Blood-induced joint damage: novel targets for therapy

Monique van Meegeren

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Blood-induced joint damage: novel targets for therapy

Bloed-geïnduceerde gewrichtsschade: nieuwe mogelijkheden voor behandeling

(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

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Prevalence of blood-induced joint damage

Blood-induced joint damage can occur due to a trauma but also during surgery when blood leaks into the joint cavity. The latter is elegantly demonstrated in an experimental canine model of osteoarthritis where transection of the anterior cruciate ligament causes exposure of the knee joint to blood leading to synovial inflammation and aggravates degenerative changes of the articular cartilage[1]. Blood-induced joint damage, also called haemophilic arthropathy, is one of the major causes of morbidity amongst haemophilia patients[2]. Haemophilia is an X-linked inherited recessive clotting disorder involving lack of clotting factor VIII (haemophilia A) or clotting factor IX (haemophilia B). About 1 in every 10,000 males worldwide is affected with haemophilia A, and about 1 in every 60,000 males with haemophilia B[3,4]. Severe haemophilia patients have less than 1% normal clotting factor activity in blood, while moderate and mild haemophilia patients have 1-5% and 5-40% clotting factor activity, respectively. Because they lack a sufficient amount of clotting factor, patients suffer from spontaneous haemorrhages of which approximately 90% occurs in the large synovial joints (elbows, knees, ankles, hips, and shoulders)[5]. Irrespective of the underlying cause, a joint bleed can lead to (severe) damage of several components of the synovial joint, including synovial tissue, articular cartilage, and subchondral bone.

Joint tissues involved

Synovial tissue

Synovial tissue is soft tissue situated between the joint capsule and the joint cavity (figure 1). It consists of two layers: the subintima (outer layer) and the intima (inner

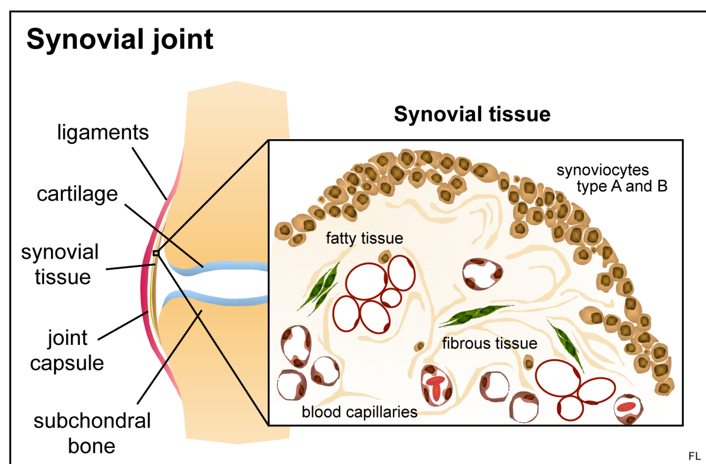

Figure 1: Schematic representation of synovial tissue in a synovial joint
At the left a schematic synovial knee joint is depicted, consisting of two bone ends covered by cartilage and attached to each other by ligaments and the surrounding joint capsule. The joint capsule is lined with synovial tissue, which is enlarged in the picture at the right. For more details, see text.

lining layer). The subintima is built up of a collagenous extracellular matrix including blood vessels, fat cells, fibroblasts, and few lymphocytes or macrophages[6]. The relative content of fat cells and fibrous tissue varies, resulting in more adipose or fibrous synovial tissue. At the location where synovial tissue is attached to the joint capsule, the sublining is mostly fibrous. The inner lining layer consists of a thin layer of 1-2 cells. These cells are divided into two types based on their cytoplasmatic contents: synoviocytes type A and type B[7]. Type A synoviocytes are macrophage like and the type B cells are fibroblast like[8]. An important function of synovial tissue is to provide packing of the joint cavity, allowing movement of adjacent bone ends, without getting damaged by stretching, rolling, and folding during joint movement. In addition, synovial tissue maintains intact non-adherent cartilage tissue surfaces, lubricates and nourishes cartilage, and controls synovial fluid volume and composition[6]. To exert these functions and to produce synovial fluid, synovial tissue needs to be highly vascularised. At the same time, these blood vessels are also the source of joint bleeds.

Articular cartilage

Articular cartilage is a hyaline cartilage covering subchondral bone in a joint as depicted in figure 2. It is avascular and aneural, depending mostly on the synovial fluid for its nutrition. Articular cartilage is composed of chondrocytes embedded in an extracellular matrix. Chondrocytes cover approximately 2% of the total cartilage volume in adults[9] and are responsible for maintenance of the matrix. The cartilage matrix consists of two major components: collagen (mainly type II) and proteoglycans. Collagen fibrils make up 10-20% of the wet weight of cartilage and provide shape and tensile strength. Proteoglycans (5-10% of the wet weight) are composed of a core protein and covalently linked sulphated glycosaminoglycans (GAGs), like aggrecan. They are responsible for a

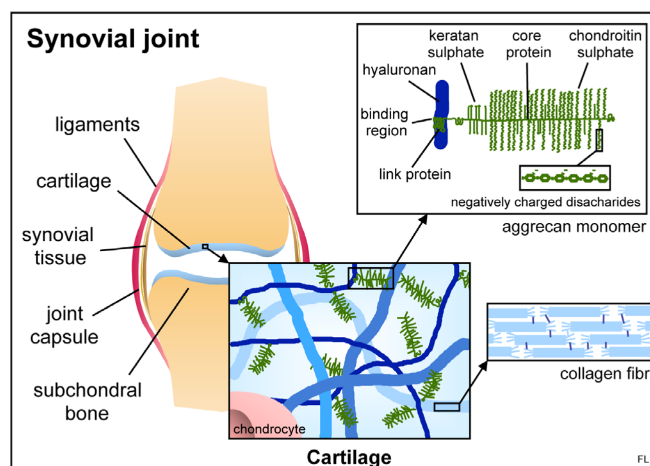

Figure 2: Schematic representation of cartilage in a synovial joint
The two bone ends of the schematic synovial knee joint at the left are covered with cartilage, which is enlarged in the picture below. Cartilage mainly consists of proteoglycans like aggrecan and collagen fibrils. For more details, see text.

negative charge that causes high osmotic pressure, thereby attracting water (68-85% of the wet weight) and resisting compressive forces on the joint cartilage. Upon loading water is forced out of the tissue and after loading water is attracted again, although swelling is limited due to the resistance of the collagen network. This intermittent hydrostatic pressure is of importance in nourishment of cartilage, in load distribution to protect subchondral bone against peak stress, and it contributes to smooth movement under high load[10-13].

The extracellular matrix is divided into subregions according to their functional role: superficial zone, middle zone, deep zone, and calcified zone. In the superficial zone chondrocytes are flattened and aligned to the surface, and produce lubricin to reduce friction in the joint[14]. Collagen fibrils are thin and parallel to the surface and only a small amount of proteoglycans is present. The middle zone has a lower rounded cell density compared to the superficial zone and high proteoglycan content. The deep zone contains even less chondrocytes as well as collagen and has maximal proteoglycan content. The calcified zone provides an intermediate zone between uncalcified cartilage and subchondral bone[15].

In healthy cartilage synthesis and degradation of the cartilage matrix are balanced, resulting in a normal turnover of cartilage matrix. In case of diseased cartilage this balance is disturbed and the extracellular matrix is broken down partly by upregulation of proteolytic enzymes such as 'matrix metalloproteinases' (MMPs) and 'a disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS)[16-18].

Subchondral bone

Subchondral bone consists of a compact subchondral bone plate supported by trabecular bone[19]. The main function is to provide support for the overlaying cartilage. Although originally a tight boundary between cartilage and bone was considered[20], more recent ideas suggest interaction between bone and cartilage[21-23]. The subchondral plate contains many arterial and venous blood vessels with tiny branches into the calcified cartilage. It is now even suggested that hollow spaces provide a direct connection between uncalcified cartilage and the marrow cavity of trabecular bone, thereby supplying nutrients to the cartilage[24].

Bone is subject to remodelling; it is resorbed by osteoclasts, followed by a phase of bone formation by osteoblasts. In healthy conditions this process is balanced such that the bone mass is maintained. Intra-articular bleedings cause changes to the bone. Most often osteophytes, bone sclerosis, and subchondral cysts are seen, but also bone erosions due to secondary synovial inflammation[25].

Pathogenesis of blood-induced joint damage

In the pathogenesis of blood-induced joint damage both inflammatory changes in synovial tissue and degenerative changes in cartilage are involved (figure 3). During natural evacuation of blood from the joint cavity red blood cells (RBC) are damaged and broken

down, leading to deposition of iron (haemosiderin) in the synovial tissue. This results in proliferation and hypertrophy of the synovium, fibrosis, and neovascularisation[26]. In response to these synovial changes infiltration of the synovial tissue with lymphocytes results in an inflammatory reaction, contributing to cartilage damage. Recently it has been demonstrated that induced joint bleeds in haemophilic mice lead to elevation of pro-inflammatory cytokines (IL-1β, IL-6, KC and MCP-1) in the synovial fluid[27], supporting the existence of an inflammatory synovial component in pathogenesis of haemophilic arthropathy. These released cytokines, especially IL-1β and TNF-α, are known for their direct effect on cartilage integrity[28].

Devastating effects of a joint bleeding are also evident independent of synovial inflammation. In vitro exposure of cartilage tissue to whole blood (50% volume/volume) for 4 days leads to disturbance of cartilage matrix turnover[29]. This is expressed by an inhibited synthesis of one of the major matrix components, aggrecan, and an increased breakdown of these molecules, important for resilience of the tissue[29]. These changes lead to in an impaired matrix integrity as measured by a diminished glycosaminoglycan content of the tissue[29]. Even after a follow-up period of 10 weeks matrix synthesis is still inhibited[30]. The in vitro experiments represent the exposure time and blood load during a natural joint bleed in humans, as 4 days is considered to be the natural evacuation time in humans and concentrations will be over 50% v/v. Disturbance of matrix turnover on the long-term is suggested to be caused by apoptosis of the chondrocyte, since inhibition of caspases, involved in the apoptotic process, results in normalization of matrix synthesis[31]. Since the hardly proliferating chondrocyte is the only cell type of cartilage and responsible for production and maintenance of the extracellular matrix, apoptosis of chondrocytes will result in a long lasting, if not permanent, impaired matrix turnover. In that condition, cartilage will be unable to handle normal loading resulting in further damage of the tissue, as supported by canine in vivo studies[32].

In vitro studies revealed that the combination of mononuclear cells (MNC) and RBC as present in whole blood, on their own can have the same effect as whole blood. A possible explanation for the irreversible damage by this combination, and with that by whole blood, is the conversion of hydrogen peroxide, produced by IL-1 activated monocytes/macrophages, and a catalyst in the form of iron supplied by RBC, into hydroxyl radicals. Scavenging these hydroxyl radicals with e.g. dimethylsulphoxide (DMSO) diminishes inhibition of matrix synthesis[33].

Translation from in vitro and animal in vivo models to human blood-induced joint damage

When choosing an animal model to study the effects of a joint bleed in vivo, there are several aspects one needs to take into account[34]. First, cartilage of smaller animals is more cellular than cartilage of larger animals (including humans)[35], thereby having a higher matrix turnover rate. Especially in studies investigating treatment modalities, a fast turnover in smaller species could bias the results, since a faster turnover is expectedly

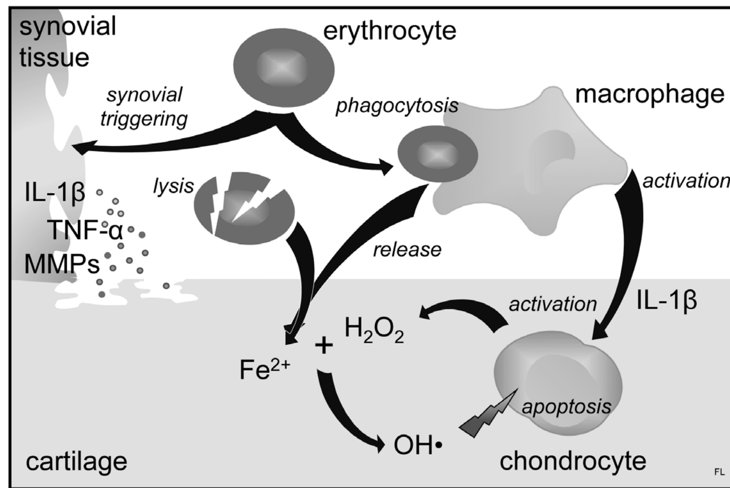

Figure 3: Proposed mechanism of blood-induced joint damage
To major components of blood are erythrocytes and monocytes/macrophages. Blood that is present in the joint cavity will trigger synovial tissue. Thereby the synovium gets inflamed and produces inflammatory cytokines, like IL-1β and TNF-α, and MMPs, which are all destructive to the cartilage.
Erythrocytes in the joint cavity can also be phagocytosed by macrophages. They get activated and produce IL-1β, which in turn leads to a higher production of H2O2 by chondrocytes. H2O2 in combination with free iron (Fe2+) reacts via the Fenton reaction and forms hydroxyl radicals (OH•). These radicals cause apoptosis of chondrocytes and thereby disturbance of cartilage matrix turnover.

related to a faster cure. Second, the thickness of cartilage varies between species; femoral condyl thickness of mice is around 0.05 mm and of humans 2-3 mm[35]. This will have influence on the impact of a joint haemorrhage on cartilage. Third, biomechanics of the animal joint should mimic those of a human joint as good as possible.
Previous research in a canine in vivo model shows that intra-articular injections of autologous blood immediately decrease cartilage matrix synthesis and content, and enhance release of matrix components[36]. This represents breakdown of the cartilage, as is also seen in comparable in vitro experiments[37]. The effects persists for a short period of time despite an, apparently ineffective, repair activity[36]. However, finally repair activity prevails and cartilage damage vanishes[30]. Clearly the effects in dogs are less severe than seen in vitro, revealing a discrepancy between in vitro and in vivo experiments. It is demonstrated that blood is cleared form the canine joint much quicker than generally observed in humans. After injection of a maximum amount of blood in the canine knee joint, the volume of blood decreases to less than 5% within 48 hours[38], while the average exposure time in humans, rats and rabbits is supposed to be 4 days[39-41]. Based on this knowledge of the canine model, it can be concluded that blood exposure in the canine model is less than in a human in vivo and in vitro situation. Moreover, since exposure to a concentration of at least 10% for more than 2 days is needed to maintain the irreversible adverse effects of blood on cartilage[42], it is likely that this blood load has not been

reached in the canine model. As such, it is important to select the correct animal model and set-up for each study for proper translation of results from basic science to clinical practice.

