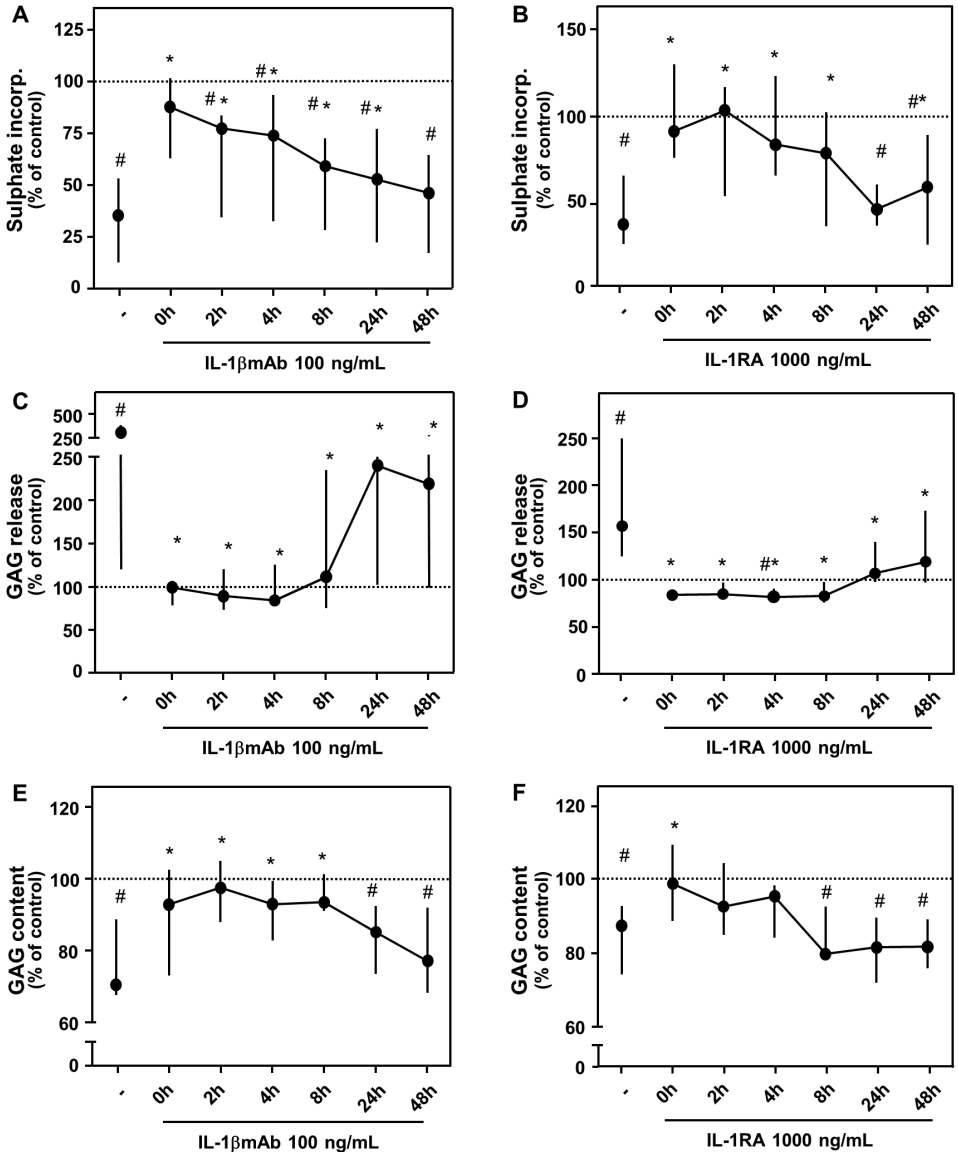

Figure 2 – Time dependent effect of antagonizing IL-1 activity

Cartilage was cultured as described in the legend of Figure 1. IL-1βmAb (A,C,E; n=7) or IL-1RA (B,D,F; n=7) was added at the moment of blood-exposure or 2 to 48h later.

Proteoglycan synthesis rate (A,B), -release (C,D) and -content (E,F) are depicted. Dotted line represents control conditions (cartilage cultures without additives).

# *P<0.05* compared with control values

* *P<0.05* compared with blood-exposed cartilage without IL-1βmAb or IL-1RA addition

Table – IL-1βmAb has no direct effect on human cartilage explants

	Control	IL-1βmAb (10 ng/mL)	*P*-value
PG synthesis rate	4.33 (2.94-8.97)	4.73 (3.42-8.74)	0.55
GAG release	1.38 (0.97-2.27)	1.21 (0.97-2.29)	0.87
GAG content	24.73 (20.56-28.08)	25.53 (22.49-27.69)	0.50

Proteoglycan turnover parameters in the absence of blood, expressed as median with interquartile range (n=7; independent cartilage donors)

No direct effect of IL-1ßmAb on healthy cartilage

IL-1 not only induces cartilage degradation, but also is important for maintaining cartilage homeostasis.[26] Therefore it was investigated whether addition of IL-1βmAb had a direct effect on healthy cartilage. Proteoglycan turnover was assessed 12 days later for long-term effects, showing no direct effects of 10 ng/mL IL-1βmAb in the absence of blood (p>0.50 for all three parameters; Table). As it is known that the impact on proteoglycan synthesis rate of IL-1β in the absence of an iron source is reversible,[7] also the short-term effect of addition of IL-1βmAb was tested. No change in proteoglycan synthesis rate was demonstrated (n=3; 4.39 (2.33-8.50) vs 4.33 (2.49-9.04) nmol/h*g). For IL-1RA it has been demonstrated previously that it has no direct effects on proteoglycan synthesis rate.[27,28]

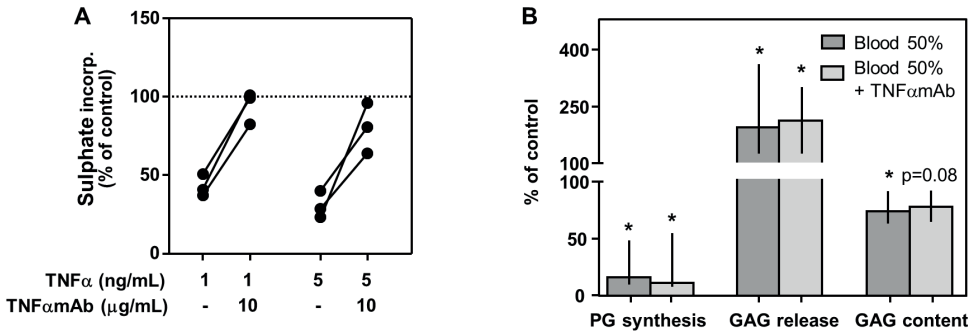

Figure 3 – Antagonizing TNFα with a monoclonal antibody cannot protect cartilage from blood-induced damage
Panel A: healthy human cartilage (n=3) was cultured for four days in the absence (control; dotted line) or presence of TNFα and a TNFα monoclonal antibody (TNFαmAb). Proteoglycan synthesis rate was determined to check the activity of the antibody. Panel B: cartilage was cultured in the absence (control; 100% level) or presence of whole blood (50% v/v) with/without TNFαmAb (10 µg/mL) (n=5). Proteoglycan turnover was determined after 12 days of recovery. * *P<0.05* compared with control values. No statistical significant effects between cultures with and without TNFαmAb were seen.

Antagonizing TNFα does not reverse blood-induced cartilage damage

To investigate the role of TNFα, we tested the influence of antagonizing TNFα with a monoclonal antibody (TNFαmAb; adalimumab) on proteoglycan turnover.

TNFα in the absence of blood clearly inhibited proteoglycan synthesis rate assessed at

day four (1 ng/mL -57%; 5 ng/mL -69%; figure 3A). Concomitant addition of TNFαmAb (10 µg/mL) reversed this effect almost completely (Figure 3A; 1 ng/mL TNFα 99% of control; 5 ng/mL TNFα 81% of control), demonstrating its functionality. However, in blood-cartilage co-cultures addition of TNFαmAb (10 µg/mL) did not affect any of the proteoglycan turnover parameters assessed after 12 days of recovery (Figure 3B). Proteoglycan synthesis rate decreased to 16% of control values, and addition of TNFαmAb did not change this (11% of control; p=0.500). GAG release increased upon blood exposure to 194% of control, and to 213% in the presence of TNFαmAb (p=0.500). Also GAG content after blood-exposure was not affected by addition of TNFαmAb (74% vs 78% of control; p=0.225).

Blood-induced chondrocyte apoptosis is limited by blocking IL-1β

Exposure of cartilage to blood for four days led to apoptosis of chondrocytes as compared to cartilage cultured in medium without additives (representative images in figure 4A&B). Addition of IL-1βmAb prevented this blood-induced apoptosis (representative image in figure 4C), whereas TNFαmAb did not affect it (representative image in figure 4D).

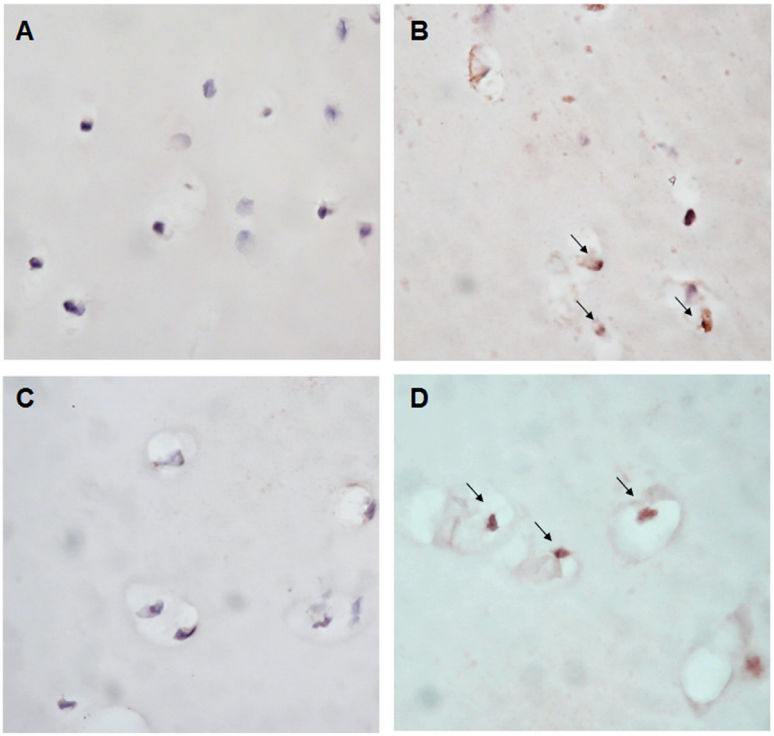

Figure 4 – Blocking IL-1 limits blood-induced chondrocyte apoptosis

Representative photo-micrographs of chondrocyte apoptosis in human articular cartilage explants cultured in medium (A) or exposed to 50% v/v whole blood (B-D) for four days. IL-1βmAb (C) or TNFαmAb (D) was added during blood-exposure.

Apoptosis of chondrocytes was determined by immune-histochemical staining with ssDNA (brown staining, indicated by arrow). Original magnification: 40x.

Blocking the activity of IL-1β limits the production of IL-6 but not TNFα

To further elucidate the role of the individual cytokines, in four-day whole-blood cultures the effect of blocking either IL-1β or TNFα on levels of other pro-inflammatory cytokines was investigated. The production of IL-1β was limited by the addition of IL-1RA but not by TNFαmAb (Figure 5A; p=0.028 and p=0.345 compared to blood only). TNFα levels were not influenced in the presence of IL-1 blocking agents (Figure 5B; both p=0.753 compared to blood only). Addition of IL-1RA or IL-1βmAb, but not TNFαmAb, limited the production of IL-6 compared to the blood culture without additives (Figure 5C; p=0.028 for both IL-1RA and IL-1βmAb; p =0.753 for TNFαmAb).

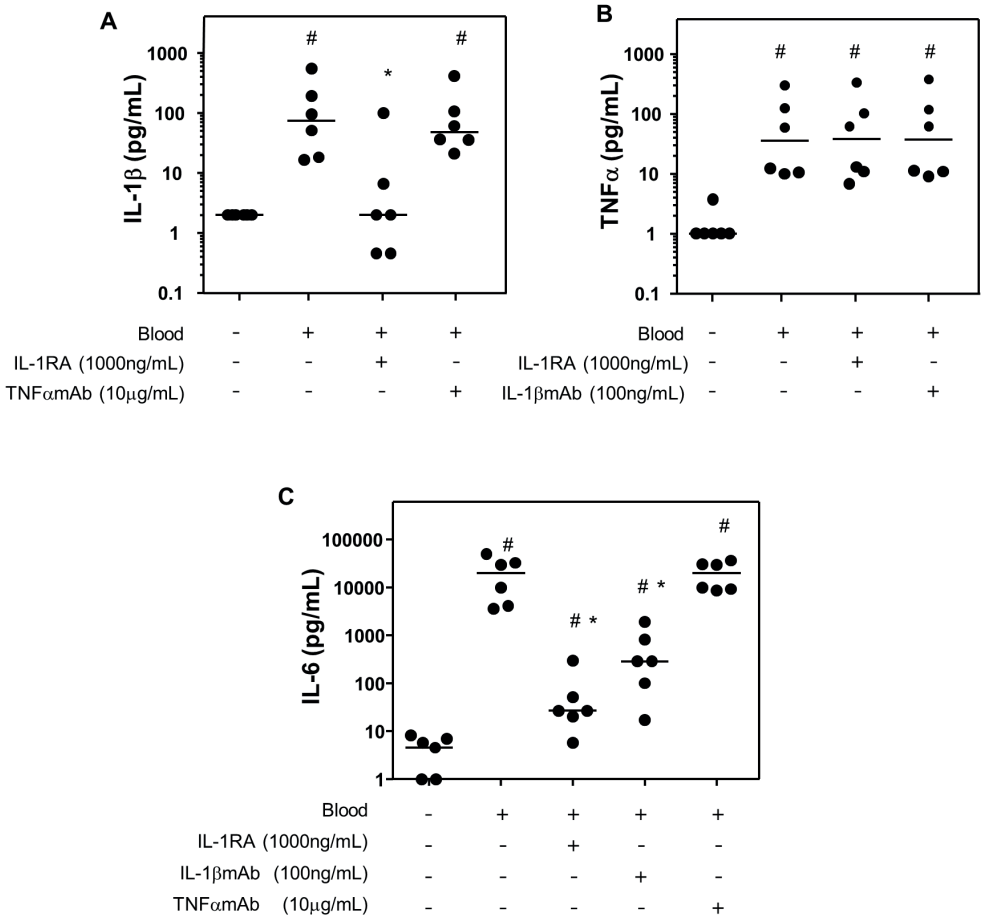

Figure 5 – Effect of antagonizing IL-1 or TNFα on pro-inflammatory cytokine production in whole blood cultures
50% v/v whole blood of 6 donors was cultured for four days. IL-1RA, IL-1βmAb or TNFαmAb was added and the production of the pro-inflammatory cytokines IL-1β (A), TNFα (B), and IL-6 (C) was measured. Data are shown as individual (dots) and median (dash) values. Control culture conditions without blood and additives is shown as well. # P<0.05 compared with control; * P<0.05 compared with blood without additions.

Discussion

This human *in vitro* study demonstrates that IL-1β and not TNFα is a key mediating cytokine in the harmful direct effects of blood on cartilage. Blocking IL-1β with either a monoclonal antibody directed against IL-1β, or the receptor antagonist IL-1RA competing with both IL-1α and IL-1β for receptor binding, reversed blood-induced cartilage damage in a dose- and time-dependent manner up to complete normalization. In contrast, blocking TNFα with a monoclonal antibody did not demonstrate any efficacy. In addition, we showed that blocking IL-1β resulted in a reduction of IL-6, whereas TNFα levels were unaffected. Blocking TNFα did not change levels of IL-1β or IL-6, advocating for two separate pathways (Figure 6). IL-1βmAb in the absence of blood did not have any effect on cartilage proteoglycan turnover, which is an important observation as IL-1β not only can induce cartilage matrix breakdown,[27,29] but also has a role in maintaining cartilage homeostasis.[26] The lack of influence on healthy cartilage is in line with previous studies showing that chondrocytes from healthy cartilage produce only low levels of pro-inflammatory cytokines,[24,25] and IL-1RA

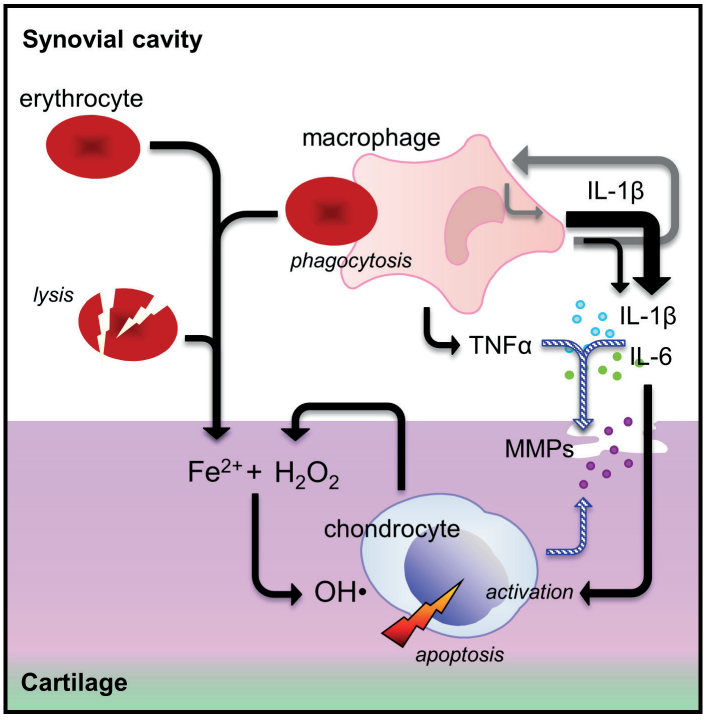

Figure 6 – Suggested role of cytokines in the pathogenesis of synovial independent blood-induced cartilage damage
Monocyte/macrophages activated by the phagocytosis of erythrocytes, produce IL-1β and TNFα, which both induce the production of proteases causing reversible cartilage damage (striped arrows). IL-1β also induces the production of IL-6 and of IL-1β itself (grey arrows). These cytokines are able to activate the chondrocyte to produce hydrogen peroxide (H_2O_2) that in the presence of iron leads to the production of hydroxyl radicals (OH·) causing chondrocyte apoptosis and as such irreversible cartilage damage, whereas TNFα is not. Local inhibition of IL-1β (directly in case of major surgery) or within the same day (in case of trauma or hemophilia induced joint bleeds) may provide a mechanism to prevent cartilage damage and with that joint destruction later on.

does not exert any effect on healthy cartilage in the absence of IL-1.[27,28]

With a molecular weight of approximately 17 kDa for IL-1RA compared to 160 kDa for the IL-1βmAb, about a 100-fold higher molar concentration of IL-1RA was necessary to elicit a similar response on blood-induced cartilage damage. This excess of IL-1RA compared to the amounts of IL-1 produced, is needed to block all IL-1 receptors on chondrocytes as well as on blood cells (especially monocytes/macrophages). Its activity relies mainly on antagonizing the activity of IL-1β, not IL-1α, as selective blocking of IL-1β with a monoclonal antibody also fully prevented cartilage destruction. Heme is a potent inducer of IL-1β secretion by macrophages,[30,31] and IL-1β production by synoviocytes is demonstrated in case of repeated joint bleeds as in hemophilic arthropathy.[32] Moreover, hemarthrosis results in hemophilic mice in elevated levels of IL-1β.[33,34] An upregulation of the nuclear factor (NF)-κB pathway, which is induced by IL-1β, has been emphasized in hemophilic mice upon joint bleeding[34] and in synovial tissue of patients with hemophilic arthropathy.[35] In experimental arthritis, blocking IL-1 prevents cartilage damage upon inflammation[36,37] as is also observed in a posttraumatic osteoarthritis model.[38] Therapeutic efficacy of IL-1RA is demonstrated in hand osteoarthritis[39] and in two cases suffering from another form of iron-induced arthropathy in case of hemochromatosis.[40]

Opposing IL-1 was most effective when performed within eight hours after blood-exposure, but still benefit was achieved when performed until 24h after exposure to blood. This is in line with the observation that IL-1 production in blood-cartilage co-culture is detected within 24h, and prevented by addition of IL-4 and IL-10 within 4-8h.[25] Addition of those anti-inflammatory cytokines after 8h does not improve cartilage turnover anymore, whereas blocking IL-1 still shows benefit several hours later. IL-1β is capable to induce its own production as well as the production of other pro-inflammatory cytokines.[9,15] So inhibiting the initial release of IL-1 by monocytes/macrophages is essential to abrogate the positive feedback loop (Figure 6).