Current treatment of blood-induced joint damage

Currently, blood-induced joint damage itself cannot be treated, only the underlying causes of a joint bleed. In case of haemophilia, the most logical and appropriate treatment is replacement of clotting factor. The average annual consumption of clotting factor prophylaxis in the Netherlands increased from 886 IU/kg in the 1970s to 1880 IU/kg in the 1990s. This resulted in a decrease in the average number of joint bleeds per year from 7.7 in the 1970s to 2.8 in the 1990s. Even more important, the increase in Pettersson score per year, representing development of joint damage in haemophilia patients, decreased from 1.0 in the 1970s to 0.4 in the 1990s[43]. This indicates that clotting factor replacement therapy prevents some joint damage due to prevention of joint bleeds. However, even with improved clotting factor replacement therapy, haemophilic arthropathy still occurs and average joint damage still deteriorates over the years.
The most important complication of clotting factor replacement therapy is formation of inhibitory antibodies against factor VIII or factor IX concentrate. The reported prevalence of inhibitors in unselected haemophilia populations is about 5-7%[44]. The number of joint bleeds per year is higher for patients with inhibitory antibody formation compared to patients without inhibitory antibodies. This may explain why currently available bypassing therapies as recombinant factor VII and activated prothrombin complex concentrate are inferior to factor VIII of factor IX concentrate[45]. It demonstrates that even though clotting factor replacement therapy is available, joints of haemophilia patients still are subjected to deterioration and as such additional treatment is required.
As mentioned before, haemophilic arthropathy is characterised by synovial inflammation as well as cartilage degradation. Synovitis can be treated by radioactive synovectomy (synoviorthesis; using ^{90}Y for the knees and ^{186}Rh for the elbows and ankles)[46] or arthroscopic synovectomy[47]. Both types of synovectomy result in reduction of the frequency and intensity of joint bleeds[46-48] and thereby limiting further deterioration of the joint. When blood-induced cartilage damage already resulted in chronic pain and disability, there are a few surgical treatment options available. The most commonly used technique for the ankle is fusion (arthrodesis) of the joint which is effective in pain relief, but at the expense of joint motion[49]. Function of a joint can be restored when total joint replacement (arthroplasty) is applied, having good results on the long term for knees, hips and elbows[50-52], except for a higher infection prevalence when compared to osteoarthritis patients.

In case of trauma or surgery a more obvious treatment would be to aspirate blood from a joint. This would prevent or at least limits development of blood-induced joint damage. Surprisingly there is no consensus on aspiration (arthrocentesis) of a joint after a joint bleed, neither in case of haemophilia nor after major joint trauma[53,54].

Outline of this thesis

Mechanism

In the first part of this thesis the mechanism of blood-induced joint damage is further clarified using *in vivo* models. It was investigated whether a more intensive blood load, accumulated at two time points, leads to more prolonged cartilage damage in a canine model than the same blood load equally distributed over a similar time period (**chapter 2**). Subsequently, it was investigated whether there is a difference in the effects of coagulating blood (as seen in trauma and surgery) or anticoagulated blood (to mimic haemophilic blood) on joint damage (**chapter 3**). In contrast to *in vitro* experiments, it is more difficult to determine minor biochemical changes in a joint due to a joint bleed *in vivo*. Biomarkers representing cartilage matrix and bone turnover as well as synovial inflammation can be detected in blood and urine. **Chapter 4** describes a study in which biochemical serum and urine markers were measured after a joint bleed in order to evaluate their usefulness in detection of early joint damage.

Treatment

The second part of this thesis focuses on potential treatment modalities for blood-induced joint damage. In **chapter 5**, it is investigated whether the use of two anti-inflammatory cytokines, interleukin (IL)-4 and -10, can prevent blood-induced cartilage damage *in vitro* when they are added during blood exposure of cartilage. However, in clinical practice it will not be feasible to administer a treatment like IL-4 and IL-10 at the same time as the onset of a joint bleed. Therefore, the potency of delayed combined IL-4 and IL-10 treatment is explored when administered at several time points after start of blood-exposure in vitro (**chapter 6**). In the *in vitro* culture system that was used synovial tissue is not available, although it has an important role in the pathobiology of blood-induced joint damage. An *in vivo* model provides interaction between all tissues. Therefore the effect of IL-4 and IL-10 on a complete joint is analysed in a murine haemophilia model (**chapter 7**). A disadvantage of IL-4 and IL-10 is that they have low bioavailability *in vivo*. One way to increase the half-life of these cytokines is to design a fusion protein. Because a fusion protein will be larger in size and glycosylated, the bioavailability will be larger *in vivo*. However, it needed to be sure that such a fusion protein is still able to counteract damaging effects of blood on cartilage *in vitro*. The results of an IL-4/10 fusion protein on protection against blood-induced cartilage damage are described in **chapter 8**. The previous chapters demonstrate that anti-inflammatory cytokines could be used to prevent damage that is caused by blood. However, a lot of patients already suffer from damaged joints. Joint distraction is a surgical treatment, which leads to both clinical and

structural effects in severe osteoarthritic patients. This treatment is successfully used as alternative to surgical treatments like ankle arthrodesis or total joint replacements. As a first step ankle joint distraction was performed on 3 haemophilia patients and the results are described in **chapter 9**.

Chapter 10 summarizes the previous chapters and places them in a broader perspective.

REFERENCES

1. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
2. Philipp C. The aging patient with hemophilia: complications, comorbidities, and management issues. Hematology Am Soc Hematol Educ Program 2010;2010:191-6.
3. Stonebraker JS, Bolton-Maggs PH, Michael Soucie J, Walker I, Brooker M. A study of variations in the reported haemophilia B prevalence around the world. Haemophilia 2011.
4. Stonebraker JS, Bolton-Maggs PH, Soucie JM, Walker I, Brooker M. A study of variations in the reported haemophilia A prevalence around the world. Haemophilia 2010;16:20-32.
5. Rodriguez-Merchan EC, Jimenez-Yuste V, Aznar JA, Hedner U, Knobe K, Lee CA, et al. Joint protection in haemophilia. Haemophilia 2011;17 Suppl 2:1-23.
6. Smith MD. The normal synovium. Open Rheumatol J 2011;5:100-6.
7. Barland P, Novikoff AB, Hamerman D. Electron microscopy of the human synovial membrane. J Cell Biol 1962;14:207-20.
8. Revell PA. Synovial lining cells. Rheumatol Int 1989;9:49-51.
9. Alford JW, Cole BJ. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. Am J Sports Med 2005;33:295-306.
10. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 1998;47:477-86.
11. Pearle AD, Warren RF, Rodeo SA. Basic science of articular cartilage and osteoarthritis. Clin Sports Med 2005;24:1-12.
12. Smith RL, Carter DR, Schurman DJ. Pressure and shear differentially alter human articular chondrocyte metabolism: a review. Clin Orthop Relat Res 2004:S89-95.
13. van Valburg AA, van Roy HL, Lafeber FP, Bijlsma JW. Beneficial effects of intermittent fluid pressure of low physiological magnitude on cartilage and inflammation in osteoarthritis. An in vitro study. J Rheumatol 1998;25:515-20.
14. Schumacher BL, Hughes CE, Kuettner KE, Caterson B, Aydelotte MB. Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints. J Orthop Res 1999;17:110-20.
15. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001:S26-33.
16. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. Arthritis Res Ther 2009;11:224.
17. Aigner T, McKenna L. Molecular pathology and pathobiology of osteoarthritic cartilage. Cell Mol Life Sci 2002;59:5-18.
18. Cawston TE, Wilson AJ. Understanding the role of tissue degrading enzymes and their inhibitors in development and disease. Best Pract Res Clin Rheumatol 2006;20:983-1002.
19. Madry H, van Dijk CN, Mueller-Gerbl M. The basic science of the subchondral bone. Knee Surg Sports Traumatol Arthrosc 2010;18:419-33.
20. Clark JM. The structure of vascular channels in the subchondral plate. J Anat 1990;171:105-15.
21. Funck-Brentano T, Cohen-Solal M. Crosstalk between cartilage and bone: when bone cytokines matter. Cytokine Growth Factor Rev 2011;22:91-7.
22. Matsui H, Shimizu M, Tsuji H. Cartilage and subchondral bone interaction in osteoarthrosis of human knee joint: a histological and histomorphometric study. Microsc Res Tech 1997;37:333-42.
23. Westacott C. Interactions between subchondral bone and cartilage in OA. Cells from osteoarthritic bone can alter cartilage metabolism. J Musculoskelet Neuronal Interact 2002;2:507-9.
24. Arkill KP, Winlove CP. Solute transport in the deep and calcified zones of articular cartilage. Osteoarthritis Cartilage 2008;16:708-14.
25. Jacobson JA, Girish G, Jiang Y, Sabb BJ. Radiographic evaluation of arthritis: degenerative joint disease and variations. Radiology 2008;248:737-47.

26. Acharya SS, Kaplan RN, Macdonald D, Fabiyi OT, DiMichele D, Lyden D. Neoangiogenesis contributes to the development of hemophilic synovitis. Blood 2011;117:2484-93.
27. Ovlisen K, Kristensen AT, Jensen AL, Tranholm M. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from haemophilic mice with experimentally induced haemarthrosis. Haemophilia 2009;15:802-10.
28. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, et al. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum 2005;52:128-35.
29. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. J Rheumatol 1997;24:1350-4.
30. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. J Rheumatol 2003;30:339-44.
31. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
32. Hooiveld MJ, Roosendaal G, Jacobs KM, Vianen ME, van den Berg HM, Bijlsma JW, et al. Initiation of degenerative joint damage by experimental bleeding combined with loading of the joint: a possible mechanism of hemophilic arthropathy. Arthritis Rheum 2004;50:2024-31.
33. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
34. Mastbergen SC, Lafeber FP. Animal models of osteoarthritis - Why choose a larger model? US Musculoskeletal Review 2009;4:11-14.
35. Stockwell RA. The interrelationship of cell density and cartilage thickness in mammalian articular cartilage. J Anat 1971;109:411-21.
36. Roosendaal G, TeKoppele JM, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum 1999;42:1033-9.
37. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
38. Jansen NW, Roosendaal G, Wenting MJ, Bijlsma JW, Theobald M, Hazewinkel HA, et al. Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. Osteoarthritis Cartilage 2009;17:433-40.
39. Niibayashi H, Shimizu K, Suzuki K, Yamamoto S, Yasuda T, Yamamuro T. Proteoglycan degradation in hemarthrosis. Intraarticular, autologous blood injection in rat knees. Acta Orthop Scand 1995;66:73-9.
40. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia 2006;12 Suppl 3:117-21.
41. Safran MR, Johnston-Jones K, Kabo JM, Meals RA. The effect of experimental hemarthrosis on joint stiffness and synovial histology in a rabbit model. Clin Orthop Relat Res 1994:280-8.
42. Jansen NW, Roosendaal G, Bijlsma JW, Degroot J, Lafeber FP. Exposure of human cartilage tissue to low concentrations of blood for a short period of time leads to prolonged cartilage damage: an in vitro study. Arthritis Rheum 2007;56:199-207.
43. Fischer K, van der Bom JG, Mauser-Bunschoten EP, Roosendaal G, Prejs R, Grobbee DE, et al. Changes in treatment strategies for severe haemophilia over the last 3 decades: effects on clotting factor consumption and arthropathy. Haemophilia 2001;7:446-52.
44. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia 2003;9:418-35.
45. Hoots WK. Arthropathy in inhibitor patients: differences in the joint status. Semin Hematol 2008;45:S42-9.
46. Rodriguez-Merchan EC, Quintana M, De la Corte-Rodriguez H, Coya J. Radioactive synoviorthesis for the treatment of haemophilic synovitis. Haemophilia 2007;13 Suppl 3:32-7.
47. Verma N, Valentino LA, Chawla A. Arthroscopic synovectomy in haemophilia: indications, technique and results. Haemophilia 2007;13 Suppl 3:38-44.

48. Yoon KH, Bae DK, Kim HS, Song SJ. Arthroscopic synovectomy in haemophilic arthropathy of the knee. Int Orthop 2005;29:296-300.
49. Rodriguez-Merchan EC. Aspects of current management: orthopaedic surgery in haemophilia. Haemophilia 2012;18:8-16.
50. Zingg PO, Fucentese SF, Lutz W, Brand B, Mamisch N, Koch PP. Haemophilic knee arthropathy: long-term outcome after total knee replacement. Knee Surg Sports Traumatol Arthrosc 2012.
51. Wang K, Street A, Dowrick A, Liew S. Clinical outcomes and patient satisfaction following total joint replacement in haemophilia--23-year experience in knees, hips and elbows. Haemophilia 2012;18:86-93.
52. Goddard NJ, Mann HA, Lee CA. Total knee replacement in patients with end-stage haemophilic arthropathy: 25-year results. J Bone Joint Surg Br 2010;92:1085-9.
53. Hermans C, De Moerloose P, Fischer K, Holstein K, Klamroth R, Lambert T, et al. Management of acute haemarthrosis in haemophilia A without inhibitors: literature review, European survey and recommendations. Haemophilia 2011;17:383-92.
54. Wallman P, Carley S. Aspiration of acute traumatic knee haemarthrosis. Emerg Med J 2002;19:50.

Chapter 2

Blood-induced joint damage: the devastating effects of acute joint bleeds versus micro-bleeds

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ABSTRACT

Objective:

A 4-day blood exposure leads to irreversible cartilage damage *in vitro*. In contrast, intermittent intra-articular blood injections twice a week during 4 weeks (mimicking micro-bleeds) in a canine model resulted in transient damage only. In this study it was evaluated whether acute joint bleeds are more harmful than micro-bleeds in a canine model of knee arthropathy.

Methods:

Seven dogs received four sequential daily intra-articular blood injections twice in 2 weeks (mimicking two acute 4-day joint bleeds). Seven other dogs received the same blood load but in a total of 8 injections intermittently over the 4-week period with at least 1 day in between (mimicking micro-bleeds over the same timespan). Contralateral knees served as controls. Ten weeks after the last injection cartilage matrix turnover and synovial inflammation were evaluated.

Results:

Only after the acute joint bleeds the release of newly formed and total (resident) cartilage matrix glycosaminoglycans (GAGs) was increased ($p=0.04$ and $p=0.01$, respectively). Furthermore, in the animals with the acute joint bleeds cartilage GAG content was decreased ($p=0.01$), and not in the animals with micro-bleeds. Mild synovial inflammation was observed in both groups (both $p<0.0001$), not different between groups.

Conclusion:

In contrast to micro-bleeds, two acute joint bleeds lead to prolonged cartilage damage independent of the level of synovial inflammation. This model suggests that micro-bleeds are less devastating than acute joint bleeds with respect to joint damage, which might be of relevance to treatment of joint bleeds in clinical practice.

INTRODUCTION

Recurrent distinct joint bleeds in case of haemophilia lead to inflammation, damage of articular cartilage, and eventually to destruction of the whole joint[1,2]. But even in the absence of evident acute joint bleeds, joint damage is observed in haemophilia patients. It has been hypothesised that this is due to chronic micro-bleeds causing deterioration of the joints without a clinically evident acute joint bleed[3].