Our group previously demonstrated that long-lasting inhibition of proteoglycan synthesis induced by lysed erythrocytes together with IL-1β is less pronounced than the effect of mononuclear cells together with erythrocytes, or whole blood.[8] TNFα was suggested as a possible factor explaining these differences, its production being demonstrated in blood-cartilage co-cultures,[24] and shown to increase hydroxyl radical formation by chondrocytes in the presence of iron.[41,42] In the present study however, blocking TNFα although completely preventing TNFα induced short-term inhibition of proteoglycan synthesis in the absence of blood (reversible changes due to upregulation of cartilage degrading enzymes[17]), did not affect long-term blood-induced cartilage damage (irreversible changes due to chondrocyte apoptosis[43]). The concentrations of TNFα produced in the blood-cartilage co-culture system (max 350 pg/mL in previous[24] and current study) are relatively low, probably too low to stimulate hydroxyl radical formation which is a prerequisite for inducing irreversible cartilage changes (in the study demonstrating this hydroxyl inducing capacity 100 ng/mL TNFα was used to stimulate chondrocytes[41]).

TNFα is produced as a membrane-bound precursor that is released through proteolytic cleavage by the TNF-converting enzyme (TACE). Adalimumab, the TNFαmAb used in this study, binds and inactivates both the membrane-bound and soluble form.[44] TNFα production by synovial tissue will also contribute to end-stage hemophilic arthropathy.[32] However, the increase in TNFα upon a joint bleeding (in a murine hemophilia model) is either absent[33] or very minimal,[34] questioning its role in the early stages of blood-induced joint damage. Probably, TNFα is mainly a mediator in synovial inflammation instead of direct cartilage destruction. Also in murine models of inflammatory arthritis and posttraumatic osteoarthritis no effect on cartilage destruction upon TNFα blockade has been found, corroborating this hypothesis.[36-38] Nevertheless, this study does not exclude a role for TNFα in inducing the reversible (short-term) changes to cartilage.

The present study advocates for IL-6 as a contributing factor to the effects of IL-1β and iron on cartilage. We show that its production is suppressed in the presence of an IL-1 blocking agent, whereas TNFα levels are unaffected. Also in osteoarthritic synovium[45] and monocyte cultures,[9] addition of IL-1RA showed a dose-dependent reduction in IL-6 production. A protective effect of blocking IL-6 on cartilage damage and synovitis upon joint bleeding in a hemophilia mouse model has been shown when administered in combination with clotting factor replacement.[46]

The main limitations of this study are related to the use of an *in vitro* model. Besides the lack of involvement of synovial tissue as mentioned earlier, also the replenishment of blood cells in an ongoing bleed is not included in this system. This is particularly important for IL-1RA treatment as cells with unblocked receptors will enter the joint and as such higher doses of IL-1RA will be required. Moreover, it needs to be investigated whether a single injection is sufficient to prevent cartilage damage, and as clinical observations demonstrate differences in vulnerability to the effects of intra-articular bleeding,[47] it remains to be investigated which patients will benefit from this treatment.

Taken together, these data suggest IL-1β to play a critical role in blood-induced cartilage damage whereas this could not be demonstrated for TNFα. Opposing the activity of IL-1β within the same day or next morning after blood-exposure, prevents the initiation of a self-amplifying loop resulting in progressive cartilage damage. As therapeutic agents opposing the activity of IL-1β are available and safe for application,[48-50] local treatment with such drugs upon a joint bleed might be relevant to prevent progressive joint damage over time. However, more (*in vivo*) evidence is needed that interfering with these pro-inflammatory cytokines released and induced upon a joint bleed halts the degenerative process.

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CHAPTER 6

A FUSION PROTEIN OF INTERLEUKIN-4 AND INTERLEUKIN-10 PROTECTS AGAINST BLOOD-INDUCED CARTILAGE DAMAGE *IN VITRO* AND *IN VIVO*

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Abstract

Background: Joint damage still causes significant morbidity in hemophilia. It results from synovial inflammation and direct cartilage-degenerating properties of blood components. Interleukin (IL)-4 and IL-10 have been shown to protect cartilage from blood-induced damage. Recently an IL4-10 fusion protein has been developed to combine the function of IL-4 and IL-10 and increase their bioavailability.

Objectives: In this study it is evaluated whether this IL4-10 fusion protein protects against blood-induced joint damage.

Methods: In vitro, human cartilage explants were exposed to whole blood and simultaneously to a broad concentration range of the IL4-10 fusion protein. Effects on cartilage matrix turnover were compared to the individual cytokines. Moreover, the influence of the fusion protein and its individual components on IL-1β and IL-6 production was investigated. In hemophilia A mice, the effect of intra-articular treatment on synovitis and cartilage damage resulting from joint bleeding was evaluated by histochemistry.

Results: In vitro, the fusion protein prevented blood-induced cartilage damage in a dose-dependent manner, with equal effectiveness as the combination of the separate cytokines. In whole blood cultures 10 ng/mL fusion protein completely blocked the production of IL-1β and IL-6 by monocytes/macrophages. In hemophilic mice, intra-articular injection of IL-4 and IL-10 neither influenced synovitis nor cartilage degeneration. In contrast, equimolar amounts of the fusion protein attenuated cartilage damage upon repeated joint bleeding, though synovial inflammation was hardly affected.

Conclusions: Overall, this study shows that the IL4-10 fusion protein prevents blood-induced cartilage damage *in vitro* and ameliorates cartilage degeneration upon joint bleeding in hemophilic mice.

Introduction

Joint bleeds cause major morbidity in hemophilia,[1] but also occur after trauma,[2] major joint surgery,[3] or as a complication of anticoagulant treatment.[4] Though even a single bleed can lead to significant joint damage, in particular repeated joint bleeds are devastating for cartilage, synovium, and bone.[5-8] In hemophilia, replacing the missing clotting factor aims at limiting these joint bleeds, but cannot fully prevent it.[9] Once arthropathy is present, conservative management is indicated to alleviate symptoms,[10,11] and to postpone orthopedic interventions, that are often necessary in advanced stages.[12] Disease-controlling agents, such as those proven effective in rheumatoid arthritis, are lacking for hemophilic arthropathy.

The pathophysiology of blood-induced joint damage involves a combination of synovium-mediated processes and direct cartilage degeneration. During natural clearing of blood components from the synovial cavity, iron accumulates within the synovial tissue, inducing inflammation, proliferation, and angiogenesis.[13-15] This subsequently leads to cartilage degeneration via the production of pro-inflammatory cytokines and matrix-degrading enzymes. Besides this, synovial-independent direct harmful effects on cartilage result from erythrocyte-derived catalytic iron in combination with interleukin (IL)-1 induced enhancement of hydrogen peroxide production by chondrocytes. Hereby hydroxyl radicals are produced in the vicinity of the cells causing chondrocyte death.[16]

It has been demonstrated previously that IL-4 and IL-10 limit blood-induced cartilage damage *in vitro*[17,18] and *in vivo*.[19] Because IL-4 and IL-10 use different signalling pathways[20] they are able to exert different, potentially additive effects. It has been postulated that combined administration of these cytokines has synergistic effects due to complementary, yet partially overlapping functionalities.[21,22] However, despite their promising effects in preclinical studies, their clinical efficacy is limited. This is considered at least in part to be the result of limited bioavailability due to rapid clearance of these small cytokines.

Recently, we developed an IL4-10 fusion protein, retaining the biologic activity of both individual cytokines. This fusion protein has clear anti-inflammatory, immune regulatory, analgesic, and chondroprotective effects in human *in vitro* models and animal *in vivo* models.[23,24] In the present study we investigated whether the IL4-10 fusion protein protects against blood-induced joint damage in a human *in vitro* model of blood-induced cartilage damage, and joint damage upon hemarthrosis in a murine *in vivo* model of hemophilic arthropathy. Its efficacy was compared to the individual cytokines and their combination.

Materials and methods

Construction of the IL4-10 fusion protein

A fusion protein of human IL-4 and IL-10 was produced by transient transfection of HEK293 cells, co-transfected with alpha2,3-sialyltransferase 5 (SIAT9, homo sapiens) to optimize glycan-capping with sialic acid. This protein consists of human IL-4 at the N-terminus, a linker sequence, and human IL-10 at the C-terminus (Supplementary figure). IL4-10 fusion protein was purified by cation exchange chromatography, and stored in sterile phosphate buffered saline (PBS), pH 7.4, at -80°C (see for further details Eijkelkamp *et al*[23]). Murine IL4-10 fusion protein was made in a similar way as the human IL4-10 fusion protein, including co-transfection with alpha2,3-sialyltransferase 5 (Supplementary figure). Purification was performed via cation exchange chromatography with additional size exclusion chromatography on a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan). Fractions containing the fusion protein were pooled, sterile filtered, dialysed against PBS, and stored at -80°C.

Experimental design in vitro study

Healthy human articular cartilage (obtained post-mortem from the humeral head; n=8; mean age 69.8±8.7 years, three males and five females) was exposed to 50% volume/volume (v/v) whole blood from healthy human donors (n=8; mean age 28.0±5.0 years, two males and six females) for four days to mimic a human joint bleed.[25] After 12 days of recovery (day 16), proteoglycan turnover was evaluated by determining proteoglycan synthesis rate, proteoglycan content and the release of proteoglycans into culture medium.

In the first set of experiments (n=5), IL4-10 fusion protein was added during blood exposure in a concentration of 0.3 pg/mL to 100 ng/mL (two steps per log unit). To compare its efficacy with the individual components, in a second set of experiments addition of 10 ng/mL of the fusion protein was compared with addition of 10 ng/mL of IL-4 and/or IL-10 (both Sigma) during blood exposure (n=8).

To study the effect of the IL4-10 fusion protein and the individual cytokines IL-4 and IL-10 on the production of pro-inflammatory cytokines, separate whole blood cultures (50% v/v in culture medium) without cartilage were performed. Blood samples from six healthy donors (age 26.7±1.6 years, two males and four females) were cultured for four days. The fusion protein, IL-4, IL-10, or a combination of both cytokines (all 10 ng/mL) was added. After four days samples were collected, centrifuged at 1,500 *g* for 10 minutes, and supernatants stored at -80°C. The concentrations of IL-1β and IL-6 were determined with commercially available ELISAs (both Invitrogen) according to the manufacturer's instructions.

To test the hypothesis that the fusion protein acts on inhibiting the pro-inflammatory activity

of monocytes/macrophages, a separate set of experiments was performed. The effect of 10 ng/mL IL4-10 fusion protein on monocytes/macrophages stimulated by lysed red blood cells (RBCs) was compared to its effect on monocytes/macrophages only (n=6; age 31.3±9.2 years, four males and two females). After four days of culture, IL-1β and IL-6 concentrations in the supernatants were measured.

Experimental design murine in vivo study

Factor VIII (FVIII)-deficient mice (B6; 129S4-F8-tm1Kaz/J; 39 males and 41 females) at least three months of age were anesthetized with isofluran/O_2 and hair over both knee joints was removed. A joint bleed was induced in the right knee on day 0 and 14 with a 30 Gauge needle inserted through the infra-patellar ligament, as previously described.[6,26] Subsequently, 3 µl PBS containing 7 pmol murine IL4-10 fusion protein (n=25), recombinant murine IL-4 and IL-10 (both 7 pmol; R&D systems; n=25), or PBS only (n=30) was injected intra-articularly on day 0, 2, 14 and 16. Intra-articular injection was performed using a 33 Gauge needle, shown not to induce bleeding.[27] Sample size calculation using Cohen's effect size was based on demonstrating a difference in cartilage degeneration in the intervention groups compared to the PBS group. The left knee of each animal served as an unaffected control. Joint diameter was measured at days 0, 2, 14, 16 and 35 using a micrometer caliper. The visual bleeding score (VBS) was assessed daily according to Valentino *et al*.[28]

Animals were euthanized by cervical dislocation on day 35, the skin over the knee joints was removed and the greatest diameter across the joint from lateral to medial measured. Knee joints were isolated and joint damage was assessed histologically. Hematoxylin-eosin (H&E) stained sections were used to score synovial inflammation according to the Valentino score.[29] For cartilage damage, Safranin-O Fast-Green (Saf-O) stained sections were assessed according to the loss of Saf-O and scored on an adapted version of the Osteoarthritis Research Society International (modified OARSI) score specific for the mouse.[27,30,31]

For specific details, we refer to the methods provided in the supplementary section.

Statistical analysis

Data analysis was performed using SPSS 21.0 (IBM, Chicago, IL, USA) software. Since data of the *in vitro* experiments were not normally distributed and this could not be achieved for all parameters by log-transformation, the Wilcoxon-signed rank test for related samples was performed to determine statistical significance between treatments and controls of cartilage or blood from the same donor sample.

For the *in vivo* experiments, continuous data were analyzed using the paired samples *t*-test and the oneway ANOVA with Bonferroni correction. Ordinal data were analyzed using the Wilcoxon-signed rank test and Pearson Chi-square test. Longitudinal changes in joint diameter and VBS were tested using a general linear model for repeated measures.

Differences were considered to be statistically significant when $p<0.05$.

Results

IL4-10 fusion protein dose-dependently protects cartilage against blood-induced damage in vitro

Exposure of human cartilage tissue to 50% v/v whole blood for four days strongly impacted proteoglycan turnover when compared to control cultures (proteoglycan synthesis -74%, p=0.043; glycosaminoglycan (GAG) release +65%, p=0.080; GAG content -16%, p=0.043; Figure 1). Addition of the IL4-10 fusion protein resulted in a dose-dependent recovery of proteoglycan synthesis rate with a sigmoidal dose-response curve. The inhibition was statistically significantly different from a concentration of 0.1 ng/mL on when compared to blood exposure only (all p=0.043). Also GAG release was reversed by addition of the IL4-10 fusion protein, with a statistically significant improvement from 0.1 ng/mL on (all p=0.043, except for 1 ng/mL, p=0.080). Moreover, GAG content improved upon fusion protein addition, being statistically significant at concentrations of 0.3 ng/mL and higher (all p=0.043, except for 1 and 100 ng/mL, both p=0.080).

IL4-10 fusion protein similarly effective as the combination of IL-4 and IL-10 in vitro

To investigate whether fusion of the individual cytokines affected its chondroprotective properties, addition of the IL4-10 fusion protein, IL-4, IL-10, and a combination of both cytokines (each 10 ng/mL) was tested in the same assay. Blood exposure again severely impacted proteoglycan turnover (synthesis -76%, p=0.012; release +59%, p=0.017; and content -10%, p=0.012; Figure 2). Addition of IL-10 or IL-4 improved both the synthesis rate (p=0.017 and p=0.012 respectively) and GAG release (both p=0.012), but did not result in a significant improvement of GAG content. The protective effect of IL-4 was stronger than that of IL-10 (p=0.036 for synthesis; p=0.012 for release).

Adding a combination of IL-4 and IL-10 (10 ng/mL each) also strongly improved proteoglycan turnover compared to blood exposure only, as did addition of 10ng/mL fusion protein (all parameters $p<0.05$). There was no difference in the effects of the combination of IL-4 plus IL-10 versus those of the IL4-10 fusion protein (p=0.401 for synthesis; p=0.611 for release; and p=0.484 for content).

IL4-10 fusion protein inhibits pro-inflammatory cytokine production

In whole blood cultures, the effect of the IL4-10 fusion protein on IL-1β and IL-6 production, being important mediators in blood-induced cartilage tissue damage, was investigated. In control culture media (containing 10% human serum), IL-1β was not detectable, whereas a very low concentration of IL-6 (68 pg/mL) was measured. In 50% v/v blood cultures both cytokines were present (109 pg/mL IL-1β; 27 ng/mL IL-6, p=0.043 and p=0.028 compared to control; Figure 3A&B), and these levels were strongly reduced by addition of the IL4-10 fusion protein, the combination of IL-4 plus IL-10 or the cytokines separately (all $p<0.05$).