Natural evacuation of blood from the joint cavity results in deposition of iron (haemosiderin) in the synovial tissue[4]. This causes proliferation and hypertrophy of the synovium, fibrosis, and neovascularisation[5,6]. As a response infiltration of synovial tissue with lymphocytes results in an inflammatory reaction, contributing to cartilage damage[4,7].

In vitro studies have shown that harmful effects of joint bleeds on cartilage are also evident independent of synovial inflammation. The combination of monocytes/macrophages and red blood cells (RBC), as present in whole blood, leads to long-lasting disturbance of cartilage matrix turnover[8]. A proposed mechanism for this irreversible damage is the conversion of hydrogen peroxide and catalytic iron, supplied by damaged RBC, into hydroxyl radicals[9]. Hydrogen peroxide is produced by chondrocytes under the influence of IL-1 formed by activated monocytes/macrophages. Hydroxyl radicals cause chondrocyte apoptosis resulting in permanent cartilage damage[10], since the chondrocyte is the only cell type of cartilage and responsible for maintenance of the cartilage matrix. It is unclear whether these mechanisms take place to a same extent as a result of recurrent distinct acute joint bleeds as well as frequently occurring sub-clinical micro-bleeds.

In humans, it is anticipated from clinical practice that after an acute clinical joint bleed blood reaches volume ratios close to 100% volume/volume (v/v) because the amount of synovial fluid is generally negligible compared to the volume of blood entering the joint. Blood is estimated to be cleared within less than a week assuming the bleed stops. This assumption is supported by rat and rabbit *in vivo* studies using intra-articular injections of blood[11,12]. As such, several *in vitro* studies have used a 4-day blood exposure of 50% v/v to mimic an acute joint bleed and to evaluate the effects and mechanisms of blood-induced cartilage damage. It appeared that the presence of this blood load for such a time period results in long lasting damage *in vitro* of human as well as canine cartilage[8,10,13,14].

In a canine *in vivo* model of blood-induced joint damage, two injections of autologous blood into their knees at day 0 and 2 caused an impaired cartilage matrix turnover as observed directly after blood exposure (day 4)[15]. However, the effects appeared transient: blood-injections given twice a week for 4 weeks did not result in long-term cartilage tissue damage (analysed 10 weeks after the last blood injection)[13]. A possible explanation for the discrepancy between *in vitro* long-term cartilage damage and the absence of prolonged damage *in vivo* could be a difference in the actual exposure load (duration and concentration) of cartilage to blood. In later experiments it appeared that blood is cleared from a canine knee much faster than anticipated originally: blood

concentrations decreased within 2 days to less than 5% v/v[16]. This is much faster than the clearance rate observed in humans as well as in rats and rabbits[11]. As such, in previous experiments canine cartilage was exposed to much lower blood loads *in vivo* than the acute joint bleeds mimicked *in vitro* with human and canine cartilage. As such it might be that lower concentrations of blood (mimicking micro bleeds) are less harmful.
Human *in vitro* studies have shown that there is a threshold for the blood load (duration and concentration) to which cartilage has to be exposed before cartilage damage becomes irreversible. A minimal blood exposure of at least 2 days with 10% v/v blood is needed to cause long-term cartilage damage[17], suggesting that micro-bleeds do not cause irreversible direct breakdown of cartilage. In the present study the effect of two acute 4 days lasting joint bleeds with two weeks interval was compared to the effect of the same blood load, but given intermittently over the 4-week period to mimic micro-bleeds.

MATERIALS AND METHODS

Animals

Fourteen skeletally mature Beagle dogs were divided in 2 groups of 7 animals each (6/8 male/female; mean age 2.1 ± 0.1 years, weighing 9-15 kg; all equally distributed). Animals were obtained from the animal laboratory of the Utrecht University, The Netherlands. They were housed in groups of 2-3 dogs, and were let out on a patio for at least 2 hours daily. They were fed a standard commercial diet with water *ad libitum*. According to strict European regulations, the Utrecht Medical Ethical Committee for animals approved the study.

Experimental design

In all dogs left knees were injected intra-articularly with 1.8 ± 0.1 mL freshly collected (vena puncture) autologous blood. During the experimental procedures, dogs were given short-term anaesthesia (Dormitor/Antisedan). Random checks on accurate injection were made by placing a second needle at the other side of the joint. In all cases a blood flow from the injected joint was demonstrated, indicating the joint was completely filled with blood. After removal of the needles, pressure was put on the puncture holes until the bleed was stopped, preventing leakage of blood outside the joint cavity.
Seven dogs received 4 blood injections in 4 successive days. This was repeated after two weeks. Based on previous studies[16] this results in 2 periods of at least 4 days continuous blood exposure with at least 20% v/v blood, mimicking 2 sequential clinically evident acute joint bleeds (figure 1; acute bleeds). Right knees of these dogs were injected at the same time points with a similar volume of saline.
In the other 7 other dogs, the same blood load was given by 8 injections with at least 1 day in between mimicking sub-clinical micro-bleeds over a same time period (figure 1; micro-bleeds). Right knees of these dogs were kept untouched. Together with the saline injected joints of the other group these joints provided a control for the possible

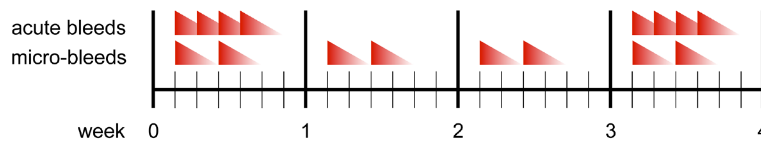

Figure 1: Schedule of blood injections for acute- and micro-bleeds
To mimic two successive clinically evident joint bleeds in 4 weeks, left knees of Beagles were injected with autologous blood for 4 subsequent days twice in 4 weeks. To mimic sub-clinical micro-bleeds over a same time period with a same overall blood load, animals were injected in their left knee twice a week during 4 weeks with at least 1 day in between the injections. As a control, right knees were either injected according to the same injection scheme with an equal volume of saline (acute bleeds) or not injected (micro-bleeds).

inflammatory responses due to the repeated injections.
All animals were sacrificed 10 weeks after the last injection. The dogs were euthanized using intravenous injection of Euthesate (Na-Pentobarbital). Both hind limbs were amputated and high-resolution photographs were taken from the synovial tissue and from the tibial and femoral cartilage surfaces. Within 3 hours after death of the animals, cartilage of condyles and plateau of both knee joints was collected. The cartilage was cut as thick as possible, excluding the underlying bone, and cut into square pieces (mean weight 3.3 ± 0.1 mg). Subsequently, the cartilage tissue samples were put in 96-well round-bottomed microtiter plates with 200 µL culture medium. 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 PAA), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled Beagle serum.

Cartilage analysis

Cartilage damage was evaluated macroscopically on high-resolution digital photographs of tibia and femur by 3 blinded observers. Severity of the damage was graded on a scale from 0-4 according to the OARSI histopathology criteria for canine osteoarthritic (OA) cartilage[18].
Biochemistry of 8 cartilage explants of predefined fixed locations from femoral condyles and tibial plateau was determined and compared with identical locations at the contralateral joint. As a measure of proteoglycan synthesis rate the *ex vivo* radioactive sulphate incorporation rate into glycosaminoglycans (GAGs) was determined. Cartilage explants were placed in fresh equilibrated (37°C, 5% CO_2 in air, 95% humidity) culture medium and 740 kBq per well $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) was added. After 4 hours of pulse labelling, the cartilage samples were washed and incubated fresh culture medium without radioactive label for 3 additional days. Culture medium and cartilage explants that were washed twice in ice cold PBS were stored at -20°C. Thawed cartilage samples were digested for 2 hours at 65°C with 2% papain (Sigma) in a buffered solution containing 50 mM phosphate buffer (pH 6.5; Baker), 2 mM Na_2-EDTA, and 2 mM N-acetylcysteine (both Sigma). GAGs were precipitated from the cartilage digest with 0.1% Alcian Blue (Sigma) and dissolved in 2% SDS (Sigma). The amount of radioactivity

was measured by liquid scintillation analysis. Radioactive counts were normalized to the specific activity of the medium, labelling time, and wet weight of cartilage (nmol/h*g).
As a measure of proteoglycan content, the amount of stained GAGs was quantified by absorptiometry at 620 nm using chondroitin sulphate (Sigma) as a reference. Results were expressed as mg GAG per wet weight of cartilage tissue (mg/g).
As a measure of retention of newly synthesized proteoglycans in the cartilage matrix, the amount of $Na_2{}^{35}SO_4$-labeled GAGs in the 3-day culture medium was assessed by precipitating the total GAG amount with Alcian Blue. The release of labelled GAGs as a measure of newly formed proteoglycans is normalised to GAG synthesis rate and expressed as percentage release of labelled GAGs in the 3 days of culture (% newly formed GAG release). The total release of proteoglycans is expressed as a percentage of the original cartilage GAG content (% GAG release).

Synovial tissue analysis
Inflammation of synovial tissue was evaluated macroscopically on high-resolution digital photographs by 3 blinded observers. Severity of the inflammation was graded on a scale from 0-5 according to the OARSI histopathology initiative developed for dogs[18].

Statistical analyses
All cartilage explants were handled individually and the average result of 8 samples was taken as representative of that joint surface. Paired Student's t-test was used to compare data of the experimental and contralateral control joints within each group. Unpaired t-test was used to analyse differences between the 'acute bleeds' group and the 'micro-bleeds' group. Data were analysed using SPSS 15.0 software and differences were considered statistically significant when $p<0.05$.

RESULTS

Acute joint bleeds causes cartilage degradation
Macroscopic cartilage damage was still minimal after blood exposure and did not differ statistically significant between the acute- and micro-bleed group according to the OARSI criteria of canine cartilage damage (data not shown).
Changes in proteoglycan turnover, a much more sensitive measurement, revealed a cartilage degenerative process. Blood-injections according to the acute bleeds injection scheme, as well as the micro-bleeds scheme, caused an increase in proteoglycan synthesis rate compared to the contralateral control joint (figure 2A; p=0.01 and p=0.02, respectively). This effect appeared to be slightly more outspoken for the acute joint bleeds than for the micro-bleeds (24% vs. 18% increase; right panel figure 2A), although this difference was not statistically significant.
An increased proteoglycan synthesis rate is the expression of initiated (ineffective) repair activity characteristic of early degenerative (osteoarthritic) cartilage[19]. The increase in proteoglycan synthesis rate was indeed ineffective, but only in the knees exposed to acute

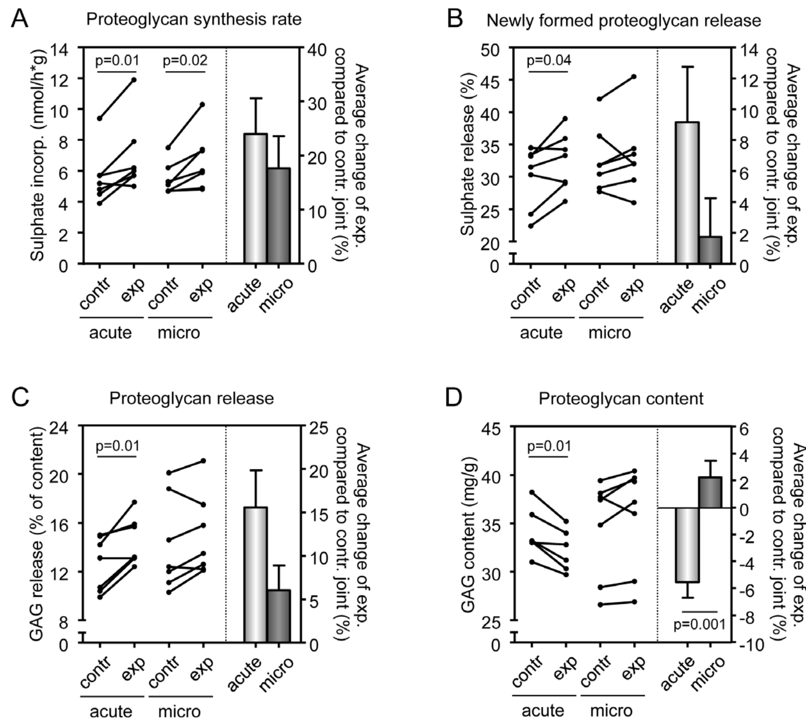

Figure 2: Parameters of cartilage damage upon acute- and micro-bleeds
Proteoglycan synthesis rate (**A**), newly formed proteoglycan release (**B**), total proteoglycan release (**C**), and proteoglycan content (**D**) were measured 10 weeks after the last acute joint bleed (n=7) or after the last micro-bleed (n=7). All these parameters are also expressed as change compared to control leg (right panel of each graph) with bars representing means ± SEM. Contr = control; exp = experimental i.e. blood-injected; acute = acute joint bleeds (white bar); micro = micro-bleeds (grey bar). P values are given in case they are below 0.05, otherwise differences were not statistically significant.

joint bleeds, since the release of newly formed GAGs (indicating a decreased retention of newly formed proteoglycans) was only statistically significantly increased after acute bleeds (figure 2B; 9%, p=0.04). After a micro-bleeds a not statistically significant change of 2% was observed. The total release of proteoglycans, primarily resident proteoglycans in addition to newly formed proteoglycans, was increased with 16% in case of an acute bleed (figure 2C; p=0.01), but only with 6% in case of micro-bleeds (ns).

Ineffective synthesis and enhanced release led to a statistically significant decrease in proteoglycan content in case of acute joint bleeds (figure 2D; p=0.01) and not in case of micro-bleeds. There was even a slight increase of 2% in proteoglycan content compared to contralateral control joints in the micro-bleeds-exposed joints, whereas there was a clear decrease of 6% in the joints exposed to acute joint bleeds when compared to contralateral control joints. This difference between both groups was statistically significant (right panel figure 2D; p=0.001).

Synovial inflammation due to acute- and micro-bleeds

Mild synovial inflammation was observed due to acute- and micro-bleeds when compared to the control knee (an increase of 1.2 versus 1.1 points on the OARSI scale, respectively, on a total scale of 5), statistically significant for both (figure 3E; both p<0.0001). There was no difference in synovial inflammation between both groups. Representative photographs demonstrate this slight increase in inflammation similar for both groups (figure 3A-D). This absence of a difference confirms that the discrepancy in cartilage damage between both groups is due to direct effects of blood on cartilage largely independent of synovial inflammation.

Intra-articular (saline) injections do not cause joint damage

There was no clear difference in cartilage and synovial tissue parameters between the control legs of both groups (figures 2A-D and 3E). Although groups were small for unpaired statistical comparison with significant inter animal variations, there was no sign of a systematic difference between the saline injected joints and the untouched joints of both groups. As such, it may be concluded that the repeated injections in the knee joint with saline did not cause alteration of cartilage biochemistry or synovial inflammation compared to knees that were not injected at all.