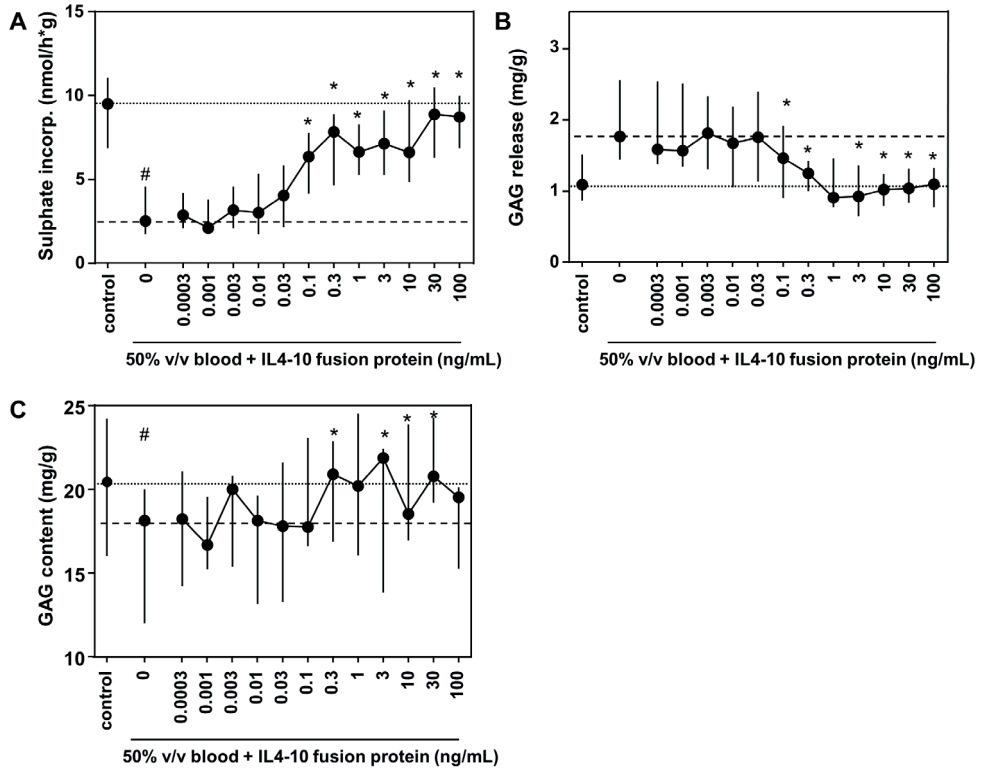


Figure 1 – Concentration dependent effect of the IL4-10 fusion protein
Healthy human cartilage (n=5) was cultured for four days in the absence (control; dotted line) or presence of 50% blood (dashed line). During blood-exposure, the IL4-10 fusion protein was added in a broad concentration range. Proteoglycan synthesis rate (A), -release (B) and -content (C) are depicted as median ± interquartile range (IQR). # *P<0.05* compared with control values, * *P<0.05* compared with blood-exposed cartilage without addition.

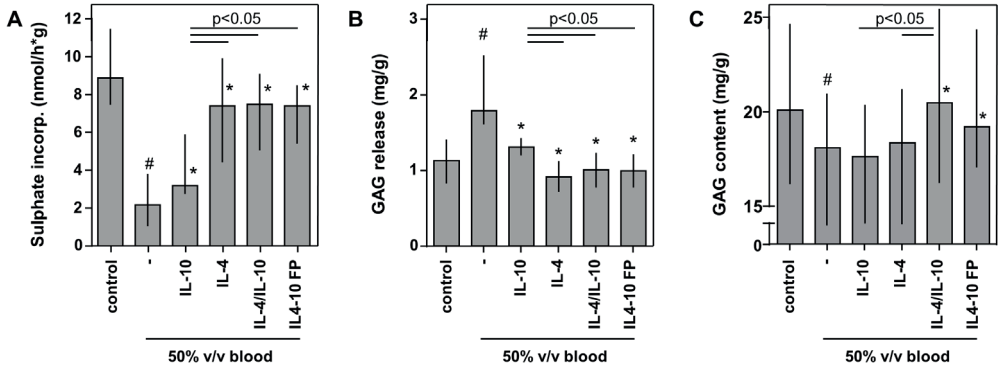


Figure 2 – Comparison of the protective effect of the IL4-10 fusion protein or the individual cytokines on blood-induced cartilage damage
Healthy human cartilage (n=8) was cultured for four days in the absence (control) or presence of 50% blood. During blood-exposure, IL-10, IL-4, the combination of both cytokines or the IL4-10 fusion protein (FP) was added (all 10 ng/mL). Proteoglycan synthesis rate (A), -release (B) and -content (C) are depicted as median ± IQR.

# *P<0.05* compared with control values, * *P<0.05* compared with blood-exposed cartilage without addition.

IL-10 alone was slightly less effective than the IL4-10 fusion protein, the combination of IL-4 and IL-10, or IL-4 alone in reducing IL-6 production (all p<0.05), but for IL-1β production no difference was found. The IL4-10 fusion protein was as effective as the combination of IL-4 plus IL-10 (p=0.180 for IL-1β and p=0.173 for IL-6).

To investigate the hypothesis that the IL4-10 fusion protein acted on CD14+ monocytes/macrophages, the effect on IL-1β and IL-6 production in four-day cultures of monocytes/macrophages alone or in combination with lysed RBCs was tested. Monocytes/macrophages stimulated with lysed RBCs produced significantly more IL-1β and IL-6 (323 pg/mL IL-1β and 3492 pg/mL IL-6) compared to medium only (11 pg/mL and 19 pg/mL respectively; both p=0.028), whereas the concentration of IL-1β and IL-6 in the supernatant of monocytes/macrophages only did not differ from medium (monocytes/macrophages produced 12 pg/mL IL-1β and 818 pg/mL IL-6, p=0.917 and p=0.116 compared to medium; Figure 3C&D).

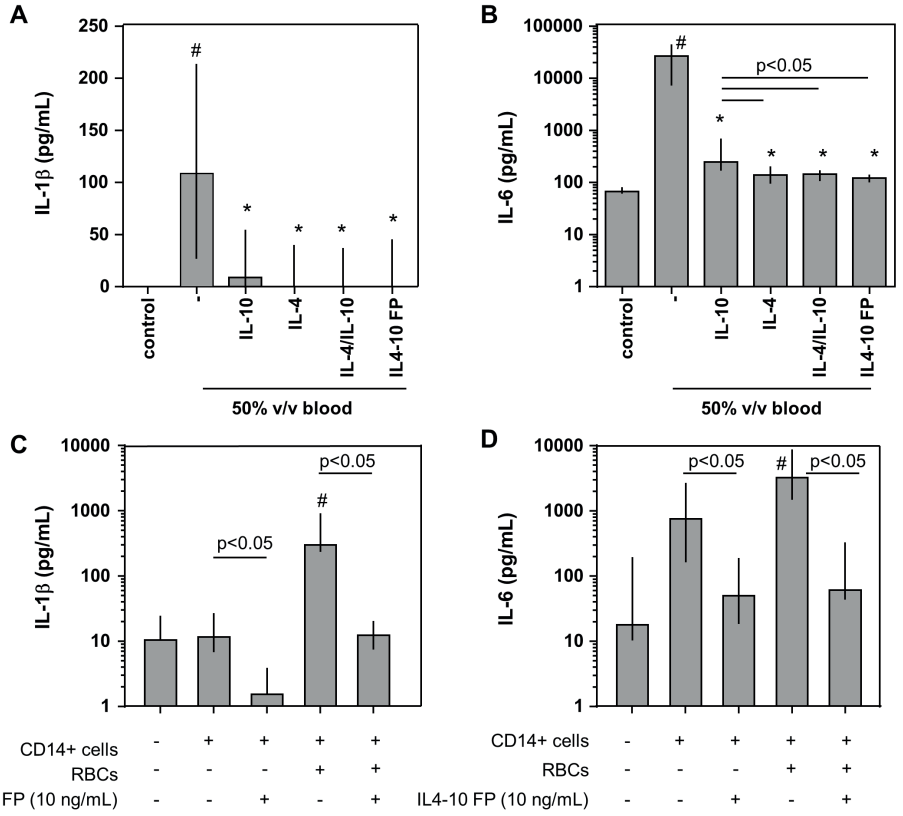


Figure 3 – Effect of IL-10, IL-4, the combination and the IL4-10 fusion protein on cytokine production

To four-day whole blood cultures (A & B; n=6) IL-10, IL-4, the combination of both cytokines or the IL4-10 fusion protein (FP) was added (all 10 ng/mL) and cytokine production was measured in the supernatants. In a separate set of experiments, CD14+ monocytes/macrophages were cultured for four days (C&D; n=6) in the absence or presence of lysed red blood cells (RBCs), 10 ng/mL of the IL4-10 FP was added and cytokine production was measured in the supernatants. Median ± IQR are depicted.

# *P<0.05* compared with control values, * *P<0.05* compared with blood 50%.

Addition of the IL4-10 fusion protein reduced this IL-1β and IL-6 production by monocytes/macrophages both when cultured in the absence or presence of lysed RBCs (IL-1β in absence of lysed RBCs 1 pg/mL, in the presence of lysed RBCs 13 pg/mL; IL-6 in absence of lysed RBCs 53 pg/mL, in the presence of lysed RBCs 65 pg/mL; all p<0.05 when compared to the same condition without fusion protein).

Effects of the IL4-10 fusion protein on joint bleeding in vivo

In hemophilic mice, a joint bleed was induced in the right knee joint twice with a two-week interval, resulting in an overall animal survival of 55% (n=17 in the PBS group, n=11 in the mouse IL-4 plus IL-10, and n=16 in the mouse IL4-10 fusion protein treated group; survival after bleeding did not significantly differ between treatment groups; p=0.355). After the punctures, the VBS and joint diameter increased equally in all treatment groups, gradually decreasing over time (Figure 4).

In all groups, the diameter of the experimental paw at the end of the study was significantly increased compared with the control paw (Table). The diameter of neither the control joint, intervention joint, nor the delta difference (experimental minus control joint) differed between treatment groups (p=0.111; p=0.617 and p=0.314, respectively).

Table – Joint diameter at day 35

	PBS	**IL-4 & IL-10**	**IL4-10 fusion protein**	***P*-value[a]**
Control	2.99 ± 0.18	3.01 ± 0.20	2.89 ± 0.09	0.111
Experimental	3.32 ± 0.30	3.44 ± 0.25	3.39 ± 0.39	0.617
***P*-value[b]**	**0.000**	**0.001**	**0.000**	

Joint diameter (mm) was measured after removal of the skin and defined as the greatest diameter across the joint from lateral to medial, expressed as mean ± SD.

[a] One-way repeated ANOVA for comparison between treatment groups.

[b] Paired *t*-test for comparison of the control with the experimental joints.

IL, interleukin; PBS, phosphate buffered saline

Synovial inflammation is not affected by intra-articular IL4-10 fusion protein in a murine joint bleeding model

The left untreated knee showed similar synovial changes in each treatment group (Figure 5C; p=0.395). Upon two sequential joint bleeds, a statistically significant increase in Valentino score was noted (p=0.000 compared to control in the PBS group), and intra-articular treatment with IL-4 plus IL-10 or the IL4-10 fusion protein did not attenuate this (still p=0.003 and p=0.001 compared to control in IL-4 plus IL-10 and IL4-10 fusion protein treated groups, respectively). There was no difference between the treatment groups in total Valentino score (p=0.339), nor in any of the subscales (Supplementary table).

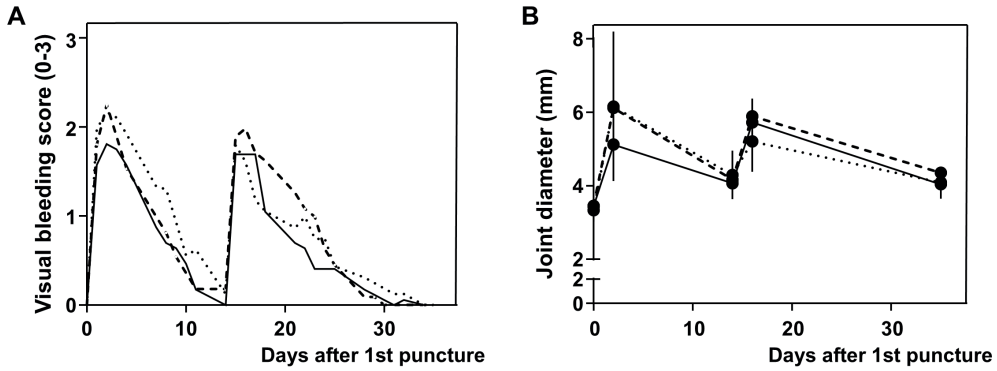


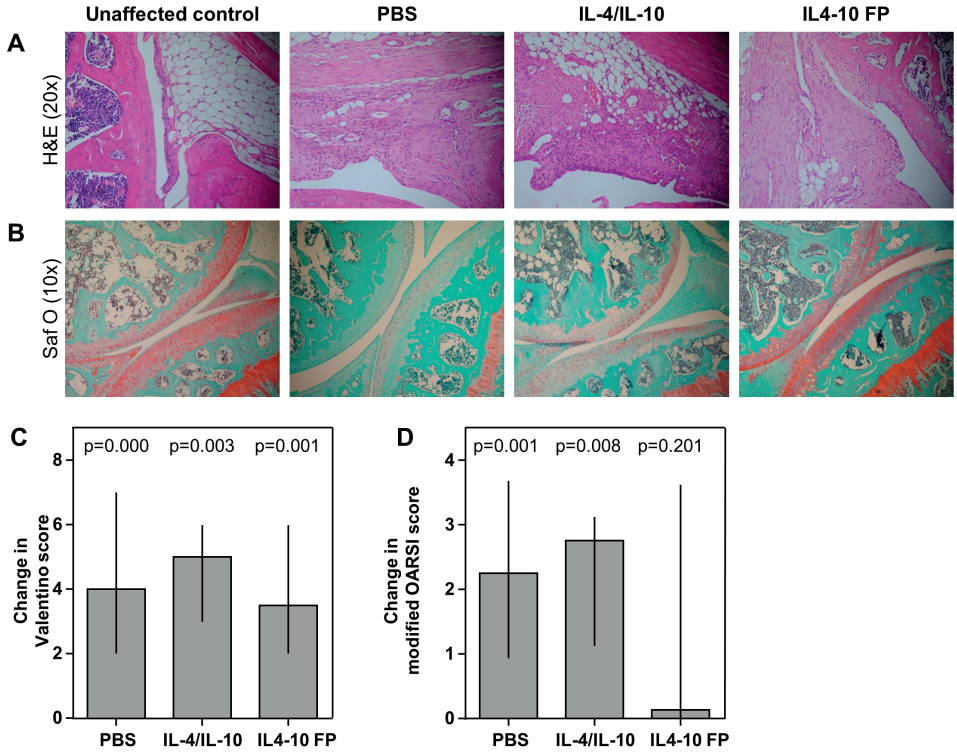
Figure 4 – Visual bleeding score and joint diameter unaffected by intra-articular treatment
Change in visual bleeding score (VBS; A) and joint diameter (B) in the intervention joint over time. (A) Visual bleeding score was scored daily by two observers blinded to the experimental condition. Data are shown as mean. (B) Joint diameter was measured with a caliper on day 0, 2, 14, 16 and 35. Data are shown as mean ± SD.
Solid line – PBS; dotted line – IL-4/IL-10; dashed line – IL4-10 fusion protein. There are no statistical differences between the three treatment groups.

Intra-articular IL4-10 fusion protein treatment attenuates blood-induced cartilage damage in vivo

Loss of Saf-O staining, representing cartilage damage, was seen in the experimental joints, significantly different compared to the contralateral joints in the PBS and IL-4 plus IL-10 groups (Figure 5D; p=0.001 and p=0.008 respectively). In contrast, there was no significant increase in the modified OARSI score in mice treated with IL4-10 fusion protein compared to the contralateral joint (p=0.201), although still an increase was noted. Comparing the modified OARSI score between the different treatment groups showed no statistically significant difference (p=0.401 for comparison of the contralateral control joints; p=0.401 for experimental joints; p=0.175 for delta). The amelioration of cartilage damage by the IL4-10 fusion protein was equally present in the femoral condyle and tibial plateau (Supplementary table).

Discussion

This study demonstrates the chondroprotective potential of an IL4-10 fusion protein in blood-induced joint damage. In a human *in vitro* model the fusion protein prevents blood-induced cartilage degeneration in a dose-dependent manner up to complete normalization. This is at least in part the result from suppressing IL-1β and IL-6 production by monocytes/macrophages, especially when activated by hemoglobin-derived iron. Addition of the (combination of the) individual cytokines showed a similar effect on cartilage damage. This cartilage-protective effect was confirmed in an *in vivo* murine joint bleeding model. Intra-articular treatment with the fusion protein in contrast to the individual components resulted



in attenuation of cartilage damage upon two successive joint bleeds. *In vivo*, synovial inflammation was not affected by injection of the individual components nor the combination.

Figure 5 – Intra-articular treatment with IL4-10 fusion protein (FP) attenuates cartilage damage but not synovial inflammation

Histological joint damage was scored at day 35 after the first joint bleeding by two observers blinded for the intervention. Representative micrographs of H&E staining used to score synovial inflammation (A) and Saf O staining for cartilage damage (B). (C) Synovial inflammation was evaluated by the Valentino score. (D) Cartilage damage was assessed by a modified version of the OARSI score. The difference between the experimental joint and contralateral control joint are depicted as median and IQR. P-values for these differences are given at the top of the graph. There are no statistical differences between treatment-groups.

IL-4 and IL-10 are regulatory cytokines demonstrating impressive results in *in vitro* and *in vivo* models of (auto-)inflammatory driven joint diseases.[22,32,33] Nevertheless, in clinical trials the promising effects of these cytokines were not seen,[34-36] possibly, at least in part, due to suboptimal bioavailability of these small molecules. IL-4 and IL-10 have a molecular weight of 18-20 kDa, below the critical size for filtration in the kidney (50-60 kDa depending on charge). Half-life of human IL-10 is about 1.9 hours after intravenous injection, and 2.7-4.5 hours after subcutaneous administration in healthy volunteers.[37-39] With a half-life of only 19 minutes after intravenous injection, IL-4 is even more rapidly cleared, as it does not form dimers whereas IL-10 does.[40] Due to its higher molecular weight of ~70 kDa,[23] the IL4-10 fusion protein is predicted to have a longer half-life than IL-4 or IL-10. This was

indeed observed in preliminary rat pharmacokinetic studies (C. Steen-Louws, unpublished observation). Moreover, the fusion protein provides an option for combined administration of both activities combined in a single molecule.[41]

The versatile properties of IL-4 and IL-10 are also demonstrated for the IL4-10 fusion protein. It reduces pro-inflammatory cytokine production in whole blood assays,[42] in cultures of human osteoarthritic cartilage, and synovial tissue.[24] Cartilage matrix turnover of osteoarthritic explants is improved by the fusion protein, and the cartilage destructive properties of osteoarthritic synovial tissue are reduced. Furthermore, *in vivo* analgesic properties are demonstrated in mouse models of chronic inflammatory pain[23] and in a large animal model of osteoarthritis.[43]

Upon joint bleeding, IL-4 and IL-10 can limit cartilage damage. They reduce inflammation via inhibition of the production of IL-1β and TNFα by inflamed synovial tissue,[44] and by synovial fluid monocytes/macrophages.[17] Hence, less chondrocyte activation and subsequent hydrogen peroxide production will occur, limiting chondrocyte apoptosis.[16,45] Additionally, a direct chondroprotective effect is suggested as blood exposure induces upregulation of the IL-4 and IL-10 receptors on chondrocytes.[17] *In vivo*, a single injection of the individual cytokines diminishes cartilage degeneration upon a joint bleed in hemophilic mice.[19]

The current study for the first time investigates the efficacy of a novel IL4-10 fusion protein on blood-induced cartilage damage. *In vitro*, the chondroprotective activity of IL4-10 fusion protein was comparable to the combination of the separate cytokines IL-4 and IL-10. A potential synergistic effect of IL-4 and IL-10 could not be demonstrated, as there was a ceiling effect; IL-4 at the concentration used already exerted maximal effects on proteoglycan turnover parameters and suppression of pro-inflammatory cytokine production. The concentrations used in the blood(-cartilage) cultures were based on previous studies demonstrating an optimal effect at 10 ng/mL.[17,18] Whole blood cultures were repeated with 10- and 100-fold lower concentrations, also showing full blockade of pro-inflammatory cytokine production by the fusion protein (data not shown). It cannot be ruled out that further lowering the concentrations of IL-4 and IL-10 may display synergistic effects in this system, as has been described previously in arthritis models.[21]

The *in vivo* potential of the fusion protein was investigated in a well-established hemophilia mouse model.[19,46,47] A previous study by our group investigating the *in vivo* efficacy of IL-4 and IL-10 showed only mild cartilage degeneration, compared to clear synovitis upon a single joint bleed.[19] Therefore, in the current study two bleeds were induced to better represent the human situation in which recurrent joint bleeds over time lead to arthropathy.[48] The drawback of a repeated injury model without clotting factor administration is an increased mortality as demonstrated by a survival of 35% after three injuries in a study by Narkbunnam *et al*.[46] In our study survival after two injuries was 55% without a difference between treatment groups. Treatment was administered intra-articularly twice after each bleed to ascertain adequate availability of the active compounds. Intra-articular delivery of active compounds like factor IX and microRNA is shown feasible in hemophilic mice,[49,50]

and this could be an interesting concept for delivery of our fusion protein, especially when formulated in a slow-release system.

The fusion protein hardly affected synovial inflammation at day 35 measured with the Valentino score. To confirm absence of efficacy on synovitis, also the Krenn score (used in rheumatoid arthritis[51]) and Goldenberg & Cohen score (degenerative joint diseases[52] were assessed. None showed an improvement upon treatment with the fusion protein or the individual cytokines (data not shown). A well-known side effect of IL-4 is an increase in inflammation as it is a chemo-attractant for macrophages and fibroblasts.[33] High levels induce accumulation of tissue macrophages and erythrophagocytosis in mice.[53] This pro-inflammatory potential is also suggested in K/BxN mice, where IL-4 appeared crucial for full development of arthritis.[54] In combination with IL-10 a better balance between the Th1 and Th2 response can be achieved[32] and therefore both cytokines were combined in the fusion protein. However, in our study the fusion protein nor the combination of IL-4 and IL-10 affected synovial inflammation at day 35 after the first joint bleed. This is in line with previous studies demonstrating that blocking inflammatory cytokines alone is insufficient to avoid blood-induced synovitis in hemophilia mice.[46] Removing blood-components from the synovial cavity obviously induces a vigorous trigger for synovial inflammation. No previous study investigating treatment upon joint bleeding in hemophilia mice, although limiting cartilage damage, could influence synovitis,[19,27,31] unless combined with clotting factor concentrate.[46,55] This is potentially due to an ineffective treatment, although it might also be the result of the vigorously increased synovial response to bleeding in mice not receiving clotting factor concentrate.

In line with the *in vitro* results, intra-articular injection of the IL4-10 fusion protein ameliorated cartilage damage *in vivo*. Such a beneficial effect was not observed with the individual cytokines, in contrast to our previous *in vivo* findings.[19] This can presumably be attributed to the increased severity of the model of repeated, rather than a single, joint bleed (average change in modified OARSI score in the vehicle group in the previous study was <1[19] whereas in the current study this was >2). Nevertheless, the IL4-10 fusion protein still alleviated cartilage damage in this repeated joint bleed model. It is likely that the better bioavailability due to its higher molecular weight, contributes to the increased efficacy of the fusion protein versus the combination of the separate cytokines.

Exploring the therapeutic potential of the IL4-10 fusion protein as disease-modifying therapy in blood-induced joint damage seems appealing, especially in combination with clotting factor replacement therapy. Besides the chondroprotective properties and suppression of pro-inflammatory cytokine production, as demonstrated in the current study, also analgesic properties are ascribed to the fusion protein.[23,24] Joint pain has a significant impact on quality of life in hemophilia patients.[56] Whether this potential analgesic effect influenced mobility of the mice, needs further investigation. It is demonstrated that loading the joint after a bleed aggravates cartilage damage,[57] which might negatively affect the chondroprotective efficacy of the fusion protein, causing an underestimation of its effect. It would have been interesting to study pain and movements in the present study as well. However, no data are

available on pain in this model, and at the start of this study the analgesic properties of the IL4-10 fusion protein were not yet known.

In conclusion, this study demonstrates for the first time that the IL4-10 fusion protein prevents blood-induced cartilage damage in a human cartilage tissue *in vitro* model and ameliorates cartilage degeneration upon repeated joint bleeds in hemophilic mice when injected intra-articularly. These data justify further investigation of the potency of the IL4-10 fusion protein in the treatment of blood-induced arthropathy.

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Supplementary methods

Cartilage culture

Healthy human articular cartilage tissue was obtained within 24 hours post mortem from the humeral head of donors with no known history of joint disorder. Collection of cartilage tissue was performed according to the medical ethical regulations of the University Medical Center Utrecht. Full thickness cartilage slices were cut aseptically, excluding the underlying bone, and kept in PBS, pH 7.4. Within one hour after dissection, slices were cut in cubic explants and weighed aseptically (range 5-15 mg; accuracy ±0.1 mg). Explants were cultured individually in a 96-wells round-bottomed microtiter plate at 5% CO_2 in air, 37°C, and 95% humidity. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with glutamine (2 mM), penicillin (100 IU/mL), streptomycin sulphate (100 µg/mL; all Paisley, UK), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled human male AB serum (Invitrogen).

For each experiment, fresh blood was drawn from healthy human donors into Vacutainer tubes (Becton Dickinson). Cartilage was exposed to 50% v/v whole blood for four days to mimic a human joint bleed,[1] and washed by two 45-minute incubations in culture medium to remove blood components. Subsequently, explants were cultured for 12 days in medium only, which was refreshed every four days.

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 8-10 cartilage explants per parameter per donor, obtained randomly and handled individually, was taken as a representative value.

Proteoglycan turnover was evaluated at the end of each experiment. To assess proteoglycan synthesis, sulphate incorporation rate into glycosaminoglycans (GAGs) was determined by addition of $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont; 74 kBq per well) at the end of the experiment. After four hours of pulse labelling, cartilage explants were washed twice in cold PBS, digested for two hours at 65°C with 2% papain (Sigma), and stored at -20°C. Proteoglycan synthesis rate was determined by precipitation of GAGs with 0.3M hexadecylpyridinium chloride monohydrate (CPC; Sigma) in 0.2M NaCl. The precipitated GAGs were dissolved in 3M NaCl and the amount of radioactivity measured by liquid scintillation counting. Radioactivity was 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).

The proteoglycan content of each explant and the release of proteoglycans into culture medium were established by staining and precipitation of GAGs with Alcian Blue (Sigma) in the papain digest or in the culture medium, respectively. Quantification of the staining was performed by absorptiometry at 620 nm using chondroitin sulphate (Sigma) as reference.

Results are expressed as milligrams of GAGs normalized to the wet weight of cartilage tissue (mg/g wet weight).

Isolation of blood cells

Mononuclear cells were isolated from lithium-heparinized peripheral blood by density centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) according to standard procedures. CD14+ monocytes/macrophages were isolated by positive selection using autoMACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In short, mononuclear cells were incubated with saturating concentrations of CD14 microbeads at 4°C for 15 minutes, washed, and suspended in PBS with 2 mM EDTA and 0.5% BSA at a final concentration of $0.4x10^4$/µl. Red blood cells (RBCs) from the same donor were isolated by filtration (purity >99%; PALL-filters, Belgium). RBCs were lysed by freezing (liquid nitrogen) and thawing (room temperature) a volume of isolated RBCs twice. A volume of 100 µl of monocytes/macrophages was co-cultured for four days with 400 µl lysed RBCs and 500 µl culture medium.

Mice and animal care

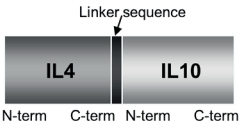
Factor VIII (FVIII)-deficient mice (B6; 129S4-F8-tm1Kaz/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house at the animal facilities of the University Utrecht. FVIII-deficient mice have a FVIII activity of <1% but lack the spontaneous bleeding phenotype as seen in the human hemophilia situation. To reduce the likelihood of combat-related bleedings, mice were housed in filter-top cages in low density (maximum of five mice per cage). They were fed a standard diet and water *ad libitum*. All animals received care in compliance with the European Convention on Animal Care and experiments were reviewed and approved by the institutional animal ethical committee.

Histological joint damage

Knee joints were isolated and fixed for at least 48 hours in 4% formaldehyde containing 2% sucrose. Subsequently, the joints were decalcified, embedded in paraffin, and cut in coronal tissue sections (5 µm). All assessments were performed by two observers blinded to the experimental conditions. Synovial inflammation was assessed on Hematoxylin-eosin (HE) stained sections according to the Valentino score.[2] The grading system is based on synovial hyperplasia (0-3), vascularity (0-3), presence of hemosiderin (0 or 1), presence of erythrocytes (0 or 1), synovial villus formation (0 or 1), and cartilage erosions (0 or 1). These scores were summed and the average of the two observers used for statistical analyses. For cartilage damage, Safranin-O Fast-Green (Saf-O) stained sections were assessed according to the loss of Saf-O and scored on an adapted version of the Osteoarthritis Research Society International (modified OARSI) score specific for the mouse[3] as described previously.[4,5] The femoral condyle and tibial plateau were scored individually, the final score for each joint was the average of both scores.

Supplementary figure

A



B

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Supplementary figure - Schematic overview of the IL4-10 fusion protein and its amino acid sequence.
The IL4-10 fusion protein consists of IL-4 at the N-terminal position and IL-10 at the C-terminal position connected via a linker sequence (A). Amino acid sequence of the human IL4-10 fusion protein (B) and murine construct (C). The linker sequence is indicated in italic and underlined, and potential N-linked glycosylation sites are indicated in bold.

Supplementary table

Supplementary table – Histological joint damage

	PBS		IL-4 & IL-10		IL4-10 FP	
	Con	Int	Con	Int	Con	Int
Valentino score						
- Hyperplasia	0.0 (0.0-1.0)	3.0 (1.5-3.0)	0.0 (0.0-1.0)	3.0 (2.5-3.0)	0.0 (0.0-0.75)	2.3 (2.0-3.0)
- Vascularity	1.0 (1.0-1.0)	2.0 (1.0-2.0)	1.0 (1.0-1.0)	2.0 (1.0-3.0)	1.0 (1.0-1.0)	2.0 (1.0-2.0)
- Hemosiderin	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
- Erythrocytes	0.0 (0.0-0.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	0.5 (0.0-1.0)	1.0 (1.0-1.0)
- Villus formation	0.0 (0.0-0.0)	1.0 (1.0-1.0)	0.0 (0.0-1.0)	1.0 (1.0-1.0)	0.0 (0.0-0.0)	1.0 (0.0-1.0)
- Cartilage erosion	0.0 (0.0-0.5)	1.0 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.0-1.0)
- Total	2.0 (1.0-3.0)	7.0 (5.0-8.0)	2.0 (2.0-5.0)	5.0 (3.0-6.0)	2.0 (1.0-3.8)	7.0 (4.1-8.0)
OARSI score						
- Femoral condyle	1.5 (0.0-2.0)	3.0 (0.5-5.0)	0.5 (0.4-1.3)	3.0 (1.3-3.8)	1.5 (0.5-3.5)	1.3 (0.4-4.0)
- Tibial plateau	0.5 (0.5-2.0)	3.0 (2.0-4.0)	0.5 (0.5-1.4)	2.0 (0.5-3.5)	0.5 (0.5-2.5)	2.0 (0.5-4.3)
- Total	1.3 (0.3-2.3)	2.4 (2.0-4.5)	0.6 (0.4-1.3)	3.0 (1.3-3.4)	1.3 (0.5-2.4)	1.3 (0.5-4.1)

Data are expressed as median (IQR)
Con, control; FP, fusion protein; IL, interleukin; int, intervention; OARSI, Osteoarthritis Research Society International; PBS, phosphate buffered saline

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CHAPTER 7

JOINT DISTRACTION, A PROMISING TREATMENT FOR SEVERE HEMOPHILIC ANKLE ARTHROPATHY;

one year follow-up data from a proof of concept open prospective study

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Abstract

Background: In hemophilia nowadays most intra-articular bleedings occur in the ankle, necessitating joint fusion in young patients. Joint distraction, an effective treatment in ankle osteoarthritis, has the advantage of preserving the original joint without compromising subsequent conservative surgeries like arthrodesis. In three cases evaluated in retrospect, good clinical efficacy and tissue structure repair was demonstrated in hemophilic ankle arthropathy.

Methods: In this prospective study data were gathered on clinical efficacy and tissue structure changes of ankle joint distraction in hemophilia patients <55 years with severe arthropathy.

Results: The study is still ongoing, one year follow-up is available for six patients (five patients with severe hemophilia, one patient with moderate hemophilia; age range 21-45 years). During treatment there were no adverse bleeding events, pin tract infection occurred in five patients effectively treated with a short course of antibiotics. Pain decreased significantly at one year follow-up (73 mm (60-80) at baseline versus 47 mm (12-58); p=0.046). Functional limitations improved (Hemophilia Activities List at baseline 53% (41-62) versus 64% (48-79%) at one year, p=0.046; Ankle Osteoarthritis Scale for disability 57 (52-73) versus 29 (15-53), p=0.028). Results of different performance tests improved considerably at one year follow-up. MRI revealed a clear trend in improvement in osteochondral changes (MRI IPSG score p=0.059), whereas soft tissue changes remained unchanged (p=1.000).

Conclusion: This first prospective study investigating the efficacy of joint distraction in hemophilic ankle arthropathy, shows clear symptomatic and functional improvement at one year follow-up as well as tissue repair, as seen in osteoarthritis patients. Although preliminary, these data indicate that joint distraction may be a promising treatment postponing more rigorous surgery like ankle arthrodesis in those patients not benefitting from conservative therapy.

Introduction

Musculoskeletal bleeding is the hallmark of hemophilia, with up to 85% of the bleeding episodes occurring in the joints.[1] Prophylactic clotting factor replacement therapy diminishes the number of bleeding episodes, but complete prevention is not possible. Patients with severe hemophilia on intermediate dose prophylaxis still experience 2-3 joint bleeds per year.[2] Most affected are the large synovial joints, especially the ankles, knees, and elbows. Whereas older patients mostly suffer from arthropathy in more than one joint, an increasing proportion of younger hemophilia patients on prophylactic therapy suffer from arthropathy in a single joint, the ankle being most frequently affected nowadays.[1] Arthropathy leads to pain, functional limitations, severely affects quality of life,[3] and often requires surgical intervention when conservative treatment fails.[4]

Currently there are two surgical treatment options for ankle arthropathy: fusion of the joint (arthrodesis) and total ankle replacement (TAR). Arthrodesis is the most performed procedure, it is very effective in pain relieve and stopping recurrent joint bleeding.[5,6] However, a major drawback is the loss of joint mobility and therewith the possibility of overloading other adjacent joints of the lower limb and foot.[5,7] Surveillance data confirm that self-reported activity increases in only 11.8% after ankle arthrodesis whereas 8.8% even report a decrease in activities.[8] For young patients, this has a great impact on their activities and participation in society. TAR has the advantage of preservation of joint motion, it has been performed in hemophilia patients with promising results, although information on long-term follow-up is scarce.[9] Concern of aseptic loosening and deep infections due to poor bone quality and increased risk of micro bleedings at the prosthesis - bone interface causes controversy about this technique in hemophilia.[9,10] Results of revision surgery are in general poor.[11] Moreover, performing ankle arthrodesis subsequent to TAR is a technical challenge.

In osteoarthritis (OA), ankle joint distraction appeared a good alternative technique to postpone arthrodesis for many years.[12] Joint distraction is a surgical procedure in which the two bony ends of a joint are gradually separated to a certain extend for a certain period of time. By fixation of the lower tibia and talus to an external frame, the damaged cartilage and peri-articular bone are mechanically unloaded allowing initiation of joint tissue repair. Both clinical benefit and structural tissue changes are observed in OA and maintained over many years.[12-14]

This treatment might be effective for hemophilic ankle arthropathy as well. It has the main advantage of preservation of the original joint without compromising subsequent conservative surgeries like arthrodesis, if still needed. A retrospective evaluation of three patients with hemophilic ankle arthropathy treated with joint distraction showed clear clinical improvement and impressive structural changes.[15]

No prospective data are available on the clinical and structural efficacy of joint distraction in hemophilia. Therefore, the aim of this single arm, interventional pilot study was to gather prospective data on the efficacy of ankle joint distraction in hemophilia. Of this study, which is still ongoing, the one year follow-up data of the first six patients are presented.