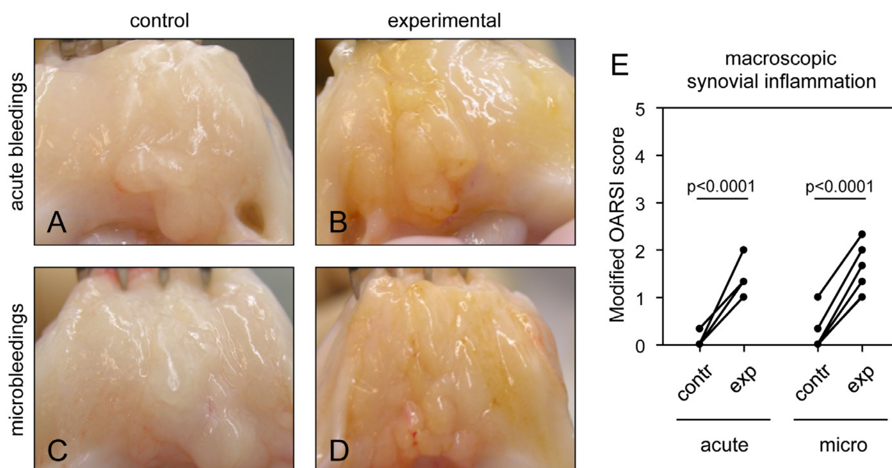

Figure 3: Macroscopic changes of the synovial tissue as a result of acute- and micro-bleeds
Beagle left knee joints were injected according to the acute bleeds protocol (**A** and **B**; n=7) or the micro-bleeds protocol (**C** and **D**; n=7). Representative pictures of control (**A** and **C**) and experimental (blood-injected) synovial tissue (**B** and **D**) 10 weeks after the last injection are shown. Acute joint bleeds as well as micro-bleeds caused synovial inflammation according to the modified OARSI score (scale 0-5) (**E**). Macroscopy was scored by 3 blinded observers and averaged. Contr = control; exp = experimental i.e. blood-injected.

DISCUSSION

The present study demonstrates that exposure to blood for at least 4 days twice in 4 weeks, representing two clinically evident acute joint bleeds, leads to long-lasting cartilage damage. In contrast, a similar blood load applied intermittently over the same time span, thereby representing sub-clinical micro-bleeds, does not result in long-term cartilage damage. Blood exposure, both by acute- and micro-bleeds, results in equal synovial inflammation, which on its own does not induce long-lasting cartilage damage in this model.

Several animal models with different outcomes are used to investigate blood-induced joint damage. A single intra-articular injection with freshly collected autologous blood in rabbit ankle or knee joints does not lead to persistent cartilage damage or loss of joint function[12,20]. A single intra-articular blood injection in a rat knee results in joint damage, although not permanent[21]. In contrast, in joints of dogs that are exposed to high levels of blood (6 intra-articular injections per week during 12-18 weeks) cartilage changes are observed[22]. Also a canine model using high pressure haemarthrosis causes cartilage damage up to 8 weeks post-operatively[23]. Indirectly it is shown that blood has harmful effects in the joint of a canine model using anterior cruciate ligament transection (ACLT) to induce OA. When joint bleeds are prevented during surgery, less synovitis, iron deposits, and cartilage degeneration are found[24].

Additionally, several haemophilic animal models have been developed to investigate joint damage due to bleeds. Haemophilic mice possess a relatively mild bleed phenotype; they mostly only bleed after trauma. A joint bleed evoked by a blunt trauma results in joint swelling because of the bleed and inflammation[25]. In a more sophisticated model a single knee puncture causes a joint bleed and eventually results in cartilage degradation in the knee joint[26]. Haemophilic dogs[27] and sheep[28] tend to bleed spontaneously, which leads to symptoms more closely mimicking humans with severe haemophilic arthropathy. However, these models are unpredictable regarding timing and total blood load.

It is advantageous to provoke controlled blood-induced joint damage models in larger animals because they allow us to investigate the pathology of blood-induced joint damage in more detail under controlled conditions. Furthermore, biochemical properties of cartilage can be evaluated more easily in larger animal models, since there is more cartilage tissue available to analyse. A reason to choose for a canine model is that canine cartilage is clearly more similar to human cartilage with respect to cartilage thickness, number of chondrocytes compared to the extracellular matrix, and the anatomy of the knee when compared to smaller rodent models[29-32].

Our canine blood-induced cartilage damage model shows that clinically evident acute joint bleeds and sub-clinical micro-bleeds result in equal synovial inflammation. This confirms that the difference in cartilage damage between both models is due to direct effects of blood on cartilage, independent of synovial inflammation. Despite the direct effects of blood on cartilage, it is known that inflammation of synovial tissue also plays a major role in blood-induced arthropathy. Deposition of iron in the synovial tissue

ultimately causes proliferation, hypertrophy, fibrosis, and neovascularisation[5,6]. As such, inflamed synovial tissue contributes to cartilage damage at a later stage[4,7]. Because of that, also micro-bleeds can deteriorate cartilage of an affected joint, but probably only over a prolonged period of time.
Cartilage damage present after two successive acute joint bleeds *in vivo* is still mild compared to the irreversible *in vitro* damage after exposure to 50% whole blood for 4 days[13]. A possible explanation for this discrepancy could be that cubic cartilage explants cultured *in vitro* are exposed to blood at all 6 sides, compared to only the articular side that is exposed to blood *in vivo*. The superficial layer of cartilage has a lower permeability when compared to the deeper layers due to a higher collagen content[33]. Furthermore, the articular surface is covered with a thin anionic protein layer representing a charge and size barrier[34]. This provides protection under healthy conditions to articular cartilage. Blood exposure to cartilage that is not protected by its superficial layer is probably the reason of more cartilage damage *in vitro* than *in vivo*. In addition, synovial tissue, which is not present in the *in vitro* culture system, could possibly produce neutralising factors (e.g. transforming growth factor β1 (TGF-β1), interleukin 10 (IL-10), and interleukin-1 receptor antagonist (IL-1ra)[35,36]) that could limit cartilage damage after exposure to blood. These conditions are restricted during *in vitro* circumstances.
Concluding, this study confirms that in a canine *in vivo* model two clinically evident acute joint bleeds within 4 weeks lead to direct cartilage damage, being a process independent of inflammation early in the degenerative process. Furthermore, this model suggests that sub-clinical micro-bleeds do not cause this direct cartilage degeneration but add to joint degeneration by inflammation in the long term. The direct devastating effects of clinical acute bleeds in contrast to sub-clinical micro-bleeds may need special attention regarding treatment of these specific bleeds (for example direct evacuation of blood from the joint).

REFERENCES

1. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. *Clin Orthop Relat Res* 1997**:** 6-11.
2. Jansen NW, Roosendaal G, Lafeber FP. Understanding haemophilic arthropathy: an exploration of current open issues. *Br J Haematol* 2008; **143:** 632-40.
3. Manco-Johnson MJ, Abshire TC, Shapiro AD*, et al.* Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *The New England journal of medicine* 2007; **357:** 535-44.
4. Roosendaal G, Vianen ME, Wenting MJ*, et al.* Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. *J Bone Joint Surg Br* 1998; **80:** 540-5.
5. Madhok R, York J, Sturrock RD. Haemophilic arthritis. *Ann Rheum Dis* 1991; **50:** 588-91.
6. Roy S, Ghadially FN. Synovial membrane in experimentally-produced chronic haemarthrosis. *Ann Rheum Dis* 1969; **28:** 402-14.
7. Ovlisen K, Kristensen AT, Jensen AL, Tranholm M. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from haemophilic mice with experimentally induced haemarthrosis. *Haemophilia* 2009; **15:** 802-10.
8. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. *J Rheumatol* 1997; **24:** 1350-4.
9. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. *Rheumatology (Oxford, England)* 2003; **42:** 784-90.
10. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. *Am J Pathol* 2003; **162:** 943-51.
11. Niibayashi H, Shimizu K, Suzuki K, Yamamoto S, Yasuda T, Yamamuro T. Proteoglycan degradation in hemarthrosis. Intraarticular, autologous blood injection in rat knees. *Acta Orthop Scand* 1995; **66:** 73-9.
12. Safran MR, Johnston-Jones K, Kabo JM, Meals RA. The effect of experimental hemarthrosis on joint stiffness and synovial histology in a rabbit model. *Clin Orthop Relat Res* 1994**:** 280-8.
13. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. *J Rheumatol* 2003; **30:** 339-44.
14. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. *Arthritis Rheum* 1999; **42:** 1025-32.
15. Roosendaal G, TeKoppele JM, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a canine in vivo study. *Arthritis Rheum* 1999; **42:** 1033-9.
16. Jansen NW, Roosendaal G, Wenting MJ*, et al.* Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. *Osteoarthritis Cartilage* 2009; **17:** 433-40.
17. Jansen NW, Roosendaal G, Bijlsma JW, Degroot J, Lafeber FP. Exposure of human cartilage tissue to low concentrations of blood for a short period of time leads to prolonged cartilage damage: an in vitro study. *Arthritis Rheum* 2007; **56:** 199-207.
18. Cook JL, Kuroki K, Visco D, Pelletier JP, Schulz L, Lafeber FP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the dog. *Osteoarthritis Cartilage* 2010; **18 Suppl 3:** S66-79.
19. Lafeber FP, van Roy H, Wilbrink B, Huber-Bruning O, Bijlsma JW. Human osteoarthritic cartilage is synthetically more active but in culture less vital than normal cartilage. *J Rheumatol* 1992; **19:** 123-9.
20. Tan AH, Mitra AK, Chang PC, Tay BK, Nag HL, Sim CS. Assessment of blood-induced cartilage damage in rabbit knees using scanning electron microscopy. *J Orthop Surg (Hong Kong)* 2004; **12:** 199-204.
21. Ravanbod R, Torkaman G, Esteki A. Biotribological and biomechanical changes after experimental haemarthrosis in the rabbit knee. *Haemophilia* 2010; **17:** 124-33.
22. Hoaglund FT. Experimental hemarthrosis. The response of canine knees to injections of autologous blood. *J Bone Joint Surg Am* 1967; **49:** 285-98.
23. Sancho FG. Experimental model of haemophilic arthropathy with high pressure haemarthrosis. *Int Orthop* 1980; **4:** 57-62.

24. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. *Arthritis Rheum* 1990; **33:** 1406-15.
25. Valentino LA, Hakobyan N, Kazarian T, Jabbar KJ, Jabbar AA. Experimental haemophilic synovitis: rationale and development of a murine model of human factor VIII deficiency. *Haemophilia* 2004; **10:** 280-7.
26. Hakobyan N, Enockson C, Cole AA, Rick Sumner D, Valentino LA. Experimental haemophilic arthropathy in a mouse model of a massive haemarthrosis: gross, radiological and histological changes. *Haemophilia* 2008; **14:** 804-09.
27. Ovlisen K, Kristensen AT, Tranholm M. In vivo models of haemophilia - status on current knowledge of clinical phenotypes and therapeutic interventions. *Haemophilia* 2008; **14:** 248-59.
28. Porada CD, Sanada C, Long CR*, et al.* Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. *J Thromb Haemost* 2009; **8:** 276-85.
29. Frisbie DD, Cross MW, McIlwraith CW. A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. Vet Comp Ortho p Traumatol 2006; 19: 142-6.
30. Griffith CJ, Laprade RF, Coobs BR, Olson EJ. Anatomy and biomechanics of the posterolateral aspect of the canine knee. J Orthop Res 2007; 25: 1231-42.
31. Mastbergen SC, Lafeber FP. Animal models of osteoarthritis - Why choose a larger model? *US Musculoskeletal Review* 2009; **4:** 11-4.
32. Stockwell RA. The interrelationship of cell density and cartilage thickness in mammalian articular cartilage. *J Anat* 1971; **109:** 411-21.
33. Muir H, Bullough P, Maroudas A. The distribution of collagen in human articular cartilage with some of its physiological implications. *J Bone Joint Surg Br* 1970; **52:** 554-63.
34. Stanescu R, Leibovich SJ. The negative charge of articular cartilage surfaces. An electron microscopic study using cationized ferritin. *J Bone Joint Surg Am* 1982; **64:** 388-98.
35. Bucht A, Larsson P, Weisbrot L*, et al.* Expression of interferon-gamma (IFN-gamma), IL-10, IL-12 and transforming growth factor-beta (TGF-beta) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA). *Clin Exp Immunol* 1996; **103:** 357-67.
36. Firestein GS, Berger AE, Tracey DE*, et al.* IL-1 receptor antagonist protein production and gene expression in rheumatoid arthritis and osteoarthritis synovium. *J Immunol* 1992; **149:** 1054-62.

Chapter 3

Coagulation aggravates blood-induced joint damage

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ABSTRACT

Objective:

Joint bleeding due to trauma, major joint surgery, or haemophilia leads to joint damage. It is unclear if there are differences between coagulating blood and anticoagulated blood with respect to joint degeneration, especially *in vivo*. Therefore, we evaluated in a canine *in vivo* model whether intra-articular blood exposure is more destructive in case of coagulating blood compared to anticoagulated blood, and whether inflammation plays a role in the cartilage damaging process.

Methods:

In 7 dogs left knees were injected with coagulating blood 4 times a week in week 1 and 4; right knees with saline. In 7 other dogs anticoagulated, heparinised blood was injected with heparinised saline as control. Ten weeks after the last injection, cartilage matrix turnover and synovial inflammation were analysed. To study inflammation-independent cartilage damage, explants of at least 6 human donors per group were exposed *in vitro* to coagulating and anticoagulated blood, plasma, and serum for 4 days (n=6). Cartilage matrix turnover was determined at day 16.

Results:

Canine knees injected with coagulating blood showed a more disturbed proteoglycan turnover than knees injected with anticoagulated blood. Synovial inflammation was only present after intra-articular injections with coagulating blood. Coagulation of blood *in vitro* resulted in more cartilage damage compared to anticoagulated blood, while plasma and serum did not alter cartilage matrix turnover.

Conclusion:

This study shows that coagulating blood causes more long-lasting *in vivo* joint damage than anticoagulated blood, thereby suggesting that, besides a haemophilic joint bleed, intra-articular blood should also be avoided during surgery and trauma to prevent joint damage.

INTRODUCTION

Intra-articular bleeding due to trauma, major joint surgery, or haemophilia leads to joint damage[1-3]. Previous *in vitro* research has shown that a single exposure of (healthy) cartilage to blood can lead to long-lasting, irreversible damage without involvement of synovial tissue[4]. The combination of monocytes/macrophages and red blood cells as present in blood is responsible for inhibition of cartilage matrix synthesis[4], because it induces apoptosis of chondrocytes by formation of hydroxyl radicals[5]. Small amounts of interleukin (IL)-1, produced by activated monocytes/macrophages, increase the production of hydrogen peroxide by chondrocytes. Hydroxyl radicals are subsequently formed by the Fenton reaction in which hydrogen peroxide reacts with erythrocyte-derived iron in the vicinity of chondrocytes[6]. This proposed mechanism is assumed to be the reason of irreversible inhibition of cartilage matrix synthesis.