Patients and methods

Patients

Six hemophilia patients suffering from severe ankle arthropathy were included between 2013 and 2016. Inclusion criteria were: hemophilia A or B, age ≥18 years and <55 years, severe complaints of ankle arthropathy in primarily the talocrural ("upper ankle") joint, insufficiently responding conservative treatment, as analgesics and physiotherapy, and causing functional limitations. Exclusion criteria were contra-indications for surgery or magnetic resonance imaging (MRI), complaints due to arthropathy in the subtalar ("lower ankle") joint primarily, psychological inabilities making it impossible to wear a distraction frame for 10 weeks or difficulty to instruct, absence of any joint space on X-ray, ipsilateral joint prosthesis, and a history of inflammatory or rheumatoid arthritis in the affected ankle. The study was undertaken in accordance with the Declaration of Helsinki, and was approved by the Medical Ethical Review Board of the University Medical Center Utrecht (no 13/193). Written informed consent was obtained from all study participants.

Ankle joint distraction

Ankle joint distraction was performed according to previous description.[12] Under general anesthesia with pre- and post-surgery administration of antibiotics (24 hours cefazoline) and clotting factor infusion (continuous infusion for 7 days) an Ilizarov external frame was inserted (Figure 1). Two Kirschner wires were drilled proximally and distally through the tibia at different angles and fixed under tension to an external ring. Four threaded rods connected these external rings, providing a firm fixation of the tibia. Two wires were drilled through the metatarsal bones and tensioned to a half-ring over the forefoot. The half-ring was connected to plates at the medial and lateral sides of the foot and extended from the half-ring around the heel. In case the subtalar joint was not affected, a Kirschner wire was drilled through the talus and fixed to the foot frame without tension, to prevent subtalar distraction. The rings around the foot and the tibia were connected by four lengthening rods bridging the upper ankle joint. The first 2 mm of distraction of the joint was performed during surgery and thereafter twice a day for 0.5 mm until a total distraction of 5 mm was achieved. At day five this was checked on a weight bearing X-ray and distraction adapted when needed. At day seven, after instructions about pin-site care and daily exercise, patients were discharged. For one week, daily clotting factor infusion was continued, thereafter resuming the regular prophylactic protocol.

In one patient, joint distraction was combined with excision of a tibial osteophyte.

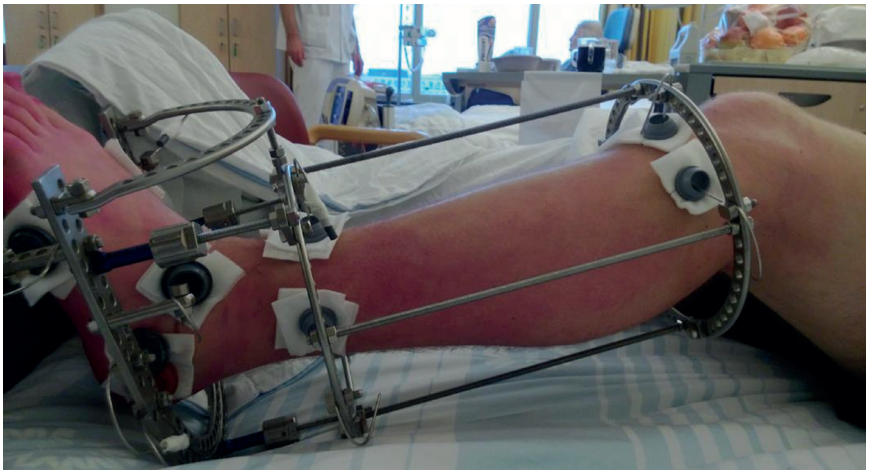

Figure 1 – External frame for ankle joint distraction

At 10 weeks, the distraction frame was removed under general anesthesia at day care surgery, after a bolus injection of clotting factor. Intensified clotting factor substitution was continued for three days. Normal full loading was gradually regained over weeks, guided by a specialized physiotherapist.

Clinical evaluation

Clinical efficacy was assessed by the use of different questionnaires: the Ankle Osteoarthritis Scale (AOS), Visual Analogue Scale (VAS) for general pain, Hemophilia Activities List (HAL), and Goal Attainment Scaling (GAS). The AOS is a validated questionnaire for ankle OA[16,17] also used to assess the effectiveness of orthopedic interventions in hemophilia,[9] assessing the level of pain and disability on a 100 mm line for nine conditions each. The HAL is a hemophilia specific, but not ankle specific questionnaire, assessing self-perceived functional ability in 42 items in seven domains.[18] Three component scores were calculated: upper extremity activities, basic lower extremity activities, and complex lower extremity activities. Additionally, an overall summarized score was calculated. Scores were normalized, where 0% represents the worst possible functional status. The GAS was included to assess clinical improvement of a specific activity indicated by the patient as an important activity limited at baseline by the ankle complaints. To measure this objectively and to enable scoring on group level, a GAS goal was established using a 6-point scale, with “0” reflecting a score indicating the patient met his or her goal as expected; scores of “+1” or “+2” indicated surpassing the desired goal, “-1” indicated inadequate goal attainment, “-2” reflects no change from baseline, and “-3” a decline compared to pretreatment. Each potential score is based on a criterion that is established collaboratively with the patient, is measurable and specific.

Performance tests

Pre- and postoperative assessment of active range of motion (ROM) of both ankle joints was

assessed by a specialized physiotherapist.
Preoperatively and at one year follow-up, three performance tests were executed: the 50 meter (m) walking test (50 MWT), the figure-8 walking test (preferred and maximum speed), and the 6 minute walking test. The 50 MWT: the patient walks in a straight line towards a designated point 50 m away from the starting point at a preferred speed. The figure-8 walking test: the patient stands besides one of two cones which were placed 8 m apart, facing towards the other cone, and starts walking at his usual pace in a figure of eight walking path around the cones, until returning to the starting position. Thereafter the test was repeated at maximum walking speed. For all tests, the outcome is the amount of time (in seconds) needed to perform the specific task. The 6 minute walking test was performed on a 35 m track in a gym. Patients were instructed to walk as much distance as possible in 6 minutes at a self-chosen speed without running or jogging. The outcome is the total distance covered in 6 minutes.

Structural changes by imaging: X-ray and MRI
Weight-bearing ankle radiographs and MRIs were made preoperatively in standard care and at one year after surgery. X-rays were scored according to the Pettersson method[19] with a maximum score of 13. Changes on MRI were quantified using the additive MRI scale for hemophilic arthropathy of the International Prophylaxis Study Group (IPSG) with a maximum score of 17.[20] All assessments were performed by a radiology resident (W.F.) with experience using the Pettersson score and MRI IPSG score.

Statistical analysis
Because of the small sample size and discrete data from the questionnaires and radiological scores, non-parametric statistics were used for analyses. Baseline data were compared to follow-up data using non-parametric tests for related samples (Wilcoxon signed rank test). Values are expressed as medians with interquartile range (IQR).
Data storage, management and statistical calculations were performed with SPSS version 21 (IBM, SPSS, Armonk, NY, USA). Graphic presentation was performed with GraphPad Prism version 7.02 (GraphPad Software, San Diego, CA, USA). *P*-values less than 0.05 (two-sided) were considered statistically significant.

Results

Patient characteristics
At present a 12 month follow-up is available for six patients (Table 1). Four patients suffer from severe hemophilia A, one from severe hemophilia B, and one from moderate hemophilia A. Four patients were on prophylactic clotting factor replacement, ankle arthropathy resulted from repeated hemarthroses in all patients. Age at time of surgery ranged from 21 to 45 years (median 29 years).

Table 1 – Baseline characteristics

	Hemo-philia	Clotting factor activity	Treatment	Affected ankle	Age at time of surgery (years)	Duration of distraction (weeks)
Case 1	B	<1%	On demand	Left	21	10
Case 2	A	<1%	Prophylaxis	Right	25	10
Case 3	A	<1%	Prophylaxis	Right	32	10
Case 4	A	<1%	Prophylaxis	Left	33	10
Case 5	A	<1%	Prophylaxis	Left	21	8
Case 6	A	2%	On demand	Left	45	10

Adverse events

There were no bleeding complications nor thrombo-embolic events during or after treatment. In one patient, the first 2 mm distraction could not be applied during surgery due to pain, therefore the complete distraction distance was achieved gradually over five days. Five patients experienced superficial skin pin site infection, in all cases treated successfully with a short course of antibiotics. One patient was readmitted at day eight after application of the frame, because he needed additional pain relief after a fall. One patient suffered from synovitis of his treated ankle at one year follow-up (unclear whether related to the treatment), requiring intensive clotting factor replacement and pain medication.

Symptomatic outcome

VAS pain improved already after six months in three patients, in one patient six months data were missing (Figure 2A). At one year follow-up VAS pain improved significantly, both compared to baseline (median 73.0 mm (60.0-79.8) at baseline versus 46.5 mm (11.5-57.5) at one year; p=0.046) and compared to six months (median 84.0 mm (75.5-90.0); p=0.043). Also, ankle specific pain, based on the AOS pain, improved from 46.7 (39.4-54.3) to 26.0 (6.2-46.4), but this was not significant (p=0.116; Figure 2B).

The AOS disability score improved from 56.5 (51.7-70.3) to 29.4 (15.0-52.8) at one year (p=0.028), resulting in a significant improvement in the total AOS score (p=0.028). Functionality measured on the HAL questionnaire improved significantly at one year follow-up (53% (41-62%) at baseline versus 64% (48-79%) at one year; p=0.046; Figure 2C), due to improvement in both basic and complex activities involving the lower extremities, whereas there was hardly any change in functionality of the upper extremities (p=0.461). After one year, four patients achieved their desired goal, in three patients the effect was even better than that (see Figure 3). The patient suffering from synovitis and pain at one year follow-up considered his situation worse than before distraction.

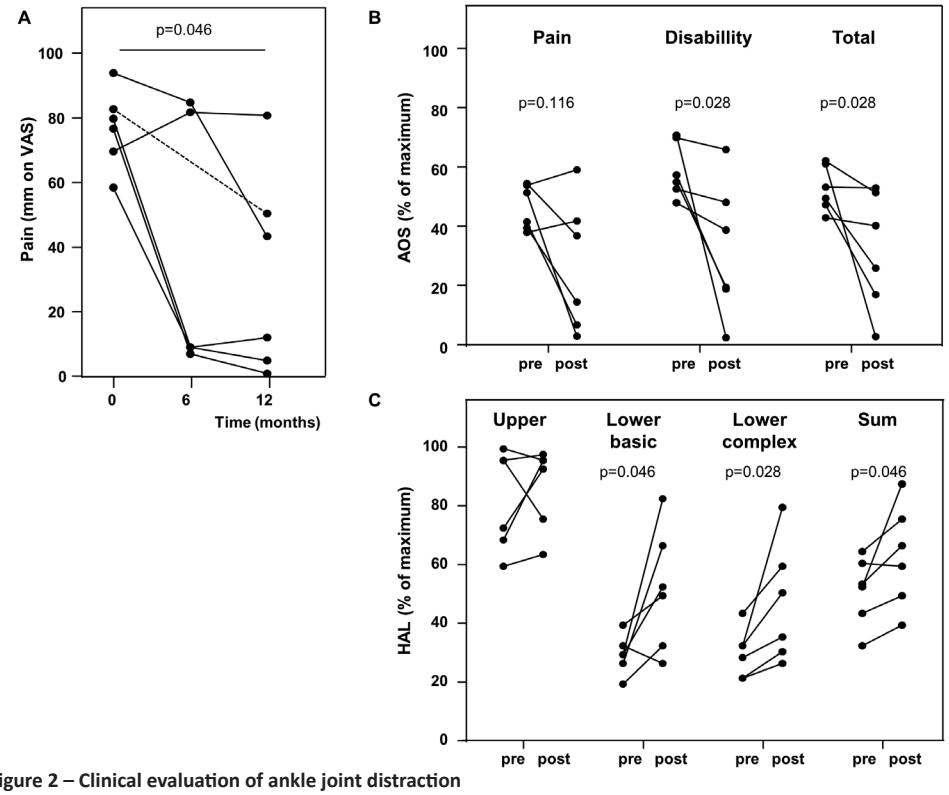

Figure 2 – Clinical evaluation of ankle joint distraction

Clinical evaluation by the Visual Analogue Scale (VAS) for pain (A; 100 mm most severe pain and 0 mm meaning no pain; dotted line indicates the patient where six months data are missing), Ankle Osteoarthritis Scale (B; AOS; 0% being the best score, 100% being the worst score), and Hemophilia Activities List (C; HAL; 100% being the best score, 0% being the worst score). Pre - at baseline; post - at one year follow-up.

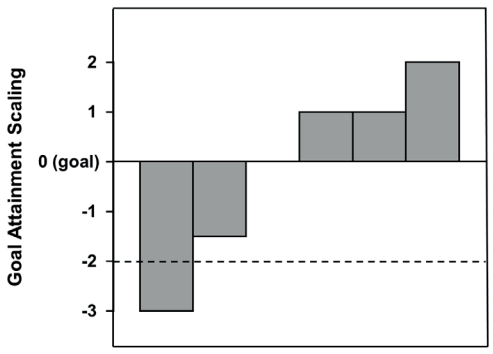

Figure 3 – Goal Attainment Scaling

Waterfall plot of change in Goal Attainment Scaling (GAS) at one year follow-up. Data are shown for each individual patient. A score of "0" at the GAS represents the predefined goal, whereas the dotted line ("-2") represents the baseline situation.

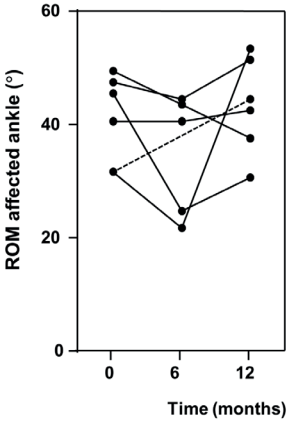

Figure 4 – Joint mobility

Joint mobility (range of motion, ROM) is presented as the full arc of ROM of the affected ankle (maximum dorsal flexion to plantar flexion). Dotted line indicates the patient where the six months data are missing.

Performance tests

ROM of the affected ankle was maintained after ankle joint distraction (Figure 4; full arc of mobility at baseline 44° (32-49°) versus 44° (36-53°) at one year follow-up. The results of the performance tests are shown in Table 2. In all patients, the figure 8 at maximum speed improved (p=0.012). Also the figure 8 at preferred speed and the 50 m walking test improved significantly. The 6 minutes walking test improved clearly in four patients, but not in the two patients not to reach their functional goal (GAS).

Table 2 - Performance tests

	Baseline	One year follow-up	*P*-value
Figure 8 at preferred speed (sec)	15.2 (13.2-18.2)	13.2 (11.7-14.8)	**0.046**
Figure 8 at maximum speed (sec)	10.8 (10.3-13.0)	9.8 (8.6-11.1)	**0.012**
50 meter walking test (sec)	33.7 (30.5-37.7)	28.7 (26.7-34.6)	**0.046**
6 minutes walking test (m)	553 (434-581)	602 (544-667)	0.116

M – meters; sec – seconds. Median (IQR)

Structural outcome

Ankle joint distraction resulted in structural changes on X-ray and MRI (Figure 5). In most patients periarticular osteoporosis was more pronounced at one year follow-up, whereas subchondral changes (cysts and irregularity) improved. This was not reflected by a significant change in the Pettersson score (p=0.414; Table 3). The MRI IPSG score decreased in four patients due to an improvement in osteochondral changes, whereas soft tissue changes remained unchanged. In one patient a clear reduction in subchondral cysts and an increase in cartilage thickness was noted, but this did was not reflected by a decrease in the IPSG score. As such, the change in MRI IPSG score showed a trend towards improvement at one year follow-up, but this did not reach statistical significance (p=0.059).

Discussion

These preliminary results of the first prospective study evaluating joint distraction in hemophilia demonstrates substantial clinical and functional improvement at one year follow-up. There were no adverse events related to bleeding, and although there was a high incidence of pin tract infection all were successfully treated with a short course of antibiotics. Patients encountered less pain and increased functionality with a preservation of ankle ROM. These clinical benefits probably result from the structural changes noted on X-ray and MRI. Although not resulting in a statistically significant change in Pettersson or MRI IPSG score, a decrease in bone cysts, bone edema, and increase in joint space width was noted.

Table 3 – Structural outcome

	Pettersson score		MRI IPSG score					
			Soft tissue		*Osteochondral*		*Total*	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Case 1	6	5	0	0	6	6	6	6
Case 2	8	6	6	7	6	4	12	11
Case 3	6	6	0	0	6	4	6	4
Case 4	6	6	0	0	6	5	6	5
Case 5	4	5	3	2	6	4	9	6
Case 6	6	6	0	0	7	7	7	7
Median	6.0	6.0	0.0	0.0	6.0	4.5	6.5	4.5
***P*-value**	0.414		1.000		0.059		0.066	

Currently, in the Netherlands the standard treatment for end-stage hemophilic arthropathy of the ankle is arthrodesis, providing substantial pain relief.[6] The main concerns of this technique are the development of degenerative changes in the adjacent joints, and the loss of joint mobility impacting functionality and activity.[21]

The only available surgical alternative at present is TAR with the advantage of preservation of joint motion, but with concerns about loosening and infection of the prosthesis, and the need for revision(s) in young patients. The mean age of hemophilia patients needing ankle surgery is at least 20 years lower than patients with OA and prosthesis revision is usually required even sooner. This is due to more intensive use of the joints by those younger patients. Long-term follow-up of TAR in hemophilia is scarce. A recent review describes a good outcome of 33 ankle replacements in 28 hemophiliacs, with a maximum follow-up of 8 years in only two patients.[4] Results of revision surgery in general are very poor and performing ankle arthrodesis subsequent to TAR is technically difficult.

Ankle joint distraction might provide an attractive alternative to postpone ankle arthrodesis or replacement therapy in young hemophilia patients with persisting, painful, conservative treatment-resistant ankle arthropathy. The primary aim of a surgical intervention is to alleviate symptoms and increase functionality. Our study shows clear (still short-term) efficacy on pain and disability when assessed with questionnaires. A predefined specific goal of the intervention was met by 67% of the patients, with 75% of them even achieving a better situation than expected. Moreover, the improved functionality was also demonstrated by a significant improvement in achievements on different performance tasks. These results are similar to the short-term efficacy of joint distraction in OA of the knee and ankle.[22,23] Herein, it is demonstrated that this benefit is maintained at a prolonged follow-up of five to ten years.[12,24] Long-term follow-up of the efficacy in hemophilic arthropathy needs to be awaited.