In addition to the direct effects of blood on cartilage, natural clearance of blood from the joint cavity leads to deposition of iron (haemosiderin) in the synovial tissue, resulting in proliferation and hypertrophy of the synovium, fibrosis, and neovascularisation[7]. Consequently, synovial tissue gets infiltrated with lymphocytes causing an inflammatory reaction, which contributes to cartilage damage[8].

Clinical observations in traumatic, non-haemophilic, haemarthroses gave the impression that intra-articular blood did not coagulate appropriately after surgery because of intra-articular fibrinolysis, even though in nearly half of the patients small soft clots were found[9]. The capacity of blood to form clots in presence of synovial fluid is confirmed by an *in vitro* study in which it has been shown that clotting time of blood is not markedly altered by the presence of synovial fluid[10]. However, dilution of blood in synovial fluid did alter the coagulation torque profile over time, decreased the final mechanical stiffness slightly, and increased the permeability of the blood clots. This could explain clinical observations of intra-articular blood clots being soft and small. The coagulation cascade involves activation of enzymes (e.g. tissue factor, thrombin, and plasmin) that may add to cartilage tissue breakdown and synovial inflammation[11-13]. As such, the question is whether coagulation of intra-articular blood is unfavourable for development of joint damage. Previous *in vitro* research showed that there is slightly more cartilage damage due to 4 days exposure to 50% volume/volume (v/v) coagulating blood compared to anticoagulated blood, although differences did not reach statistical significance[14]. Since it is known that a molecular crosstalk exists between coagulation and inflammatory pathways via proteinase-activated receptors (PAR)[15,16], coagulation of blood could cause additional joint damage via synovial tissue inflammation.

These *in vitro* experiments suggest that coagulating blood due to joint trauma or major joint surgery could cause more joint damage than absent or delayed coagulation of blood in case of haemophilia after a joint haemorrhage. However, this has never been demonstrated *in vivo*, whereas it may have clinical implications for treatment of a joint bleed in haemophilia as well as after trauma or major surgery. Therefore, in the present study it is evaluated in a canine *in vivo* model whether intra-articular blood exposure is

more destructive in case of coagulating blood when compared to anticoagulated blood, and whether inflammation plays a role in the cartilage damaging process.

MATERIALS AND METHODS

Canine *in vivo* experiments

Fourteen skeletally mature Beagle dogs (6 males and 8 females, mean ± SEM age 2.1 ± 0.1 years, weighing 7-15 kg) were obtained from the animal laboratory of the Utrecht University, The Netherlands. They were housed in groups of 2-3 dogs, and were let out on a patio for at least 2 hours daily. They were fed a standard commercial diet with water *ad libitum*. The Utrecht Medical Ethical Committee for animals approved the study.
In all dogs both knee joints were injected with a mean ± SEM volume of 1.9 ± 0.1 mL of fluid, until the intra-articular cavity was completely filled. The injections occurred under short-term anaesthesia (Dormitor/Antisedan), 4 times a week in week 1 and week 4. In 7 dogs the left knee was injected with freshly collected autologous coagulating blood, and the right knee with a similar volume of saline. In the other 7 dogs freshly collected autologous anticoagulated blood (17 IU/mL heparin sodium; Leo) was injected, while the contralateral knee was injected with the same volume of saline and heparin concentration as injected in the experimental joints. Appropriateness of the intra-articular injection of blood was randomly confirmed by placing a second needle in the contralateral side of the joint through which blood flowed after injection. When this occurred, the second needle was removed and the proper volume of blood was injected. The animals were killed 10 weeks after the last injection, using intravenous injection of Euthesate (Na-Pentobarbital). Both hind limbs were amputated and within 3 hours joints were opened under laminar flow conditions. High-resolution photographs were taken from synovium of the suprapatellar pouch and from cartilage of condyles and plateau of both knee joints. Cartilage and synovial tissue were collected aseptically. The cartilage was cut as thick as possible, excluding the underlying bone, and cut into square pieces (mean ± SEM weight 3.3 ± 0.1 mg). Subsequently, the cartilage tissue samples were put in 96-well round-bottomed microtiter plates with culture medium. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin sulphate (100 µg/mL; all PAA), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled Beagle serum.

Canine cartilage and synovium analysis

Cartilage damage was evaluated macroscopically on high-resolution digital photographs of tibia and femur by 3 blinded observers. Severity of the damage was graded on a scale from 0-4 according to the OARSI guidelines for evaluation of canine cartilage damage macroscopically[17].
For biochemical analyses, 8 cartilage explants of predefined fixed locations from femoral condyles and from tibial plateau were used and compared with samples of identical locations from the contralateral (control) joint. All cartilage explants were handled

individually and the average result of 8 samples was taken as representative of each joint surface. Proteoglycan synthesis rate and release of newly formed proteoglycans were evaluated *ex vivo* by ^{35}sulfate incorporation rate and release of radioactive-labelled glycosaminoglycans (GAGs), respectively[18]. In short: 740 kBq of $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) was added to each cartilage explant. After 4 hours of pulse labelling of sulphated GAGs, the cartilage samples were washed thoroughly under culture conditions and cultured for an additional 3 days without label. Cartilage explants were digested in papain (Sigma) and GAGs from the papain digest and from the 3-day culture medium were precipitated and stained with Alcian Blue (Sigma). The amount of radioactivity was measured by liquid scintillation analysis and the amount of blue staining was quantified at 620 nm using chondroitin sulphate (Sigma) as a reference. Both radioactivity of cartilage and 3-day culture medium were normalised to the specific activity of the pulse medium, labelling time, and wet weight of cartilageProteoglycan synthesis rate is expressed as nanomoles of sulphate incorporated per gram wet weight of cartilage tissue (nmol/h*g). The release of newly formed proteoglycans is expressed as a percentage release of newly formed proteoglycans during 3 days of culture (% of proteoglycan synthesis rate). Proteoglycan content is expressed as mg GAG per wet weight of cartilage tissue. The total release of proteoglycans is expressed as a percentage of the original content (% of GAG content).

Synovial inflammation was evaluated macroscopically on high-resolution digital photographs by 3 blinded observers. Severity of inflammation was graded on a scale from 0-5 according to the OARSI guidelines[17]. Three samples of synovial tissue were fixed in 4% phosphate-buffered formalin for histochemistry. Deparaffined sections were stained with haematoxylin-eosin and scored for degree of inflammation by 2 blinded observers on a range of 0-18 according to the OARSI guidelines for histological evaluation of canine synovial inflammation[17].

Furthermore, the catabolic effects of inflamed synovial tissue on cartilage were tested. Two samples of synovial tissue per joint were cultured for 3 days, after which culture supernatant was stored at -80°C. Hip cartilage of another set of dogs was processed as described above and exposed to a concentration of 20% v/v of the synovial tissue culture supernatants for 4 days. Proteoglycan synthesis rate and -release of the cartilage were measured as described before.

Human *in vitro* experiments

Healthy human full-thickness articular cartilage tissue was obtained *post mortem* from humeral heads within 24 hours after death of the donor, according to the medical ethical regulations of the University Medical Center Utrecht. The donors (n=6, mean ± SEM age 67 ± 6 years, 4 males and 2 females) had no known history of joint disorders. Human cartilage was found equally sensitive to blood compared to canine cartilage[19]. Cartilage was cut aseptically and the underlying bone was excluded. Slices were kept in phosphate-buffered saline (PBS, pH 7.4). Within one hour after dissection, slices were cut in small full thickness cubes and weighed aseptically (range 5-15 mg, accuracy ±

0.1 mg). Explants were cultured individually in a 96-wells round-bottomed microtiter plate containing 200 µL medium per well in an incubator (at 5% CO_2 in air, 37°C, and pH 7.4). Culture medium was the same as described in the canine *in vivo* experiments, only Beagle serum was replaced by 10% heat inactivated pooled human AB^+ serum.

For each experiment, fresh blood was drawn from healthy human donors (n=6, mean ± SEM age 25 ± 1 year, 3 males and 3 females) in vacutainer tubes, either a plasma tube (367880, Becton Dickinson) or a serum tube (367820, Becton Dickinson) to obtain anticoagulated and coagulating blood as well as plasma and serum (both centrifuged at 1300 *g* for 10 minutes), respectively. Explants were exposed to 50% v/v coagulating or anticoagulated whole blood, plasma, or serum for 4 days, which is considered to be the natural evacuation time of blood from the joint cavity[20] and mimics the 4 day blood exposure in the canine *in vivo* experiments. After this exposure, cartilage explants were washed twice under culture conditions for 45 minutes to remove all additives and cultured for an additional 12 days without additives. Medium was refreshed every 4 days.

Human cartilage analysis

Each experiment was performed with cartilage from a single donor. The mean value of 10 cartilage explants per parameter per donor, obtained randomly and handled individually, was taken as a representative value for the cartilage of that donor per condition to correct for possible biological variations between samples. Proteoglycan synthesis rate, -release, and -content were evaluated at the end of each experiment as described previously[21]. In short: As a measure of proteoglycan synthesis rate $^{35}SO_4$ incorporation rate was determined after 4 hours of pulse labelling, using hexadecylpyridinium chloride monohydrate (Sigma) to precipitate the GAGs from a papain digest of the human cartilage tissue. Determination of total proteoglycan release and -content was according to the procedures described above for the canine *in vivo* experiments.

Determination of DNA content

Cellularity of canine and human cartilage explants was established by measuring the DNA content using the fluorescent dye Hoechst (33258; Calbiochem), while calf thymus DNA (Sigma) was used as a reference. For analysis of the DNA content, papain digests were diluted to the appropriate concentrations and analyses were performed as described previously[22]. Results were expressed as mg DNA per wet weight of cartilage tissue. In all experiments there was no difference in DNA content per wet weight tissue between left and right joints within each group or between treatment groups, and as a result there was no difference in cellularity after exposure to blood (data not shown). Therefore results are expressed per wet weight of cartilage tissue.

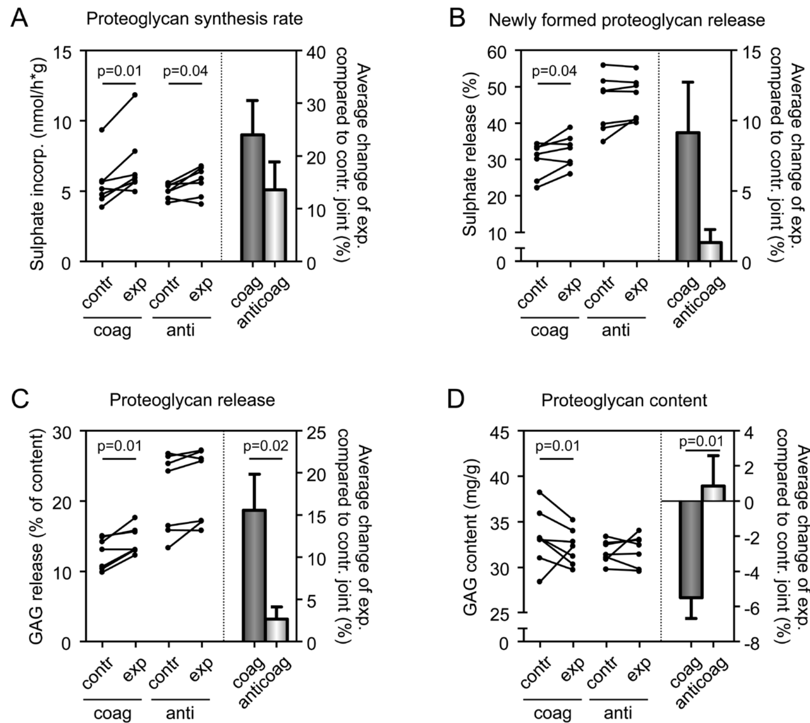

Figure 1: Proteoglycan turnover of cartilage after exposure to blood
Proteoglycan synthesis rate **(A)**, release of newly formed proteoglycans **(B)**, total proteoglycan release **(C)**, and proteoglycan content **(D)** were measured 10 weeks after the last injection with coagulating blood (n=7) or anticoagulated blood (n=7). All parameters are also expressed as percentage change of the blood-injected leg compared to the control leg, calculated for each animal and averaged for 7 animals (right panel of each graph). Bars represent mean ± SEM. Contr = control; exp = experimental i.e. blood-injected; coag = coagulating blood (grey bars); anti/anticoag = anticoagulated blood (white bars).

Calculations and statistical analyses

The n-values in this study indicate the number of animal joints or human cartilage and blood donors. The canine *ex vivo* data were analysed using a paired Student's t-test to compare data of the experimental and contralateral control joints within each group. An unpaired t-test was used to analyse differences between the group injected with coagulating blood and the group injected with anticoagulated blood. We compared absolute values of the control and experimental joints of both groups, as well as the change between experimental and contralateral control joints between groups. The human *in vitro* data were analysed using a paired Student's t-test for related samples. Differences were considered to be statistically significant when $p \leq 0.05$. SPSS 15.0 software was used for analyses.

RESULTS

Intra-articular coagulating blood alters proteoglycan turnover

Macroscopic cartilage damage was minimal after blood exposure and did not differ between both groups according to the OARSI criteria of canine cartilage damage (data not shown). However, changes in proteoglycan turnover, a much more sensitive measure, revealed a cartilage degenerative process. Proteoglycan synthesis rate was increased in the experimental joints of both groups (24% in coagulating blood group vs. 14% in anticoagulated blood group) as a characteristic of (ineffective) cartilage repair activity (p=0.01 and p=0.04, respectively; figure 1A). The relative effect (change between experimental and control joint) was higher after exposure to coagulating blood, but not significantly different between both groups (right panel of figure 1A).

Retention of newly formed proteoglycans is represented by release of newly formed proteoglycans (figure 1B). This release increased by 9% only after intra-articular injections with coagulating blood (p=0.04). Clearly the relative effect was greater for the coagulating blood exposure than for the anticoagulated blood exposure, although not statistically significant (approximately 4-fold; right panel of figure 1B).

Total proteoglycan release was increased by 16% after exposure to coagulating blood comparing experimental joints with contralateral joints (p=0.01; figure 1C). Anticoagulated blood did not demonstrate a statistical significant increase in total

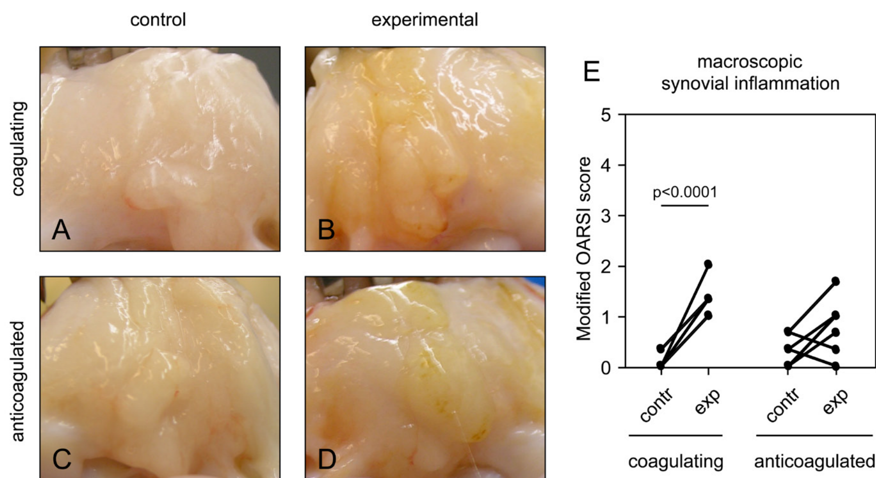

Figure 2: Macroscopic changes as a result of blood injections in the knee joint
Beagle left knee joints were injected with coagulating blood **(B)** or anticoagulated (heparinised) blood **(D)**, while control right knees were injected with the same amount of saline **(A)** or heparinised saline **(C)**. Representative pictures of synovial tissue 10 weeks after the last injection are shown. Only blood injection with coagulating blood, and not with anticoagulated blood, caused statistically significant synovial inflammation compared to its control knee (modified OARSI score, scale 0-5) **(E)**. Macroscopic changes were scored by 3 blinded observers and averaged. Contr = control; exp = experimental i.e. blood-injected (n=7; note that some data points overlap).

proteoglycan release when compared to the contralateral control joints. Moreover, the relative change was more than 5-fold higher in the group injected with coagulating blood than in the group injected with anticoagulated blood (p=0.02; right panel of figure 1C). Unexpectedly, we measured a greater release of both newly formed proteoglycans and total proteoglycans in cartilage of animals whose knees were injected with heparin as a control or with anticoagulated blood containing heparin than in cartilage of animals not injected with heparin but with saline and coagulating blood (in all cases p≤0.003; figure 1B and 1C).

Proteoglycan content was decreased in the experimental joint compared to the contralateral control joint after injections with coagulating blood (6%; p=0.01; figure 1D) and not after injections with anticoagulated blood. The change between experimental and control joints was different for both groups (p=0.01; right panel of figure 1D).

Intra-articular coagulating blood causes synovial inflammation

Representative photographs of the macroscopic appearance of synovial tissue are given in figure 2A-D for controls and experimental joints of coagulating and anticoagulated blood-injected joints. Macroscopically the synovial tissues showed no sign of inflammation due to the repeated injections with saline (figure 2A; OARSI score = 0.1; as a control for coagulating blood in figure 2B) or heparinised saline (figure 2C; OARSI score = 0.3; as a control for anticoagulated blood in figure 2D). Individual values are depicted in figure

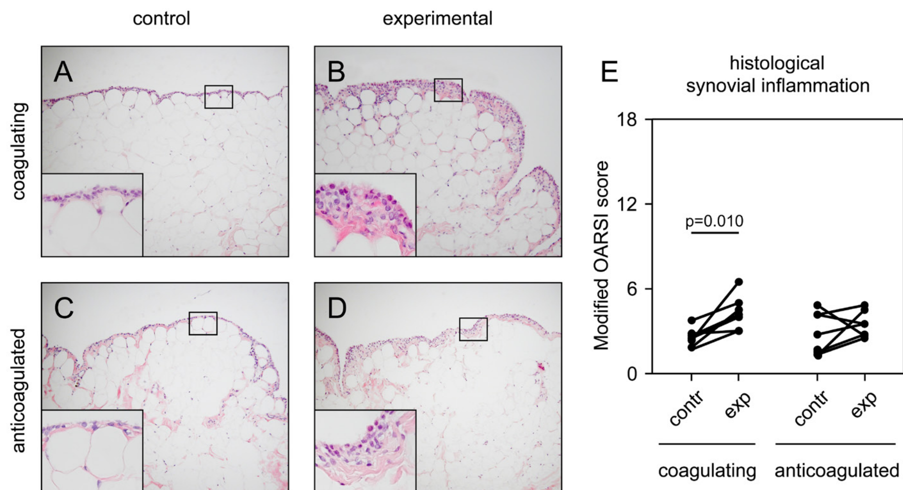

Figure 3: Histological changes of synovial tissue after blood injections in the knee joint
Representative (for the average score) light micrographs of control (saline: score 2.5 **(A)** and heparinised saline: score 2.9 **(C)**) and experimental synovial tissue (coagulating blood: score 4.3 **(B)** and anticoagulated blood: score 3.5 **(D)**) 10 weeks after the last injection are shown. Microscopic grading was based on the amount of lining cell layers, hyperplasia, and cell infiltration according to the modified OARSI score (scale 0-18). Injection with coagulating blood resulted in synovial inflammation compared to its control knee, whereas injection with anticoagulated blood did not (both n=7) **(E)**. Histological changes were scored by 2 blinded observers and averaged. Original magnification: 12.5x.

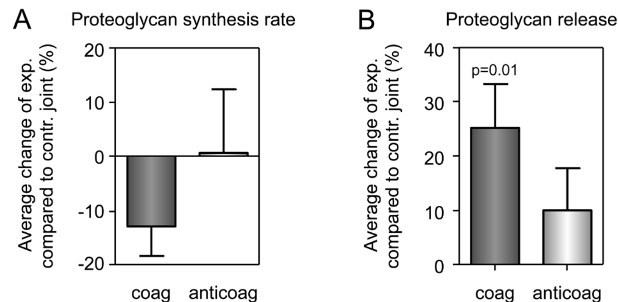

Figure 4: Catabolic properties of synovium culture supernatants on canine cartilage
Healthy canine hip cartilage was exposed for 4 days to 20% v/v synovial tissue culture supernatant from canine knees injected with coagulating or anticoagulated blood. Proteoglycan synthesis rate **(A)** and -release **(B)** were determined directly after this culture period. Bars represent the percentage change of the blood-injected leg compared to the control leg ± SEM, calculated for each animal and averaged for 7 animals. Coag = coagulating blood (grey bars); anticoag = anticoagulated blood (white bars).

2E. Mild synovial inflammation was observed by injection of anticoagulated as well as coagulating blood but only statistically significant upon injections with coagulating blood (p<0.0001 compared to control leg; figure 2E).
Figure 3A-D shows representative light micrographs of control (saline and heparinised saline; A and C, respectively) and experimental joints injected with coagulating and anticoagulated blood (B and D, respectively). Corroborating the macroscopic findings, there is no histological difference in synovial inflammation after repeated injections with saline or heparinised saline (figure 3E; OARSI score = 2.5 and 2.9, respectively). Injection with coagulating blood resulted in an increase of the synovial inflammation score with 72% compared to contralateral control joints (figure 3E; p=0.01), whereas intra-articular injection with anticoagulated blood did only cause an increase of synovial inflammation score with 21% (p=0.38) assessed by histology.
Catabolic properties of synovial tissue supernatants were tested on canine hip cartilage. The proteoglycan synthesis rate was only decreased by supernatant from knees injected with coagulating blood, although not statistically significant (figure 4A; p=0.17). Proteoglycan release was increased by addition of synovium culture supernatant, but only statistically significant with supernatant of synovial tissue exposed to coagulating blood and not when exposed to anticoagulated blood (figure 4B; p=0.01 and p=0.22, respectively).

Coagulating blood changes proteoglycan turnover more than anticoagulated blood *in vitro*

The differences in damaging effects of coagulating and anticoagulated blood on cartilage could depend on the different levels of synovial inflammation as seen in the canine *in vivo* experiment, thereby adding to the direct effects of blood on cartilage. To study whether coagulating blood results also in inflammation-independent cartilage damage, coagulating and anticoagulated blood were tested *in vitro* in the absence of synovial

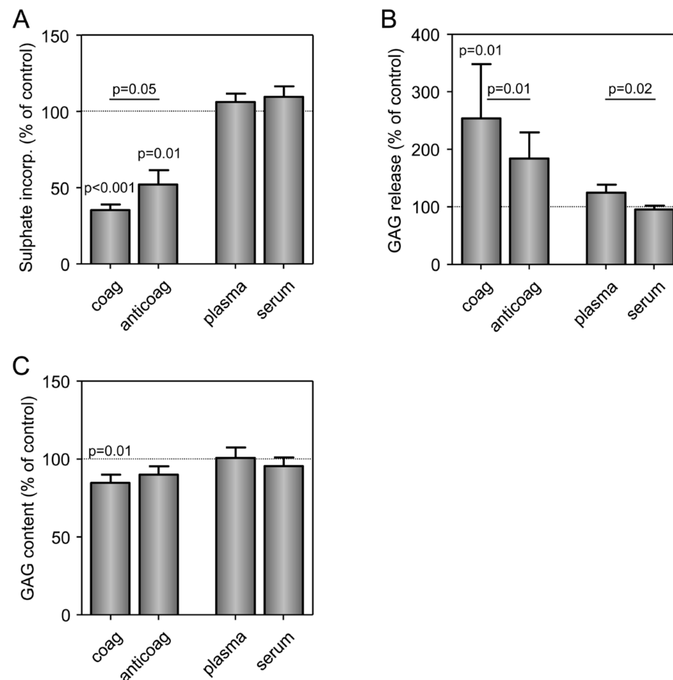

Figure 5: *In vitro* exposure of human cartilage to coagulating and anticoagulated blood, and to plasma and serum

Human articular cartilage was exposed to 50% v/v coagulating and anticoagulated whole blood, plasma, or serum for 4 days. Proteoglycan release **(B)** was measured directly after blood-exposure. Proteoglycan synthesis rate **(A)** and -content **(C)** were determined after a recovery period of 12 days. Bars represent mean percentage of control ± SEM (at least n=6). Values were compared to controls and considered to be statistically significant when p≤0.05. Coag = coagulating blood; anticoag = anticoagulated blood.

tissue. It appeared that human articular cartilage tissue explants were more sensitive to damage in terms of inhibition of proteoglycan synthesis rate by coagulating than by anticoagulated blood (figure 5A). Coagulating blood limited proteoglycan synthesis rate with 65% compared to control, while synthesis rate was reduced with only 48% in case of exposure to anticoagulated blood (p<0.001 and p=0.01, respectively; p=0.05 for the difference between both). Release of proteoglycans from the cartilage in the culture medium is enhanced with 178% after exposure to coagulating blood (p=0.01 compared to control; figure 5B), and only with 84% after exposure to anticoagulated blood (p=0.46). The difference between both is also statistically significant (p=0.01). A decrease of proteoglycan synthesis rate and an increase in proteoglycan release eventually led to a decrease of proteoglycan content of cartilage explants with 15% after exposure to coagulating blood (p=0.01; figure 5C) and with 10% after exposure to anticoagulated blood (p=0.08).

To determine the different effects between absence and presence of clotting factors without interference of blood cells, cartilage explants were exposed for 4 days to 50%

plasma and 50% serum. Figure 5A-C shows that proteoglycan synthesis rate, -release, and -content were not statistically significant altered by the components on their own after a recovery period of 12 days when compared to control values. Proteoglycan release, however, was slightly enhanced after exposure to plasma when compared to exposure to serum (p=0.02, figure 5B).

DISCUSSION

In this study it is shown that intra-articular injected coagulating blood causes more cartilage damage when compared to anticoagulated blood. *In vitro* exposure of cartilage to blood without synovial involvement demonstrated that at least part of the effect of coagulation was direct on cartilage and independent of inflammation. Interestingly, in absence of MNC and RBC, plasma and serum did not alter cartilage matrix turnover on the long term suggesting that the effects of components of the coagulation cascade, mostly present in plasma, are only transient and not long lasting.

Intra-articular injection with coagulating blood led to a decrease of total proteoglycan content of 6%. Since the average rate of tibial cartilage volume loss is reported to be 5% per year in osteoarthritic patients[23], a loss of proteoglycan content of 6%, one of the major components of articular cartilage, at 10 weeks after exposure to blood is clinically relevant. Analysis was performed at a single time point, and therefore it remains unknown whether or not the effects sustain for a longer period as well. This limits direct translation to long-term clinical implications.

The use of heparin to prevent blood from coagulating is rather artificial when compared to the absence of clotting factor VIII or IX in case of haemophilic blood. To control for this, the contralateral joints of the anticoagulated blood-injected joints were injected with saline containing similar amounts of heparin. It appeared that control knees injected with heparinised saline cause a higher release of proteoglycans compared to saline. A possible explanation could be that the heparin, being a GAG, interfered with the assay measuring proteoglycan release. However, the amounts of injected heparin might be considered negligible in this GAG-assay after 10 weeks follow-up because the biological half-life of heparin is short: 0.5-3 hours[24]. It is more plausible that heparin caused an enhanced release of proteoglycans from cartilage, which is in agreement with equine *in vitro* experiments.

Equine cartilage explants cultured in presence of heparin showed an increased GAG loss, indicating that heparin stimulates articular cartilage breakdown[25]. This could be explained by induction of production of matrix metalloproteinases (MMPs) by heparin, as has been demonstrated in human heart fibroblasts[26]. Especially MMP-1 (type-I collagenase) and MMP-2 (type IV collagenase, gelatinase A) are induced without affecting TIMP-1, the naturally existing tissue inhibitor of metalloproteinases. These MMPs could add to a cascade of proteinase activation that causes enhanced degradation of cartilage matrix. Based on the above, also a higher release of GAGs from cartilage would be expected after injection of anticoagulated (heparinised) blood compared to coagulating blood.