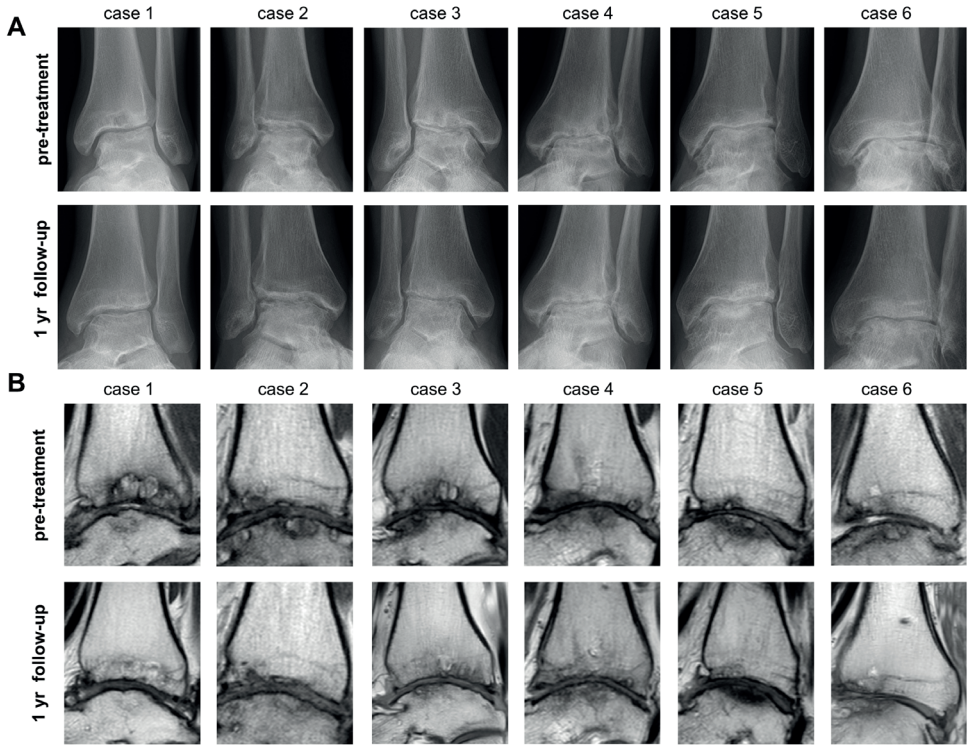

Figure 5 – Structural changes on X-ray and MRI
Structural improvement on X-ray *A) and MRI (B; representative images of sagittal PD-weighed TSE images) one year after ankle joint distraction.

Ankle joint distraction is an intense treatment. Patients are admitted to the hospital for one week, requiring continuous clotting factor infusion, followed by a week of increased clotting factor replacement at home. Thereafter, their normal clotting factor substitution regimen is resumed. Due to a very limited mobility, work participation is limited, but most patients manage to resume some of their activities during the distraction period. After ten weeks, mobility is gradually regained in a patient-tailored program under guidance of a specialized physiotherapist, as well as weight bearing. One patient suffered from synovitis during this rehabilitation period. This was the patient that also had an osteophyte removed. At time of surgery, there were no clinical or radiological signs of synovitis. At one year follow-up, MRI did not show synovial swelling anymore after therapy with intensified clotting factor replacement and a specific cyclo-oxygenase-2 inhibitor. Clinically, he still suffered from pain and was restricted in his mobility.

A major advantage of ankle joint distraction is the preservation of joint mobility without compromising subsequent conservative surgeries like arthrodesis, if still needed. ROM of the ankle at one year follow-up was maintained, whereas arthrodesis would result in a loss in arc of motion of 17°, with a greater loss in younger patients.[8] It remains to be elucidated whether joint distraction can best be performed in early or late stages of ankle

arthropathy. In knee OA, it is suggested that cartilaginous thickness one year after distraction depended on the degree of joint damage at baseline with better outcomes in more severe OA.[25] Irrespectively, each year with preservation of joint mobility gained before definitive ankle fusion, is valuable in young hemophilia patients. Long-term follow-up is needed to determine the duration of the effects of ankle joint distraction.

Distraction therapy is thought to renew joint homeostasis by stimulating the intrinsic regenerative capacity of the joint. The exact underlying basis for the clinical benefit obtained is not fully elucidated yet. Unloading is suggested to provide a favorable mechanical environment for cartilage repair by reducing mechanical stress while maintaining intermittent changes in hydrostatic pressure important for nutrition of the chondrocytes.[26] This stimulates proteoglycan synthesis,[27] and decreases the production of pro-inflammatory cytokines.[28,29] Recently, also an increased attachment of mesenchymal stem cells to cartilage is observed in joint distraction, further attributing to cartilage repair.[30] Reduced mechanical stress on cartilage is also due to a diminished subchondral sclerosis after joint distraction, whereas bone density in cystic lesions increases which correlates with clinical improvement.[13] This is in line with the structural changes observed in our study, showing increased periarticular osteoporosis in contrast to a resolution of bone cysts.

External fixators are used previously in hemophilia to correct flexion deformities, to support arthrodesis of infected joints, for treatment of open fractures, and osteoclasis (surgical fracture to correct position).[31-33] Also in these cases no bleeding was reported after temporary clotting factor supplementation during application and removal of the fixation frame. This is in line with our experience demonstrating no bleeding adverse events. In contrast, the incidence of pin tract infections was high (83%), in all cases rapidly resolving after initiation of antibiotics. This is a well-known complication in the use of an (Ilizarov) external fixator, with reported infection rates ranging between 1% for major infections to 80% for minor infections.[34] Our infection rate was comparable. Nevertheless, this is of potential concern in patients after joint replacement. In case of knee OA, no problems during and after placement of a total knee prosthesis secondary to knee joint distraction has been reported.[35]

In conclusion, these preliminary data suggest that joint distraction may offer a surgical alternative for severe hemophilic ankle arthropathy, with the potential to postpone ankle arthrodesis or TAR and with that preserve joint mobility and functionality. This study is still ongoing, additional data have to be awaited to determine the position of joint distraction in surgical treatment of severe ankle arthropathy in young patients with hemophilia.

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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

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Summary

The hallmark of severe hemophilia is a spontaneous bleeding tendency with most of the episodes occurring in the joints.[1,2] This leads to cartilage damage, synovitis and bone destruction, with severe impact on mobility and quality of life.[3] Similar destruction can be caused by joint bleeds resulting major trauma or joint surgery. In the last decades, significant steps forward have been made to elucidate the mechanisms of blood-induced joint damage. A principal role for iron and inflammation in the pathophysiology is demonstrated, but targeted therapies are lacking. The mainstay of treatment will always be the prevention of joint bleeding by clotting factor replacement therapy, although this cannot fully prevent joint bleeding. As such, arthropathy as a result of the limited remaining joint bleeds will still occur. At present the only other therapeutic options are orthopedic surgery in end-stage arthropathy, and conservative treatment aiming at preservation of function and pain relief to postpone orthopedic surgery as long as possible. Disease-modifying and joint saving therapies to interrupt the perpetuating process of blood-induced joint damage are urgently warranted.

This thesis aimed to contribute to our understanding of the pathophysiology of blood-induced joint damage, and to translate this knowledge into improved prevention and treatment of hemophilic arthropathy. Emphasis lays on the role of iron and inflammation herein. A literature study and cross-sectional cohort study were conducted to gain more insight in the role of iron in the pathogenesis. To improve the detection of joint damage in an early stage an explorative canine and human *in vivo* study was performed. For the establishment of new treatment modalities, several human *in vitro* studies and a murine *in vivo* study were conducted to investigate different anti-inflammatory and structure modifying therapies, including a pilot interventional study in hemophilia patients to explore a new orthopedic approach.

Iron in the pathophysiology of hemophilic arthropathy

A central role for iron in the pathophysiology of hemophilic arthropathy is demonstrated previously.[4,5] Likewise, in hereditary hemochromatosis joint destruction is caused by iron overload. A comparison between these types of arthropathy was never conducted before, but could provide more insight in the role of iron in inducing joint damage. **Chapter 2** describes a literature review in which both disorders were compared on their clinical and histological characteristics, and preclinical studies were reviewed on the influence of iron on different joint components. This study demonstrated that in both diseases arthropathy is characterized by cartilage degeneration, subchondral bone changes with osteophyte and cyst formation, osteoporosis, and synovial inflammation and proliferation. However, synovial inflammation and proliferation are much more explicit in the arthropathy as a result of hemophilia compared to hemochromatosis. Other substantial differences are the occurrence of chondrocalcinosis radiographically and calcium pyrophosphate dihydrate deposition disease in hemochromatosis, versus a rapid progression with joint deformity

and neovascularization in hemophilia. The detrimental effects of iron to all components of the joint are confirmed by preclinical studies. Iron induces synovial inflammation and hyperplasia, chondrocyte death, and impaired osteoblast function. These effects, particularly the synovial changes, are aggravated in the presence of a pro-inflammatory signal, which is prominent in hemophilic arthropathy and minimal in hemochromatosis. As such, this study confirmed that the devastating effects of iron on the joint are aggravated by inflammation. In case of a joint bleed both signals are present resulting in a rapidly progressive joint disease. Clinically, clear variability is observed in the severity of joint damage resulting from a similar number of bleeds (clinical observations,[6,7]). It could be hypothesized that co-inheritance of an *HFE* mutation in hemophilia increases iron exposition to the joint and therewith aggravates joint damage after a bleed, as suggested previously.[8] Moreover, polymorphisms in *HMOX1*, the gene coding for heme-oxygenase (the rate-determining enzyme in heme degradation), might play a role as they are associated with more severe joint damage in rheumatoid arthritis.[9] **Chapter 3** describes the results of a large cross-sectional cohort study investigating the impact of these genetic variations on the severity of hemophilic arthropathy. In a cohort of 252 patients with severe and moderate hemophilia the presence of an *HFE* gene mutation (Cys282Tyr and His63Asp) and polymorphisms in the $(GT)_n$-repeat length in the *HMOX1* promoter region were analyzed. Severity of arthropathy was determined by assessing damage on X-ray.[10] Analysis was adjusted for well-known factors impacting the development of hemophilic arthropathy: hemophilia severity, annualized joint bleeding rate, clotting factor consumption, age at evaluation, age at entry at the Van Creveldkliniek, and year of birth. Although carrying an *HFE* mutation significantly increased serum iron levels and transferrin saturation, this did not result in increased joint damage. In conclusion, this cross-sectional cohort study did not support the hypothesis that severity of hemophilic arthropathy is associated with *HFE* mutations or *HMOX1* polymorphisms.

Translation

Assessing arthropathy on X-rays is suitable for moderate-severe hemophilic arthropathy. However, the introduction of early prophylactic clotting factor replacement therapy has improved joint outcome substantially.[11,12] X-rays are relatively insensitive to detect early joint changes. Other imaging modalities also have limitations; the sensitivity of ultrasound for bone and cartilage defects is limited, whereas magnetic resonance imaging (MRI) is expensive, and for both ultrasound and MRI examining the six main joints is time consuming. As such, there is a need for new methods to sensitively and easily evaluate joint damage over time. A good cross-sectional correlation is demonstrated for biochemical markers of bone and cartilage turnover with radiographic joint damage.[13] This correlation was demonstrated for urinary C-terminal telopeptide of type II collagen (uCTX-II), serum cartilage oligomeric matrix protein (sCOMP), serum cartilage cleavage product C1,2C (sC1,2C), and serum chondroitin sulfate 846 (sCS846).

In **chapter 4** we evaluated whether these biomarkers changed upon a joint bleed. In hemophilia patients reporting a joint bleed, biomarkers were analyzed immediately after the bleed and up to two weeks later. A temporary increase in uCTX-II and sCS846 was seen five days after joint bleeding. To verify the potential of these biomarkers in detecting the harmful effects of a joint bleed, a joint bleed was induced in dogs by intra-articular blood injections and biosamples were collected thereafter. Again an increase in uCTX-II was detected after a week. Moreover, sCOMP increased already within two days after a bleed. We concluded that biochemical markers are sensitive to changes in joint tissue turnover induced by a single bleed, and as such might be useful in monitoring the impact of a joint bleed and in evaluation of treatment of such bleeds.

Treatment

The last part of this thesis focused on potential new treatment modalities for blood-induced joint damage. As shown in chapter 2, the devastating effects of iron are amplified by an inflammatory response. In **chapter 5** the role of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α in the pathogenesis of blood-induced cartilage damage were unraveled, and the therapeutic potential of antagonizing these cytokines was examined in human *in vitro* cultures. It was demonstrated that a recombinant human IL-1β monoclonal antibody (IL-1βmAb) or IL-1 receptor antagonist (IL-1RA) protected cartilage from blood-induced damage in a dose- and time-dependent manner. IL-1β and IL-6 production in whole blood cultures was reduced, while TNFα production remained unaffected. Interestingly, addition of a TNFα monoclonal antibody, although demonstrated to inhibit the direct (transient) effects of TNFα on cartilage, showed no effect on blood-induced (prolonged) cartilage damage. As such, these results suggested that opposing the activity of IL-1β within a couple of hours after blood-exposure, might prevent the initiation of a self-amplifying loop resulting in progressive cartilage damage. Further (*in vivo*) studies are needed to prove the therapeutic potential of targeting IL-1β to halt the degenerative process after a joint bleed.

Another approach to limit inflammation shown successful in preclinical studies of blood-induced joint damage is the use of the anti-inflammatory cytokines IL-4 and IL-10. These cytokines showed a protective effect in *in vitro* cartilage-blood co-cultures,[14,15] even when administered within 4-8 hours after blood-exposure.[16] The combination of both cytokines was also tested *in vivo* in a hemophilia mouse model showing amelioration of cartilage damage, although its effect was limited.[17] Moreover, IL-4 and IL-10 had no effect on synovial inflammation. It was hypothesized that a limited bioavailability of these small molecules potentially caused the lack of effect. Therefore, a fusion protein of IL-4 and IL-10 was designed. Due to an increased molecular mass, further increased by the ability of the fusion protein to form dimers, glomerular filtration of the molecule will be limited and therewith bioavailability will increase. In **chapter 6** we tested whether a fusion protein of both cytokines did affect its chondroprotective potential *in vitro*, and its efficacy *in vivo*. We showed that

the IL4-10 fusion protein was able to prevent blood-induced cartilage degeneration in a dose-dependent manner up to complete normalization *in vitro*. IL-1β and IL-6 production by monocytes/macrophages was suppressed, especially when these cells were activated by hemoglobin-derived iron. Its effect was similar to addition of the (combination of the) individual cytokines, showing a preserved functionality after fusion. Its cartilage-protective effect was confirmed in an *in vivo* murine joint bleeding model. Intra-articular treatment with the fusion protein in contrast to the individual components resulted in attenuation of cartilage damage upon two successive joint bleeds. Surprisingly, synovial inflammation was not affected by any of the treatments. The lack of effect of the individual cytokines on cartilage was in contrast to a previous study,[17] and might be contributed to an adaptation of the *in vivo* model. Two bleeds instead of one were induced to better represent the human situation in which recurrent joint bleeds over time lead to arthropathy.[18] Treatment was administered intra-articularly twice after each bleed to ascertain adequate availability of the active compounds. We concluded that exploring the therapeutic potential of the IL4-10 fusion protein as disease-modifying therapy in blood-induced joint damage seems appealing, especially in combination with clotting factor replacement therapy, which will better represent the human situation.

These anti-inflammatory approaches aim to prevent (progression of) joint damage. It will be inadequate in end-stage arthropathy, which despite prophylactic treatment still occurs, with the ankle being the most affected joint.[2] In these cases surgical treatments are indicated to relieve pain and improve functionality. The current surgical options for severe ankle arthropathy are arthrodesis and total ankle replacement, both procedures being successful to alleviate pain but with the limitation of losing joint motion in case of arthrodesis and a limited life span of a prosthesis with poor results after revision surgery. An alternative surgical technique, ankle joint distraction, has shown clinical benefit and structural tissue changes in osteoarthritis.[19] Experience in hemophilia is limited with only three cases evaluated in retrospect.[20] In **chapter 7** preliminary results of the first prospective study evaluating joint distraction in hemophilia are described. In six patients substantial clinical and functional improvement at one year follow-up was demonstrated. Patients encountered less pain and increased functionality with preservation of the ankle range of motion. There were no adverse events related to bleeding, and although there was a high incidence of pin tract infection all were successfully treated with a short course of antibiotics. The clinical benefits probably resulted from the structural changes as noted on X-ray and MRI. A decrease in bone cysts, bone edema and increase in joint space width was noted. Although preliminary, these data support the option of joint distraction as a surgical alternative for severe hemophilic ankle arthropathy. It has the potential to postpone ankle arthrodesis and with that preserve joint mobility and functional ability. This study is still ongoing, additional data and a longer follow-up are needed. For young patients with severe hemophilic ankle arthropathy not benefitting from conservative therapy joint distraction may be a promising treatment postponing more rigorous surgery like ankle arthrodesis.

General discussion

Although our understanding of the pathophysiology of hemophilic arthropathy has increased substantially over the past decades, this has not yet resulted in targeted therapies or patient tailored treatment. Clinically, the effects of joint bleeds are heterogeneous, the severity of joint damage in patients with a similar bleeding history varies, and also late stage effects differ with some patients developing chronic synovitis, and others suffering from osteochondral degeneration.
A better understanding of these differences might improve patient tailored treatment. Moreover, unravelling the pathophysiology further and determining the key factors in inducing blood-induced joint damage might lead to new targets for joint saving therapy.

Joint damage after a bleed
The frequency of joint bleeding as well as the damage resulting from a joint bleed varies among hemophilia patients. In a landmark study by Manco-Johnson *et al*[11] joint damage was assessed by MRI in boys with hemophilia at six years of age. A remarkable finding was that some boys showed changes on MRI without displaying evidence of joint bleeds, whereas others reported many bleeds without structural damage visible.[11] These unexpected MRI changes are absent in physically active healthy controls, suggesting they are hemophilia specific.[21] Whether this is due to so called micro-bleeds, subclinical bleeds resulting in joint damage without displaying the clinical symptoms of a bleed, remains matter of debate.[18]
Another explanation might lay in different sequelae of a bleed. Genetic variations may modify the response to a joint bleed, for instance if this alters iron control or the inflammatory response. The role of genetic variations in iron handling on the severity of hemophilic arthropathy could not be confirmed in the study described in chapter 3. Although iron has a pivotal role in the pathogenesis of hemophilic arthropathy, in chapter 2 we also demonstrated that its joint-damaging effect is more pronounced in the presence of an inflammatory milieu. Genetic variations impacting the inflammatory response in hemophilia patients were recently suggested to result in a predisposition to joint damage.[22] In this study the impact of over 10,000 genetic markers on joint range of motion was investigated in children and adolescents with hemophilia A. An association for 25 genetic markers mainly involved in inflammation and immune modulation was demonstrated. This hypothesis is further supported by a series of Indian hemophilia patients showing an association of the HLA B27 phenotype, associated with seronegative spondylarthropathy, with chronic synovitis.[23]
Other hypothetical determinants influencing the susceptibility to blood-induced joint damage are polymorphisms in genes encoding pro- and anti-inflammatory cytokines, other HLA phenotypes, or natural heterogeneity in (anti-)fibrinolytic proteins such as antiplasmin. Some of these polymorphisms are investigated in inhibitor formation,[24-26] but their impact on blood-induced joint damage has not been unambiguously established.

Uncoupling of inflammation and cartilage degeneration

Another intriguing clinical observation is a dissociation in the severity of synovial and osteochondral changes.[27,28] In chronic synovitis, massive synovial reactions are often accompanied by relatively preserved cartilage tissue, whereas in other patients a minimal synovial response comes along with severe cartilage degeneration and bone damage. This implies that inflammation and erosive changes are not simply coupled.

In hemophilic mice, none of the treatment strategies given upon joint bleeding, although limiting cartilage damage, could influence synovitis,[5,29,30] unless combined with clotting factor concentrate.[31,32] This is in line with our findings in chapter 6 showing an effect of the IL4-10 fusion protein on cartilage damage, but not on synovitis. The necessity of clotting factor concentrate to limit synovial changes suggests that ongoing bleeds, or subclinical bleeds, are essential for the development of synovitis. This is supported by a canine study comparing the impact of acute joint bleeds with micro-bleeds.[33] In this study the same blood load was injected over a similar time span, but the injections were either given mimicking two acute bleeds, or dispersed over the time span to mimic micro-bleeds. Synovial inflammation was similar in both groups, but cartilage damage was significantly lower in the micro-bleed group. Furthermore, chronic synovitis is more frequent in patients in resource-poor countries, whereas the degenerative cartilage and bone changes still occur in young patients in resource-rich countries despite prophylactic clotting factor replacement therapy and intensive treatment at time of a bleed. This implies that if the amount of blood in a joint exceeds a certain threshold, even for a short period of time, a cascade is initiated ultimately leading to progressive cartilage degeneration which may take years to become clinically evident. A parallel to features of degenerative osteoarthritis can be made. Mechanical stress supports this progressive damage,[34] and potentially weight-baring activities are initiated more rapidly in patients treating their occasional bleeds aggressively.

Another hypothesis is that the key regulators in the degenerative pathway of cartilage and bone damage differ from the factors responsible for synovitis. In rheumatoid arthritis, TNFα is suggested important in initiating synovial inflammation, whereas IL-1β seems more crucial for cartilage degeneration.[35,36] In chapter 5, this pivotal role for IL-1β compared to TNFα in blood-induced cartilage damage is confirmed. In a murine study, it is suggested that synovial inflammation is limited by a TNFα antagonist when administered at time of a bleed in combination with clotting factor concentrate.[32] The effect of IL-1β blockade on hemophilic synovitis has not been investigated yet.

This two-compartment model can explain why some interventions only limit cartilage damage and not synovitis, and underlines the importance of targeted therapy on an individual level or a combined approach.

Improving research tools

Unravelling the different pathways and key players in the synovium-mediated and cartilage-mediated processes is a challenge. For this purpose, the use of *in vitro* models seems most

suitable, but these models have several limitations. The most distinct one is the lack of co-cultures of cartilage and synovium, whereas this seems so important (as described above). Therefore, animal models are used frequently to study the processes involved in blood-induced joint damage, but these models also have limitations. In hemophilic mice, joint bleeding results in clear synovitis and bone changes, whilst cartilage damage is relatively mild. Moreover, the observed bony changes consist of osteoporotic changes,[37] extensive osteophyte formation and bony enlargement already after a single bleed,[38] but lack cyst development. In a recently described hemophilia rat model, cyst formation after joint bleeding was demonstrated, but the frequency was low and the cysts small.[39] An advantage of the rat model is the increased size compared to the current mice models, enabling (to a certain extent) consecutive blood drawings and more *ex vivo* investigations requiring separate cartilage, synovium, and bone samples. This size-related benefit is even more explicit in larger animal models like the dog. Dogs with naturally occurring hemophilia are used in preclinical trials of human factor VIII (FVIII) and FIX products as well as gene therapy,[40] but are less suitable to study joint damage due to a low joint bleeding rate, costs, their long lifespan and the requirement of *ex vivo* studies. Another approach is to inject autologous blood into the joint of a non-hemophilic dog to mimic a joint bleed.[41] However, a restriction of this model is that the clearance of blood from the joint is several times more rapid than in mice and human. As always, it is necessary to select the proper animal model for each study. Another appealing approach to identify the biological processes that follow bleeding into the joint of patients with hemophilia is the measurement of biochemical markers of joint tissue turnover. They have the potential to provide dynamic information on tissue turnover and to detect changes in an early phase. It is demonstrated that some of these markers correlate with radiographic joint damage[13] and change immediately after a joint bleed (chapter 4), but it remains to be investigated whether this correlates with radiographic progression of joint damage. These markers have the potential to reveal the supposed impact of subclinical bleeds on joint tissue. In theory, they can be used to monitor disease progression and treatment efficacy more closely and dynamically than the present joint outcome methods that are insensitive to small and early changes in a joint and provide information only on the cumulative result. This is essential for future clinical trials investigating new therapies for hemophilic arthropathy.

New targets for treatment

Currently, for hemophilia patients treatment to stop or turn around the processes resulting in synovitis and cartilage damage is lacking. Clotting factor substitution is a very expensive treatment not ubiquitally available. And even with clotting factor replacement, joint damage will occur as full substitution is not feasible. As such, there is an urgent need for affordable treatments abrogating the vicious circle in the pathogenesis of hemophilic arthropathy.

Based on the current knowledge of the pathogenesis of hemophilic arthropathy new treatment options preferably are directed against iron deposition, inflammation, hyperfibrinolysis, cartilage damage and/or bone remodeling (Table).

Table – Potential targets for therapy based on the pathogenesis of HA

Target	Intervention	Stage in research in HA (administration route)	Effect		
			Cartilage	*Synovium*	*Bone*
Iron	Iron chelators				
	• Deferoxamine[42]	*In vivo* (i.a.)	+	+	?
	• Deferasirox[5]	*In vivo* (p.o.)	+	-	?
Inflammation	Anti-inflammatory				
	• IL-4&IL-10[16,17,29]	*In vitro + in vivo* (i.a.)	+	-	?
	• IL-1β[ch 5]	*In vitro*	+	?	?
	• TNFα[ch 5]	*In vitro + in vivo* (i.a.)	-	+	?
Fibrinolytic system	Anti-fibrinolytics				
	• Antiplasmin[30]	*In vivo* (i.a.)	+	+	?
	• Amiloride[30]	*In vivo* (i.a., p.o.)	-	-	?
	• PAR-inhibitor[43]	*In vitro + in vivo* (i.a.)	+	+	?
Bone remodelling	Anti-resporptive				
	• Bisphosphonates[44]	*In vivo* (p.o.)	?	?	+
	• RANK-L inhibition	*Not studied yet*			
Angiogenesis	• VEGF inhibitors	*Not studied yet*			
Regenerative approaches	• MSCs[45-47]	*In vitro + in vivo* (i.a.)	+	+	?
	• Bone marrow derived cell tranplantation[48]	*Case series*	+	?	+
	• Joint distraction[20, ch 7]	*Case series*	+	-	+
	• Bone marrow stimulation	*Not studied yet*			
	• Osteochondral auto/ allograft	*Not studied yet*			
	• Chondrocyte implantation	*Not studied yet*			

+ beneficial effect, - no beneficial effect, ? effect unknown
Ch, chapter; HA, hemophilic arthropathy; i.a., intra-articular; IL, interleukin; p.o., per os; MSCs, mesenchymal stem cells; PAR, protease activated receptor; RANK-L, receptor of nuclear factor (NF)-κB ligand; TNFα, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

Iron chelating agents

As shown in chapter 2 iron has a pivotal role in the pathophysiology of hemophilic arthropathy, 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 based on histopathological assessment.[42] In another study, hemophilic mice were randomized to receive placebo or oral deferasirox prophylactically 8 weeks before and 5 weeks after joint bleed induction. Although deferasirox had no effect on synovitis, it limited cartilage damage significantly.[5]

Interestingly, deferasirox acts as a potent nuclear factor-κB (NF-κB) inhibitor in leukemia cells exhibiting hyperactivity of the NF-κB pathway by an iron-independent mechanism and without relying on the reactive oxygen species scavenging properties of the drug.[49] As the NF-κB pathway is induced in hemophilic arthropathy,[50] deferasirox may have two underlying pathways to exhibit its beneficial effect, a phenomenon not shared by other chelators.

Anti-inflammatory therapy

Research focusing on the effect of anti-inflammatory therapy in hemophilic arthropathy is a fast moving field of research but has not reached the stage of clinical studies yet (Table). An IL-6 receptor antagonist when added to clotting factor replacement after joint bleeding is shown to limit synovitis *in vivo*.[31] A significant decline in synovial hyperplasia, hemosiderin deposits and infiltration of macrophages is demonstrated. This is also demonstrated for a TNFα antagonist when administered at time of a bleed in combination with clotting factor concentrate.[32] In chapter 5 we showed that a TNFα monoclonal antibody did not ensue cartilage protection in an *in vitro* model of blood-induced cartilage damage. On the contrary, antagonizing IL-1 either by a recombinant human IL-1β monoclonal antibody or IL-1 receptor antagonist had a cartilage protective effect. Blocking a pro-inflammatory response by administration of the anti-inflammatory cytokines IL-4 and IL-10 has shown impressive effects in human *in vitro* and animal *in vivo* models. Besides suppressive effects on pro-inflammatory cytokine production and an experimentally demonstrated cartilage-protective efficacy[14,51,52] (chapter 6), also an analgesic activity is demonstrated for the IL4-10 fusion protein.[53,54]

Several anti-inflammatory agents are successful and registered for treatment in other inflammatory joint diseases, but translation into clinical trials regarding blood-induced joint damage is still awaited. Blocking IL-1 has been successfully applied in conditions varying from rheumatoid arthritis and gout to auto-inflammatory syndromes.[55] In addition, in two cases of iron-induced arthropathy of the hand due to hemochromatosis, a disorder sharing several characteristics with HA,[56] therapeutic efficacy of an IL-1 receptor antagonist (IL-1RA) has been demonstrated.[57] 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[58] and posttraumatic osteoarthritis.[59] Clearly, in future clinical studies, these anti-inflammatory strategies have to be tested in addition to clotting factor administration.

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.[30] In contrast, treatment with amiloride, a specific urokinase-type plasminogen activator (uPA)-inhibitor, was not effective. The effect of plasmin can also be reduced by silencing protease activated receptors (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.[43] *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.

Anti-resorptive agents
Bone damage is a late phenomenon of hemophilic arthropathy. Bisphosphonates diminish the activity of osteoclasts, resulting in maintained or increased bone mineral density (BMD).[60] In one relatively small prospective study, ten hemophilia patients with an increased risk of fracture were monthly treated with the oral bisphosphonate ibandronate.[44] After follow-up of 12 months, ibandronate significantly improved spinal BMD and reduced bone resorption. Inhibition of the receptor activator of nuclear factor-κB-ligand (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.[61] To our knowledge, this treatment modality has not been studied in hemophilia patients.

Other approaches
In theory, inhibition of neoangiogenesis using vascular endothelial growth factor (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[62-64] and ocular diseases characterized by neoangiogenesis.[65,66] So far, no preclinical or clinical research has been conducted focusing on hemophilic arthropathy.
A different approach is to pursue cartilage regeneration via bone marrow stimulation, osteochondral auto-allograft transplantation, administration of mesenchymal stem cells (MSCs) and growth factors, and joint distraction.[20,45-47,67] In hemophilia, only joint distraction and administration of MSCs are investigated. Genetic engineered MSCs expressing coagulation factor were injected intra-articularly in hemophilic mice.[46] A beneficial effect on cartilage and synovium was demonstrated, but it remains questionable whether the therapeutic effect was a result of the expression of coagulation factor or attributable to the MSCs itself. Another murine study showed some beneficial effects on cartilage structure and function, a decrease in TNFα and IL-1β, and a slight increase in IL-4.[47] In five hemophilia patients, bone marrow-derived cells were transplanted into the ankle and the effects analyzed retrospectively.[48] Treatment was combined with synovectomy and arthroscopic debridement, with the use of autologous platelet-rich fibrin, so it is hard to judge what the exact benefit of the stem cell therapy was. At a mean follow-up of two years, an improvement in symptoms, functional ability, and signs of regeneration of cartilage and bone was demonstrated. The last approach tested in hemophilia is ankle joint distraction[20] (chapter 7), showing an improvement in pain, increased functionality with preservation

of the ankle range of motion, and structural changes on X-ray and MRI. Changes in bone tissue consisted of a decrease in cysts and edema. Cartilage regeneration was suggested by increase in joint space width. For all these approaches, more studies are needed to determine their position in the treatment of arthropathy.

Challenges for future research

It is still a long way before targeted therapies will translate into clinical practice and are 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.[68] Most important, assessing the viability of new targets remains a challenge.

First, research so far is mainly conducted in *in vitro* and animal *in vivo* models. The main limitation of *in vitro* work is studying joint tissue, e.g. cartilage, lacking the context of surrounding structures and 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. In the murine models homogeneous mice (with the same genetic background, similar age, and environmental factors) are used, while patients form a heterogeneous group exposed to different factors that modify disease outcome. Besides, there is no universal and reliable outcome measure available which predicts efficacy in humans.

A second obstacle to overcome is the route of administration. In the previously described animal models, anti-fibrinolytics and anti-inflammatory modalities are administered via intra-articular injection. With clotting factor substitution, hemophilic arthropathy is more and more a disease restricted to a single joint. As such, it makes sense to implement the therapy locally ensuring the affected site is reached and adequate levels of the active compound are achieved, limiting unnecessary (toxic) side effects when systemically added, at relatively high dose to reach sufficient concentrations in the joint. Conversely, an intra-articular injection is not preferred considering the bleeding tendency of hemophilia patients. Also, the issue of determining the optimal time frame for administering 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*. A pretreatment period requires continuous treatment since a joint bleeding cannot be predicted. Treatment on demand would be more convenient and ameliorates compliance. A related dilemma is the treatment of established hemophilic arthropathy, will the aforementioned targeted therapies still be beneficial? In order to answer this, special attention should be paid to improving non-invasive diagnostics methods to prospectively monitor joint damage, and to detect early stage hemophilic arthropathy. Since a single bleeding already causes joint damage, quantifying early changes is essential to evaluate possible treatment strategies. Further research must address these issues.

For the therapeutic use of small molecules like anti-inflammatory cytokines their short half-life could limit their effectivity. After intravenous administration the half-life of IL-10 is

1.9 hours,[69] and for IL-4 this is only 19 minutes.[70] By fusing both cytokines, the molecular weight is increased and therewith the threshold for glomerular filtration is passed. Chapter 6 demonstrated a conserved functionality for the IL4-10 fusion protein both *in vitro* and *in vivo*. Other half-life extension strategies are fusion to recombinant albumin or to the Fc-region of human IgG, attachment of polyethylene glycol (PEG), glycosylation or sialylation, or the use of a slow-release system.[71] Also local delivery of the active substance using gene therapy or modulation of processes by microRNAs is an attractive approach.[72] Ideally, the gene used in gene therapy is only activated in case of a bleed, which can be achieved using an inflammation-induced transgene expression by genetic engineering.[73-76] More research is needed to investigate whether these approaches are effective and safe.

Overall conclusion

The current thesis shows that the irreversible joint damage in hemophilia results from the devastating combination of iron and inflammation. The harmful effects of iron to all components of the joint are aggravated in the presence of a pro-inflammatory signal. Two genetic polymorphisms in iron control, *HFE* mutations and *HMOX1* polymorphisms, cannot explain the heterogeneity in the severity of joint damage observed in adult hemophilia patients. Biochemical markers might be used to sensitively and prospectively detect small changes in joint tissue turnover caused by a joint bleed. The degenerative process of blood-induced cartilage damage is hierarchically dominated by the pro-inflammatory effect of IL-1β, not TNFα. A fusion protein of the anti-inflammatory cytokines IL-4 and IL-10 is shown to prevent blood-induced cartilage damage *in vitro* and *in vivo*. For patients with advanced joint damage, joint distraction seems a promising technique to alleviate pain, improve joint function, and induce structural tissue changes.

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ADDENDUM

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CURRICULUM VITAE

NEDERLANDSE SAMENVATTING

Introductie

Hemofilie is een zeldzame, erfelijke bloedingsziekte veroorzaakt door een tekort aan stollingsfactor VIII (hemofilie A) of stollingsfactor IX (hemofilie B). De ernst van de hemofilie wordt gedefinieerd aan de hand van de resterende stollingsfactoractiviteit. Bij ernstige hemofilie is dit minder dan 1% in vergelijking met de gezonde populatie, bij de matig-ernstige vorm 1-5% en bij milde hemofilie tussen de 5-40%. De ziekte komt vrijwel uitsluitend voor bij mannen, omdat het gen voor zowel stollingsfactor VIII als IX gelegen is op het X-chromosoom. Er wordt geschat dat 1 op de 5 000 tot 10 000 jongens geboren wordt met hemofilie, 80-85% hiervan lijdt aan hemofilie A.
Hemofilie wordt gekenmerkt door bovenmatig bloeden na een trauma of ingreep, in geval van ernstige hemofilie kunnen spontane bloedingen optreden. 70-80% van deze spontane bloedingen treedt op in de gewrichten, voornamelijk in de enkels, knieën en ellebogen. De heupen en schouders zijn minder vaak aangedaan en bloedingen in kleinere gewrichten zijn zeldzaam.
Om onherstelbare gewrichtsschade te voorkomen moeten gewrichtsbloedingen zo snel mogelijk worden behandeld met intraveneuze toediening van stollingsfactor. Bij patiënten met ernstige hemofilie wordt met preventieve behandeling getracht bloedingen te voorkomen. Ondanks regelmatige toediening van stollingsfactor blijkt het onmogelijk deze bloedingen geheel te voorkomen. Herhaalde gewrichtsbloedingen leiden op lange termijn tot ernstige gewrichtsschade met schade aan het synovium (de zachte binnenbekleding van de gewrichtsholte), kraakbeen en het onder het kraakbeen gelegen (subchondraal) bot: hemofilie artropathie.
Behalve bij hemofilie kunnen ook gewrichtsbloedingen na een (sport)letsel of grote chirurgische ingrepen leiden tot bloed-geïnduceerde gewrichtsschade (schade ten gevolge van bloedblootstelling).