However, the use of heparin seems minor compared to the effects of blood coagulation on GAG release.
Intra-articular injections with coagulating blood cause more synovial inflammation when compared to injections with saline. Additional *in vitro* experiments demonstrate that the damaging effects of coagulating and anticoagulated blood on cartilage are at least partly independent of synovial involvement. This seems in contrast to work of Jansen *et al.* who demonstrated that there is no statistically significant difference between cartilage proteoglycan turnover resulting from exposure to coagulating blood and that resulting from anticoagulated blood *in vitro*[14], although the trend in those data is comparable to our data. The limited amount of experiments performed in that study might explain the lack of statistical significance. Proteoglycan synthesis rate of cartilage cultured *in vitro* decreases when cartilage gets damaged due to blood-induced apoptosis of chondrocytes[5]. In contrast, proteoglycan synthesis rate is increased in *ex vivo* cultured cartilage of the experimental canine legs. Literature demonstrates that an increased proteoglycan synthesis rate is an expression of initiated (ineffective) repair activity characteristic of early degenerative (osteoarthritic) cartilage[27]. The increase in proteoglycan synthesis rate was indeed ineffective after administration of coagulating blood, since the release of newly formed proteoglycans was increased as well. This corroborates that an increased proteoglycan synthesis observed in the *in vivo* experiment is ineffective and represents a degenerative process as is also observed in the *in vitro* experiments.
A possible explanation why anticoagulated blood causes less blood-induced joint damage than coagulating blood, is that anticoagulation by heparin inhibits thrombin formation through activation of antithrombin[28]. The heparin-antithrombin complex inactivates clotting enzymes including thrombin (factor IIa) and factors Xa, IXa, XIa, and XIIa, thereby preventing fibrin formation and thrombin-induced activation of factor V and VIII[29]. Thrombin itself is directly damaging to cartilage, as it enhances release of proteoglycans from human and bovine cartilage explants when cultured *in vitro*[11]. Thus inhibition of thrombin formation by anticoagulation reduces proteoglycan release. In addition, it is known that thrombin is a mediator of inflammation and has a role in the pathogenesis of rheumatoid arthritis[30,31]. This explains why anticoagulation of blood during a joint haemorrhage limits synovial inflammation due to reduction of thrombin production when compared to inflammation of the synovium after exposure to coagulating blood in which thrombin is formed.
An important difference between serum and plasma is that serum lacks fibrinogen because it has been converted into fibrin by thrombin. Furthermore, it has been demonstrated that the concentration of plasminogen (inactive form of plasmin) in serum is 38% lower than in plasma[32]. Plasmin, like thrombin, enhances release of proteoglycans from human and bovine cartilage explants when cultured *in vitro*[11]. Based on the above it was expected that plasma, containing higher levels of components of the coagulation cascade, would be more harmful to cartilage than serum. However, the *in vitro* experiments of this study show that exposure of cartilage explants to 50% serum and plasma does not cause long-lasting, irreversible cartilage damage, although the

release of proteoglycans is higher after exposure to plasma when compared to serum. Previous research showed that plasma is able to reduce cartilage proteoglycan synthesis rate while serum was not; however, these effects were analysed directly after blood exposure and therefore possibly transient[4].
During serum preparation in BD vacutainer collection tubes (EDTA and citrate) the coagulation system and platelets are activated and thrombin is produced. Several platelet proteins, like VEGF, FGF-b, RANTES, as well as several chemokines and growth factors, are increased in serum[33]. In contrast, serum of healthy controls contained lower levels of many cytokines, like IL-1 and TNF-α, whereas MMP-1, 8, and 13, involved in cartilage breakdown, did not differ between serum and plasma[33]. These data suggest that both serum and plasma contain different components causing transient cartilage damage. Apparently the long-lasting effects do not primarily depend on the soluble mediators of the coagulation cascade but on the mechanism of chondrocyte apoptosis, in which catalytic iron from red blood cells is a prerequisite[5,6]. However, as suggested by the present *in vivo* study, components of the coagulation cascade add to the vulnerability of cartilage. This could possibly add to the transient damage or to the increase of hydrogen peroxide production by chondrocytes, eventually leading to persisting cartilage damage. This study shows that coagulation of blood after a joint bleed, as would be the case in trauma or surgery, causes more lasting damage to a joint than in case of anticoagulation, as seen in haemophilia patients. Intra-articular anticoagulation might limit the harmful effects of blood in a human joint. However, this could never be applied in haemophilia patients, since they need clotting factor to stop bleedings. In all cases it might be even more important to aspirate blood from a joint as soon as possible to prevent joint damage.

REFERENCES

1. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
2. Lafeber FP, Miossec P, Valentino LA. Physiopathology of haemophilic arthropathy. Haemophilia 2008;14 Suppl 4:3-9.
3. Valentino LA. Blood-induced joint disease: the pathophysiology of hemophilic arthropathy. J Thromb Haemost 2010;8:1895-902.
4. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
5. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
6. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
7. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. Clin Orthop Relat Res 1997:6-11.
8. Roosendaal G, Vianen ME, Wenting MJ, van Rinsum AC, van den Berg HM, Lafeber FP, et al. Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. J Bone Joint Surg Br 1998;80:540-5.
9. Harrold AJ. The defect of blood coagulation in joints. J Clin Pathol 1961;14:305-8.
10. McCarty WJ, Luan A, Siddiqui M, Hansen BC, Masuda K, Sah RL. Biomechanical properties of mixtures of blood and synovial fluid. J Orthop Res 2011;29:240-6.
11. Furmaniak-Kazmierczak E, Cooke TD, Manuel R, Scudamore A, Hoogendorn H, Giles AR, et al. Studies of thrombin-induced proteoglycan release in the degradation of human and bovine cartilage. J Clin Invest 1994;94:472-80.
12. Busso N, Morard C, Salvi R, Peclat V, So A. Role of the tissue factor pathway in synovial inflammation. Arthritis Rheum 2003;48:651-9.
13. Yang YH, Hall P, Milenkovski G, Sharma L, Hutchinson P, Melis E, et al. Reduction in arthritis severity and modulation of immune function in tissue factor cytoplasmic domain mutant mice. Am J Pathol 2004;164:109-17.
14. Jansen NW, Roosendaal G, Bijlsma JW, Degroot J, Lafeber FP. Exposure of human cartilage tissue to low concentrations of blood for a short period of time leads to prolonged cartilage damage: an in vitro study. Arthritis Rheum 2007;56:199-207.
15. Esmon CT. Interactions between the innate immune and blood coagulation systems. Trends Immunol 2004;25:536-42.
16. Cirino G, Vergnolle N. Proteinase-activated receptors (PARs): crossroads between innate immunity and coagulation. Curr Opin Pharmacol 2006;6:428-34.
17. Cook JL, Kuroki K, Visco D, Pelletier JP, Schulz L, Lafeber FP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the dog. Osteoarthritis Cartilage 2010;18 Suppl 3:S66-79.
18. Marijnissen AC, van Roermund PM, TeKoppele JM, Bijlsma JW, Lafeber FP. The canine 'groove' model, compared with the ACLT model of osteoarthritis. Osteoarthritis Cartilage 2002;10:145-55.
19. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. J Rheumatol 2003;30:339-44.
20. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia 2006;12 Suppl 3:117-21.
21. Lafeber FP, Vander Kraan PM, Van Roy JL, Huber-Bruning O, Bijlsma JW. Articular cartilage explant culture; an appropriate in vitro system to compare osteoarthritic and normal human cartilage. Connect Tissue Res 1993;29:287-99.
22. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988;174:168-76.

23. Wluka AE, Stuckey S, Snaddon J, Cicuttini FM. The determinants of change in tibial cartilage volume in osteoarthritic knees. Arthritis Rheum 2002;46:2065-72.
24. McAvoy TJ. The biologic half-life of heparin. Clin Pharmacol Ther 1979;25:372-9.
25. McCarthy HE, Singer ER, Davies Morel MC. The effect of sodium heparin on equine articular cartilage. Vet J 2001;162:73-9.
26. Tyagi SC, Kumar S, Katwa L. Differential regulation of extracellular matrix metalloproteinase and tissue inhibitor by heparin and cholesterol in fibroblast cells. J Mol Cell Cardiol 1997;29:391-404.
27. Lafeber FP, van Roy H, Wilbrink B, Huber-Bruning O, Bijlsma JW. Human osteoarthritic cartilage is synthetically more active but in culture less vital than normal cartilage. J Rheumatol 1992;19:123-9.
28. Bjork I, Lindahl U. Mechanism of the anticoagulant action of heparin. Mol Cell Biochem 1982;48:161-82.
29. Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, et al. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. Chest 2001;119:64S-94S.
30. Morris R, Winyard PG, Blake DR, Morris CJ. Thrombin in inflammation and healing: relevance to rheumatoid arthritis. Ann Rheum Dis 1994;53:72-9.
31. Varisco PA, Peclat V, van Ness K, Bischof-Delaloye A, So A, Busso N. Effect of thrombin inhibition on synovial inflammation in antigen induced arthritis. Ann Rheum Dis 2000;59:781-7.
32. Cederholm-Williams SA. Concentration of plasminogen and antiplasmin in plasma and serum. J Clin Pathol 1981;34:979-81.
33. Stroncek D, Slezak S, Khuu H, Basil C, Tisdale J, Leitman SF, et al. Proteomic signature of myeloproliferation and neutrophilia: analysis of serum and plasma from healthy subjects given granulocyte colony-stimulating factor. Exp Hematol 2005;33:1109-17.

Chapter 4

Biomarkers to identify joint damage after a joint haemorrhage; an explorative human and canine *in vivo* study

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ABSTRACT

Objective:

A panel of four biomarkers for cartilage degradation is associated with overall radiographic joint damage in haemophilia patients. We investigated whether these biochemical markers can detect development of joint damage immediately after a joint haemorrhage.

Methods:

Urinary CTX-II, serum COMP, serum C1,2C, and serum CS846 were measured in samples of 8 haemophilia patients collected within 2 days, after 3-5 days, and about 90 days after the patient reported a joint bleed (considered baseline). In parallel, the same biomarkers were measured in 7 dogs before they received an experimental joint bleed (induced by intra-articular blood injections over 5 days), immediately after this bleeding episode, and 1 and 13 weeks later.

Results:

The levels of all four biomarkers were elevated 3-5 days after a joint bleed in haemophilia patients when compared to baseline levels (at 90 days after the bleed), although not statistically significant. A combined score of the four biomarkers was increased when compared to levels directly after the bleed ($p=0.024$). One week after the experimentally induced canine joint bleed, the same panel of biomarkers (except sCOMP) was increased; uCTX-II and the combined biomarker score being statistically significantly different from the time point directly after the bleed ($p=0.007$ and $p=0.02$, respectively).

Conclusion:

This study demonstrates that a single joint bleed elevates biochemical markers of joint tissue damage, both in haemophilia patients and after an experimentally induced canine joint bleed. Due to the great variation in patient and bleeding characteristics in a small number of patients, these effects were only approaching statistical significance. Irrespectively, this explorative study warrants further research on the value of biochemical markers to detect the destructive properties of a joint bleed.

INTRODUCTION

Haemophilic arthropathy is one of the major causes of morbidity amongst haemophilia patients and most often occurs in elbow, knee, and ankle joints[1]. Several tissues of the joint are affected: synovial tissue gets inflamed, cartilage gets damaged and finally lost, whereas bone gets eroded and remodelled[2]. These changes in a joint can be visualized by ultrasonography, Magnetic Resonance Imaging (MRI), or radiography[3]. Ultrasonography is a cheap method to perform, but needs specific skills of a trained observer and is characterised by significant inter-observer variation. It is especially useful in detecting changes in soft tissue or to demonstrate joint effusions. Furthermore, this technique has its limitations regarding quantification of the observed changes. MRI provides information on all aspects of haemophilic arthropathy; both soft and hard tissues of the joint can be visualised. Specific semi-quantitative scoring systems for MRI of haemophilic joints have been developed[4]. However, the method is expensive and time consuming, which hampers the use for frequent follow up of patients. The most commonly used technique is radiography, which is a more economical and convenient method. The advantage of this imaging technique is that the resolution is significantly higher than the resolution of the other two techniques. The disadvantages are that it is difficult to determine early and minor changes of haemophilic arthropathy, since soft-tissue changes cannot be detected, and that cartilage breakdown and loss can only be visualised indirectly through loss of joint space width. Moreover, in contrast to the two other 3D methods, X-rays provide only a 2D overlay view of the 3D joint. Nevertheless, the use of radiographs is widespread. The World Federation of Haemophilia prefers evaluation of radiographs by use of the Pettersson score. This score is determined by examining radiographs of the six main joints (elbows, knees, and ankles), with a sum score ranging from 0-78 points[5]. However, the Pettersson score might not be sensitive enough to detect early small changes[6,7].

The three techniques described provide information of the cumulative joint damage over time, and only supply information after significant changes in joint damage. It is favourable to detect minor changes in the joint and changes in an earlier phase of arthropathy, preferably in a dynamic way. This would provide tools to treat the patients more adequately after a joint bleed and not only after significant damage has occurred and can be visualised by ultrasonography, MRI, or radiographs. A possible way to detect small joint changes in a dynamic way is by measuring biochemical markers of joint tissue turnover.

Turnover of joint tissues like cartilage, bone, and synovial tissue leads to release of small molecules into the synovial fluid. Subsequently, they are reabsorbed in the blood and can be detected in serum or urine after clearance by the kidney. A biomarker is of use when it is sensitive to differences between people with a joint disease and healthy controls, and when it varies during different stages of the disease in line with the severity or stage of the disease. Biomarkers are most valuable when they can reflect actual damage to the joint tissues. They have already been investigated more extensively in osteoarthritis

and, although to a lesser extent, also in rheumatoid arthritis patients. Several systemic biochemical markers for joint metabolism have been evaluated in osteoarthritis, systematically reviewed by van Spil *et al*[8]. From this study it became clear that currently available biochemical markers perform inconsistently between studies. However, from all markers CTX-II and COMP were studied most extensively and performed best. Cartilage Oligomeric Matrix Protein (COMP) is a non-collagen biomarker for cartilage degradation present in articular cartilage and other tissues like the synovial membrane, ligaments, tendons, and meniscus. A meta-analysis on studies in osteoarthritis patients demonstrates that sCOMP is elevated in these patients and correlates with disease progression[9]. CTX-II (C-terminal telopeptide of type II collagen) is a biomarker for cartilage degradation as it is a degradation product of C-terminal telopeptides of type II collagen, although more recently it is speculated that the origin of CTX-II might be bone degradation instead of, or at least in addition to, cartilage degradation[10]. Nevertheless, CTX-II is associated with progression of OA[11] and RA[12]. Two other biomarkers that have been studied less intensively and show varying results, are C1,2C and CS846. C1,2C is a cleavage product of both collagen type I and type II; thereby reflecting cartilage, but also bone degradation. CS846 is the 846 epitope of aggrecan chondroitin sulphate and a marker of proteoglycan (and hence cartilage) degradation.
As far as we are aware of, biochemical markers in urine and/or serum have only been studied once for haemophilia patients. Jansen et al. reported for a relatively small cohort of 36 patients, that urinary CTX-II, serum COMP, serum C1,2C and serum CS846 correlate with the subscore reflecting joint space narrowing (viz. cartilage loss) and with the total Pettersson score reflecting overall disease progression[13]. Moreover, a combined panel of uCTX-II, sCOMP, and sCS846 showed the best correlation with the overall radiographic joint damage. Thus despite the limited usefulness of these biomarkers in OA at present, these biochemical markers appeared to be associated with the degree of haemophilic arthropathy. The reason for this could be the relatively fast progression of this type of arthropathy as compared to arthropathy in OA. As such, we were interested whether these biomarkers also reflect the increase in joint damage, that is expectedly induced immediately after a joint bleed[14] in a dynamic way. Therefore, in this explorative study we investigated whether a joint bleed results in a (transient) rise in biochemical markers representing cartilage breakdown.

MATERIALS AND METHODS

Human blood and urine samples

Blood and urine samples were collected from 8 randomly selected haemophilia patients within 2 days, after 3-5 days, and about 90 days after the patient reported a joint bleed in their knee, elbow or ankle. Ten mL of blood was collected into vacutainer tubes (Becton Dickinson). A total of 8 IU recombinant clotting factor concentrate (factor VIII of IX, as appropriate) was added to the blood samples to allow proper coagulation. The samples were kept at room temperature for at least 1 hour, and subsequently centrifuged at

1500xg, aliquoted and stored at -80°C. Urine samples were kept at 4°C for a maximum of 8 hours, and then aliquoted and stored at -80°C. All samples were taken in the morning, but not fasted, nor at a fixed time point of the morning. Thereby the circadian changes in biomarker levels over the day and changes due to food intake were neglected[15-17]. This is because it was not feasible in practice and it was hypothesized that levels would rise significantly due to the joint damage induced by the joint bleed. The study was performed according to the Declaration of Helsinki and was approved by the Medical Ethics Review Board of the University Medical Center Utrecht. All patients gave written informed consent.