Bloed-geïnduceerde gewrichtsschade
Het ontstaan van gewrichtsschade na een bloeding wordt gekenmerkt door verschillende processen: ontsteking van het synovium (synovitis) en directe schade aan het kraakbeen, uiteindelijk gevolgd door botveranderingen.

Synovitis
Het synovium is een dunne laag cellen die de binnenkant van het gewricht bekleedt. Het is verantwoordelijk voor het interne 'milieu' van het gewricht. De functie van het synovium is de productie van synoviale vloeistof, een stroperige (visceuze) vloeistof die zorgt voor een soepele beweging van het gewricht en die de voedingsstoffen bevat voor het kraakbeen. Daarnaast is synovium verantwoordelijk voor het opruimen van afvalstoffen

uit de gewrichtsholte, waaronder bloed. In dit opruimproces spelen ontstekingscellen een belangrijke rol. Deze verplaatsen zich na een bloeding naar het gewricht en ruimen in ongeveer 3-4 dagen het bloed op. Eén van de schadelijke stoffen die bij dit opruimproces vrijkomt uit de rode bloedcellen is het ijzerbevattende 'heem', afkomstig uit hemoglobine. Dit heem kan zich ophopen in het synovium, zeker in het geval van herhaalde bloedingen. Synovium met zichtbare ijzerophoping produceert aanzienlijk meer ontstekingsstoffen (cytokines) dan synovium zonder ijzerophoping. Bovendien stimuleert ijzer de celdeling waardoor het synovium dikker wordt. In dit verdikte en ontstoken synovium worden nieuwe, kwetsbare bloedvaten aangemaakt. Hiermee is een vicieuze cirkel ontstaan: het verdikte synovium is meer vatbaar voor mechanische schade, en de fragiele, nieuwgevormde bloedvaten zijn kwetsbaarder, wat leidt tot nieuwe bloedingen. Ongeveer 25% van de patiënten met ernstige hemofilie ontwikkelt zo een 'target joint', een gewricht waarin drie of meer bloedingen optreden in een periode van zes maanden.

Kraakbeenschade

Kraakbeenschade na een bloeding is het gevolg van veranderingen van het synovium, maar wordt ook veroorzaakt door directe, synovium-onafhankelijke effecten van bloed op kraakbeen.

Kraakbeen is een speciale vorm van bindweefsel die de uiteinden bedekt van de botten die een gewricht vormen. Het is bestand tegen grote mechanische belasting (duw- en trekkrachten). In het kraakbeen liggen relatief weinig cellen (chondrocyten). Kraakbeen is niet doorbloed, de chondrocyt is voor zijn voedingsstoffen afhankelijk van de synoviale vloeistof. De belangrijkste taak van de chondrocyt is de aanmaak en het onderhoud van de kraakbeenmatrix. De kraakbeenmatrix bestaat grotendeels uit water (70-80%), en daarnaast uit collageen (10-20%) en proteoglycanen (5-10%). Collageen zorgt voor de vormvastheid van kraakbeen en biedt weerstand aan trekkrachten. Proteoglycanen zijn sterk negatief geladen deeltjes en zorgen daarmee voor een hoge osmotische gradiënt waardoor water het kraakbeen ingetrokken wordt. Op deze manier heeft het gewrichtskraakbeen, net als een spons, veerkracht onder belasting. Bij belasting wordt water uit het kraakbeen gedrukt en na ontlasten wordt dit weer aangetrokken. Het collageennetwerk voorkomt een ongelimiteerde zwelling van het kraakbeen.

In gezond kraakbeen is er een balans tussen de aanmaak en afbraak van de kraakbeenmatrix. Deze balans wordt zelfs na een korte blootstelling aan bloed al blijvend verstoord. De afbraak van proteoglycanen neemt sterk toe en de aanmaak van nieuwe proteoglycanen wordt onderdrukt. Bloedblootstelling activeert de chondrocyt waardoor deze waterstofperoxide gaat produceren. In aanwezigheid van ijzermoleculen uit de rode bloedcellen wordt dit waterstofperoxide omgezet in de zeer schadelijke hydroxyl radicalen die leiden tot celdood van de chondrocyt. Hierdoor kan geen nieuwe kraakbeenmatrix meer worden aangemaakt, verliest het kraakbeen zijn weerstand en vormvastheid, en treedt versnelde slijtage op.

Een enkele bloeding is reeds schadelijk voor het gewricht, het herhaaldelijke karakter van de bloedingen kenmerkend voor ernstige hemofilie leidt tot zeer ernstige gewrichtsschade (hemofilie arthropathie). Ondanks preventieve toediening van stollingsfactor is het niet mogelijk om gewrichtsbloedingen en gewrichtsschade helemaal te voorkomen.
De doelstellingen van dit proefschrift zijn het vergroten van het inzicht in het mechanisme van bloed-geïnduceerde gewrichtsschade, het ontwikkelen van een nieuwe methode om vroege schade op te sporen, en het onderzoeken van mogelijkheden om deze schade tegen te gaan en te behandelen.

Samenvatting van de hoofdstukken

Rol van ijzer in bloed-geïnduceerde gewrichtsschade
IJzer speelt een belangrijke rol in het ontstaan van bloed-geïnduceerde gewrichtsschade. De erfelijke aandoening hemochromatose, veroorzaakt door een genetische mutatie in het *HFE* gen, leidt tot ijzerstapeling in het gehele lichaam en kan ook leiden tot gewrichtsschade (artropathie). **Hoofdstuk 2** beschrijft een literatuuronderzoek waarin de kenmerken van beide vormen van gewrichtsschade (erfelijke ijzerstapeling versus hemofilie) zijn vergeleken om de rol van ijzer bij het ontstaan van gewrichtsschade verder te ontrafelen. Het blijkt dat de artropathie bij zowel hemofilie als bij hemochromatose wordt gekenmerkt door kraakbeenslijtage, veranderingen van subchondrale bot, botontkalking en synoviale ontsteking en verdikking. De synoviale veranderingen zijn veel meer uitgesproken in hemofilie dan in hemochromatose. De progressie van de gewrichtsschade bij hemofilie is sneller en kan leiden tot standsafwijkingen. Bovendien is de vaatnieuwvorming in het synovium specifiek voor hemofilie arthropathie. Bij hemochromatose vindt daarentegen chondrocalcinose (ophoping van kalkkristallen in het kraakbeen) en vorming van calciumpyrofosfaat kristallen plaats, wat niet wordt gezien bij hemofiliepatiënten. Het schadelijke effect van ijzer op het gewricht wordt bevestigd door preklinische studies (laboratorium- en dierstudies). IJzer stimuleert ontsteking en celdeling van het synovium, leidt tot celdood van de chondrocyten en vermindert de functie van osteoblasten (botcellen). Deze effecten, in het bijzonder de synoviale veranderingen, worden versterkt in de aanwezigheid van ontsteking. In geval van een gewrichtsbloeding zijn zowel ijzer als ontsteking aanwezig wat resulteert in snel progressieve gewrichtsschade.

Bij patiënten met hemofilie verschilt de mate van gewrichtsschade die ontstaat na een vergelijkbaar aantal gewrichtsbloedingen. Erfelijke verschillen tussen hemofiliepatiënten kunnen hierin een rol spelen. Een mogelijkheid is dat door een *HFE* mutatie de ijzerblootstelling aan het gewricht wordt verhoogd waardoor de gewrichtsschade na een bloeding verergert. Een ander potentiële genetische factor is een variatie in *HMOX1*, het gen dat codeert voor heem-oxygenase. Dit is het enzym dat bepalend is voor de snelheid van de afbraak van heem. Een verlaagde afbraaksnelheid van heem zorgt voor een langere

aanwezigheid van ijzer in het gewricht en kan zo tot meer schade leiden na een bloeding. Variaties in dit gen zijn geassocieerd met de mate van gewrichtsschade bij reumatoïde artritis. In **hoofdstuk 3** is in een grote groep hemofiliepatiënten onderzocht wat de invloed van deze genetische variaties is op de ernst van de hemofilie artropathie. In 252 patiënten met ernstige en matige hemofilie is de aanwezigheid van een *HFE* mutatie (Cys282Tyr en His63Asp) bepaald evenals de genetische variatie in het *HMOX1* gen (hiervoor is de lengte van de (GT)n-repeat in de promotor regio bepaald). Aan de hand van röntgenfoto's van de ellebogen, enkels en knieën is de mate van hemofilie artropathie vastgesteld. Andere factoren die in de analyse zijn meegenomen zijn: de ernst van de hemofilie, het aantal bloedingen per jaar, het stollingsfactorverbruik, de leeftijd ten tijde van de röntgenfoto's, de leeftijd waarop patiënten naar de Van Creveldkliniek kwamen, en het geboortejaar. Van deze factoren is bekend dat zij de ontwikkeling van hemofilie artropathie beïnvloeden. Hoewel het dragerschap van een *HFE* mutatie duidelijk de ijzerconcentratie in serum verhoogt, resulteert dit niet in een toename van de gewrichtsschade. Ook de variaties in het *HMOX1* gen blijken niet geassocieerd te zijn met de mate van gewrichtsschade.

Biomarkers

Het gebruik van röntgenfoto's ter beoordeling van artropathie is bruikbaar bij enigszins gevorderde gewrichtsschade, maar is minder geschikt om vroege gewrichtsschade op te sporen. Andere radiologische technieken hebben ook beperkingen. Schade aan bot en kraakbeen is lastig vast te stellen met echografie, het gebruik van MRI (magnetic resonance imaging) is duur, en voor zowel echografie als MRI geldt dat het veel tijd kost om zes gewrichten in beeld te brengen. Er is dus behoefte aan nieuwe methoden die de consequenties van een gewrichtsbloeding veel eerder kunnen aantonen. Stoffen die vrijkomen bij de aanmaak en afbraak van kraakbeen en bot kunnen gemeten worden in bloed of urine en zo dienen als biochemische markers (biomarkers) van gewrichtsschade. Voor een aantal van deze biomarkers is een goede correlatie aangetoond met de radiologische gewrichtsschade bij hemofiliepatiënten. Dit geldt voor C-terminal telopeptide van type II collageen (gemeten in de urine, uCTX-II), serum cartilage oligomeric matrix protein (sCOMP), cartilage cleavage product C1,2C (sC1,2C) en serum chondroitine sulfaat 846 (sCS846).

In **hoofdstuk 4** is onderzocht wat het effect is van een gewrichtsbloeding op deze biomarkers. Na een gewrichtsbloeding zijn bij hemofiliepatiënten deze biomarkers op verschillende tijdstippen gemeten. uCTX-II en sCS846 stijgen kortdurend op dag vijf na de gewrichtsbloeding. Ook in een diermodel wordt een stijging gevonden in uCTX-II een week na de bloeding. Bovendien stijgt sCOMP binnen twee dagen na de bloeding. Deze studie leert ons dat biomarkers gevoelig zijn voor de veranderingen in bot- en kraakbeenmetabolisme veroorzaakt door een gewrichtsbloeding. Dit geeft de mogelijkheid om de schade door een gewrichtsbloeding te monitoren en het effect van een behandeling te evalueren.

Behandeling

Het laatste deel van dit proefschrift richt zich op mogelijke behandelstrategieën voor bloed-geïnduceerde gewrichtsschade. Zoals in hoofdstuk 2 is aangetoond worden de schadelijke effecten van ijzer versterkt door ontsteking. In **hoofdstuk 5** is onderzocht wat de rol van de ontstekingsbevorderende cytokines IL-1β en TNFα is op het ontwikkelen van bloed-geïnduceerde kraakbeenschade. Het blokkeren van de activiteit van IL-1 door toediening van een monoklonaal antilichaam (IL-1βmAb) of door het blokkeren van de IL-1 receptor (IL1-RA) kan bloed-geïnduceerde kraakbeenschade voorkomen. In beide gevallen is het effect dosis- en tijdsafhankelijk.

Een TNFα monoklonaal antilichaam is in staat de tijdelijke effecten van TNFα op kraakbeen te blokkeren, maar blijkt geen effect te hebben op de blijvende kraakbeenschade na bloedblootstelling. Het blokkeren van de IL-1β activiteit binnen enkele uren na bloedblootstelling zou effectief kunnen zijn om progressieve kraakbeenschade te voorkomen. Om de effectiviteit van IL-1 blokkade op bloed-geïnduceerde gewrichtsschade verder te onderzoeken zijn meer (in vivo) studies nodig.

Een andere aanpak om ontsteking te beperken is het gebruik van de ontstekingsremmende cytokines IL-4 en IL-10. Eerdere (preklinische) studies naar hun effect op bloed-geïnduceerde gewrichtsschade waren succesvol en ook in een eerdere muisstudie werd een beschermend effect op kraakbeenschade aangetoond. Een effect op synoviale ontsteking ontbrak echter. Mogelijk is een (te) snelle klaring van deze kleine moleculen de oorzaak voor het beperkte effect. De ontwikkeling van een fusie-eiwit van IL-4 en IL-10 heeft gezorgd voor een hogere moleculaire massa en daarmee is de verwachting dat de uitscheiding door de nieren afneemt en de biologische beschikbaarheid toeneemt. In **hoofdstuk 6** is onderzocht of het fuseren van beide cytokines de werkzaamheid heeft beïnvloed (in vitro en in vivo). Deze studie toont aan dat het IL4-10 fusie-eiwit in staat is bloed-geïnduceerde kraakbeenschade te voorkomen met een vergelijkbare werkzaamheid als (een combinatie van) de individuele cytokines. In een hemofilie muismodel wordt de kraakbeenbeschermende functionaliteit bevestigd, maar ook hier wordt geen effect gezien op synoviale ontsteking. Behandeling van een gewrichtsbloeding met het IL4-10 fusie-eiwit kan gewrichtsschade dus gedeeltelijk beperken. Toekomstige studies moeten uitwijzen of het in combinatie met stollingsfactor behandeling ook effect heeft op synoviale ontsteking.

Het onderdrukken van ontsteking is gericht op het voorkomen van/verminderen van gewrichtsschade. In vergevorderde vormen van artropathie zal dit echter niet afdoende zijn. Ondanks preventief gebruik van stollingsfactor komt schade nog steeds voor, in het bijzonder in de enkel. Operatieve ingrepen ter verlichting van de pijn en verbetering van de functionaliteit zijn dan noodzakelijk. De huidige opties voor ernstige enkelartropathie zijn het vastzetten van het gewricht (artrodese) of gewrichtsvervanging (prothese). Beide opties zijn effectief in vermindering van de pijn. Bij artrodese gaat dit echter ten koste van de beweeglijkheid van het enkelgewricht en voor enkelvervanging geldt dat de

levensduur van de prothese beperkt is en de resultaten van revisiechirurgie tegenvallen. Een alternatieve optie is enkeldistractie, een techniek waarbij de enkel gedurende 10 weken uit elkaar wordt getrokken met behulp van pennen, in de hoop dat zich nieuw kraakbeen vormt. Deze techniek is in artrose succesvol toegepast, maar de ervaring in hemofilie is nog beperkt. **Hoofdstuk 7** beschrijft de eerste resultaten van een nog lopende studie waarin enkeldistractie wordt onderzocht in (tot nu toe) zes hemofiliepatiënten. Na een jaar wordt een duidelijke verbetering van de klachten en functionaliteit gezien. De pijn neemt af, er is toename van de functionaliteit en de beweeglijkheid van de enkel blijft behouden. Er zijn geen bloedingscomplicaties opgetreden. Wel heeft het merendeel van de patiënten een infectie gehad van de huid rond de botpennen, die in alle gevallen succesvol behandeld is met een korte antibioticumkuur. Structurele veranderingen zijn al na een jaar zichtbaar op röntgenfoto's en MRI's: een afname van botcysten en botoedeem, en een verwijding van de gewrichtsspleet. Hoewel deze studie slechts zes patiënten beschrijft en de follow-up nog kort is, zijn deze data veelbelovend. Enkeldistractie is veelbelovend als alternatief voor jonge hemofiliepatiënten met ernstige enkelartropathie. Zo kan een artrodese worden uitgesteld en blijft beweeglijkheid van het gewricht behouden.

Conclusie

Dit proefschrift maakt duidelijk dat ijzer en ontsteking leiden tot onherstelbare gewrichtsschade bij hemofilie. De schadelijke effecten van ijzer worden versterkt in de aanwezigheid van ontstekingsbevorderende signalen. De variatie in gewrichtsschade die wordt gezien in volwassen hemofiliepatiënten kan niet worden verklaard door twee genetische variaties die de ijzerhuishouding beïnvloeden (aanwezigheid van een HFE mutatie of genetische variatie in HMOX1). Biochemische markers kunnen kleine veranderingen in bot- en kraakbeenmetabolisme na een gewrichtsbloeding waarnemen. In het proces van bloed-geïnduceerde kraakbeendegeneratie speelt IL-1β wel en TNFα geen belangrijke rol. Een fusie-eiwit van de ontstekingsremmende cytokines IL-4 en IL-10 is in staat bloed-geïnduceerde kraakbeenschade te voorkomen. Enkeldistractie kan bij reeds gevorderde gewrichtsschade uitkomst bieden, aangezien het zorgt voor pijnverlichting, functieverbetering en weefselherstel.

Kortom, dit proefschrift heeft ons inzicht in het ontstaan van bloed-geïnduceerde gewrichtsschade vergroot en een basis gelegd voor nieuwe behandelstrategieën om hemofilie artropathie te voorkomen en te behandelen.

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CURRICULUM VITAE

Lize van Vulpen was born on the 9th of August 1983 in Bunnik, the Netherlands. After graduating secondary school at Christelijk Gymnasium Utrecht (2001) she started medical school at the University of Utrecht and obtained her medical degree (*cum laude*) in August 2007. She started her clinical career at the department of Internal Medicine at the Sint Antonius Hospital in Nieuwegein. In April 2008 she started her residency Internal Medicine alternately at the University Medical Center Utrecht (prof. dr. D.H. Biesma, prof. dr. M.M.E. Schneider, and prof. dr. H.A.H. Kaasjager) and the "Diakonessenhuis Utrecht" (dr. A.F. Muller).

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