Canine blood and urine samples

Additionally, 7 skeletally mature Beagle dogs (3 males and 4 females; mean age 2.1 ± 0.1 years, weighing 9-15 kg) were obtained from the animal laboratory of the Utrecht University, The Netherlands. They were housed in groups of 2-3 dogs, and were let out on a patio for at least 2 hours daily. They were fed a standard commercial diet with water ad libitum. The Utrecht Ethical Committee for animals approved the study. The left knees of these dogs were injected intra-articularly with 1.8 ± 0.1 mL freshly collected (vena puncture) autologous blood for five subsequent days to mimic a major joint bleed. During the experimental procedures, dogs were given short-term anaesthesia (Dormitor/ Antisedan). Random checks on accurate injection were made by placing a second needle at the other side of the joint. In all cases a blood flow from the injected joint was demonstrated, indicating the joint was completely filled with blood. After removal of the needles, pressure was put on the puncture holes until the bleeding was stopped, preventing leakage of blood outside the joint cavity. Blood and urine samples were collected about a week before the intra-articular blood injections (baseline), directly after the major joint bleed consisting of five days with intra-articular blood injections (week 0), 1 week after the joint bleed, and after 13 weeks. All samples were collected in the morning, but were non-fasted. Also in this canine study fasting and circadian changes were considered of inferior relevance. To collect serum samples, 10 mL of blood was collected in vacutainer tubes (Becton Dickinson) and allowed to coagulate for at least 1 hour at room temperature. Subsequently samples were centrifuged at 1500xg for 10 minutes, aliquoted, and stored at -80°C. Urine samples were kept at 4°C for a maximum of 8 hours, and then aliquoted and stored at -80°C. Cartilage damage in these animals was evaluated ex vivo by determination of turnover of proteoglycans, one of the major components of cartilage, as previously described[18].

Biomarker assays

All biomarkers were measured in duplo using standard ELISA kits according to the manufacturer protocol. In human urine samples CTX-II (CartiLaps; Nordic Bioscience) was meausured and corrected for creatinine levels (Cayman), whereas C1,2C (IBEX), CS846 (IBEX), and COMP (Anamar) were detected in serum samples. In the canine urine samples CTX-II (Pre-Clinical CartiLaps; Nordic Bioscience; specific for animals including

Table 1: Patient characteristics

case	type of haemophilia	clotting factor level	prophylaxis /on demand	age at time of the bleed	clotting factor consumption (IU/kg/year)	number of joint bleeds per year	number of affected joints	number of joint prostheses	Pettersson score
1	A	5%	on demand	18	300	0	1	0	5
2	A	<1%	on demand	71	1300	4	6	0	68
3	A	2%	on demand	49	200	2	4	0	20
4	B	<1%	prophylaxis	32	1300	9	2	0	12
5	A	<1%	prophylaxis	58	6000	14	4	2	68
6	A	3%	on demand	63	500	8	4	0	26
7	A	<1%	on demand	33	1300	20	1	0	5
8	A	<1%	prophylaxis	56	3800	13	4	2	66

dogs) was measured and corrected for creatinine levels (Cayman). In the canine serum samples C1,2C (IBEX; cross reactivity with dogs), CS846 (IBEX; cross reactivity with dogs), and COMP (Novatein Biosciences; specific for dogs) were determined.

Calculations and statistical analysis

Levels of the individual biomarkers were compared to baseline and to each other using a (two-sided) paired Student's t-test. Combined biomarker scores were calculated as well[13]. Each biomarker received the same weight by computing the average level of each biomarker in the total group of patients at baseline. For each patient the ratio of its biomarker level at a certain time point to this mean value was determined. The mean of these ratios for the four different biomarkers for each patient, averaged for all patients, was plotted and tested using a (two-sided) paired Student's t-test. Differences were considered to be statistically significant when $p<0.05$. SPSS 15.0 software was used for analyses.

RESULTS

Biomarkers for detection of joint damage upon a joint bleed in haemophilia patients

Cartilage degradation biomarkers were measured in 8 patients whose characteristics are described in table 1. Seven out of the 8 patients suffered from haemophilia type A, and one from haemophilia type B. Patients had moderate (1-5% clotting factor level, n=3) or severe haemophilia (<1%, n=5), for which they received clotting factor replacement therapy on demand or as prophylaxis. The age at the time of the joint bleed ranged from 18-71 years old. There was also a large variation in average clotting factor consumption (200-6000 IU/kg/year), the average number of joint bleeds per year (0-20), the number of affected joints which are included in the Pettersson score (0-6), the number out of these joints which has been replaced by a prosthesis (0-2), and the total Pettersson score (5-68).

Table 2: Proteoglycan turnover after an experimentally induced joint bleed in a dog

	Mean ± SEM	P-value
Change PG synthesis rate (%)	24 ± 7	0.011
Change of newly formed GAG release (%)	9 ± 4	0.043
Change of percentage GAG release (%)	16 ± 4	0.009
Change of GAG content	-5 ± 1	0.007

Baseline levels of the biomarkers could not be determined previous to a joint bleed, since the occurrence of a joint bleed in haemophilia patients cannot be predicted. Therefore the values of the biomarkers around 90 days after the joint bleed were considered baseline levels, and levels shortly after the joint bleed were normalised to these supposed baseline levels. In figure 1A a representative patient (number 2 from table 1) illustrates that levels of uCTX-II, sC1,2C, and sCS846 were not increased yet within the first 2 days after the joint bleed and as such comparable to the levels at day 90. Concentrations were elevated when measured 3-5 days after the joint bleed as compared to levels at day 90. sCOMP level of this patient was already increased within 2 days after the joint bleed to a level comparable to that after 3-5 days.

Figure 1B depicts the mean value of all patients for each biomarker as a percentage of baseline values (set at 90 days after the joint bleed). The level of uCTX-II measured within the first 2 days after the joint bleed was higher, but not statistically significant when compared to baseline (116%; p=0.801). After 3-5 days this level was further increased to 130% of baseline level, still not statistically significant (p=0.294), and also not statistically significant compared to the uCTX-II concentration measured within 2 days (p=0.125) Nevertheless, a tendency toward a relevant increase over time shortly after the bleed might be appreciated.

The concentration of sCOMP did slightly increase in the first 2 days after a joint bleed (104% compared to baseline levels; p=0.510). At 3-5 days after the joint bleed a still small, nearly statistically significant, increase was detected compared to baseline values (109%; p=0.060). There was no statistically significant difference in sCOMP between the two time points (p=0.631).

Within the first 2 days after a joint bleed, mean levels of sC1,2C did not increase (100% of baseline value; p=0.803). When sC1,2C levels were measured again 3-5 days after the joint bleed, levels were found to be slightly, but not statistically significant, increased to 109% of baseline values (p=0.348). The difference between sC1,2C concentrations at day 2 and 3-5 was approaching statistically significant difference (p=0.095).

The change in level of sCS846 was comparable to that of sC1,2C. Within the first 2 days after the joint bleed the concentration was not increased when compared to baseline (100%; p=0.827). After 3-5 days this level increased to 107% (p=0.262), but not statistically significantly different compared to the level measured within 2 days (p=0.131).

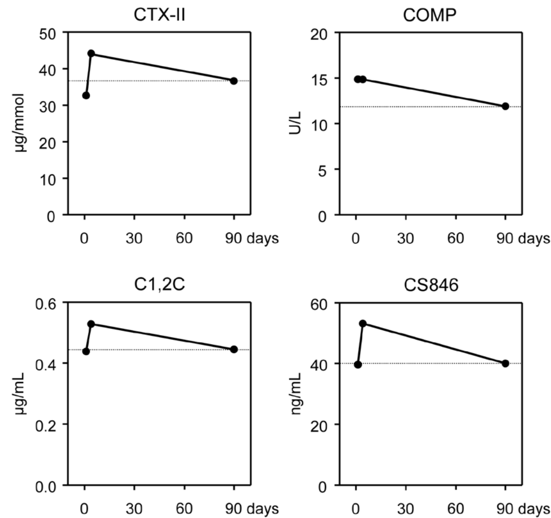

Figure 1A: A representative profile of biomarker levels as measured over time after a human joint bleed
Biomarker levels of a representative patient (number 2; table 1) were measured in blood or urine samples within 2 days, after 3-5 days, and about 90 days after the patient reported a joint bleed. uCTX-II was measured in urine samples and corrected for creatinine levels; COMP, C1,2C, and CS846 were measured in serum samples. Biomarker concentrations measured a prolonged period of 90 days after the haemorrhage were considered baseline levels (represented by the dotted line). The level of sCOMP increased already within the first 2 days after a joint bleed. The levels of uCTX-II, sC1,2C, and sCS846 increased only after 3-5 days.

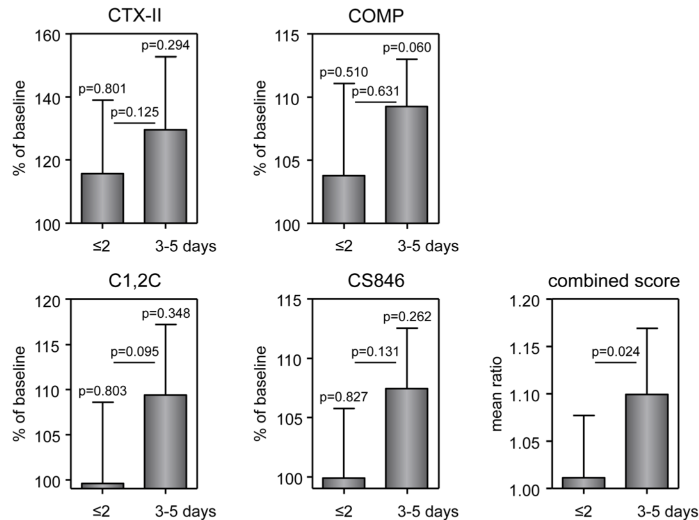

Figure 1B: Average biomarker levels observed after a human joint bleed
Blood and urine samples were collected from eight haemophilia patients within 2 days, after 3-5 days after the patient reported a joint bleed. Biomarker levels measured 90 days later were considered baseline levels and put at 100%. CTX-II was measured in urine samples and corrected for creatinine levels; COMP, C1,2C, and CS846 were detected in serum samples. Additionally, a combined score of four biomarkers is depicted as ratio of the mean baseline concentration, thereby giving each biomarker the same weight. Mean values ± SEM are depicted.

The combined biomarker score represents the mean score of 4 biomarkers that are expressed as a ratio of the mean baseline concentration, thereby giving each biomarker the same weight. With this combined score it is clearly demonstrated that biomarker levels 3-5 days after a joint bleed were statistically significantly increased compared to levels measured after 2 days (ratio of 1.10 compared to a ratio of 1.01; p=0.024).

Biomarkers for detection of cartilage damage upon an experimental joint bleed in dogs
The observed increase in biochemical markers upon a joint bleed in this heterogeneous population tempted us to study these biochemical markers in a more homogeneous group by experimental induction of a joint bleed in a canine model of blood-induced joint damage. Thirteen weeks after the joint bleed, cartilage damage was evaluated by the change in cartilage proteoglycan turnover of blood-exposed joints compared to control joints. Table 2 shows that after a joint bleed proteoglycan (PG) synthesis rate was increased by 24%, the newly formed glycosaminoglycans (GAG) release by 9%, and the total release of GAGs by 16%. Moreover, total GAG content was decreased by 5% due to a joint bleed. All four parameters were statistically significantly changed and demonstrate very mild, but clear development of cartilage destruction upon induction of a joint bleed.

Figure 2 shows that directly after the experimental joint bleed uCTX-II level was similar to that of baseline before a bleeding was induced (99%, p=0.611). However, one week later the uCTX-II level increased to 175%, which was statistically significantly different from values measured directly after the experimentally induced joint bleed (p=0.007). However, compared to baseline values it did not reach statistical significance (p=0.119). Thirteen weeks after the joint bleed uCTX-II level dropped again statistically significantly (p<0.001 compared to week 1) to a value slightly below baseline.
Mean sCOMP level was slightly elevated to 114% of baseline level (p=0.531), already immediately after the experimentally induced canine joint bleed. After 1 week the sCOMP level was decreased again comparable to baseline level (101%, p=0.926), sustaining this level up to 13 weeks.
The concentration of sC1,2C measured directly after the joint bleed was slightly lower than baseline, but not statistically significant (92%; p=0.096). One week after the joint bleed, sC1,2C level was increased when compared to directly after the experimentally induced joint bleed (p=0.087), approaching statistical significance but not different from baseline (106%, p=0.457). At 13 weeks sC1,2C level dropped again (96%; p=0.390 compared to baseline), but not statistically significant when compared to week 1 (p=0.128).
sCS846 level directly after an experimentally induced canine joint bleed was lower than baseline (86%; p=0.336). After 1 week sCS846 concentration was increased to 146% (p=0.131); not being statically significantly different from baseline (p=0.334). After 13 weeks the level dropped to baseline again (95%; p=0.589 compared to baseline), not statistically significant when compared to week 1 (p=0.287).
The combined biomarker score, giving each biomarker the same weight, increased

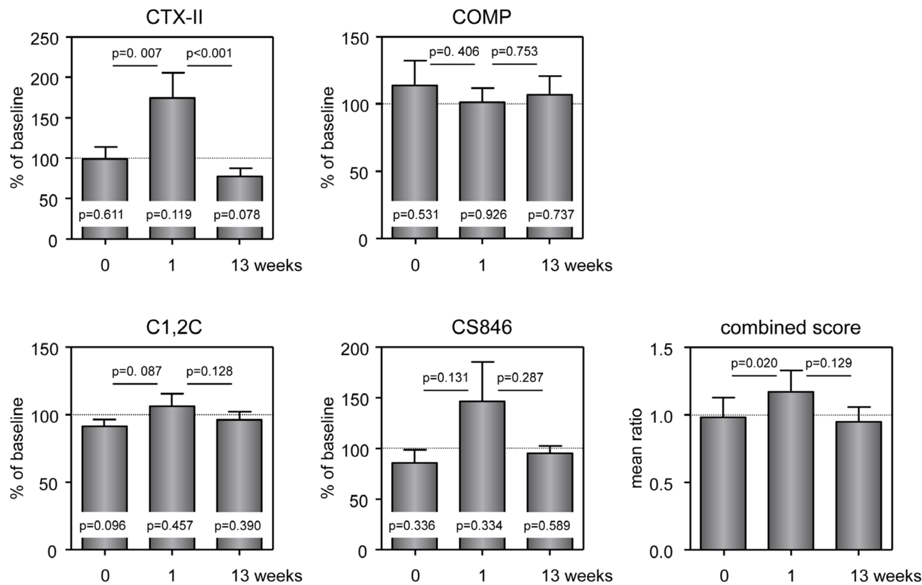

Figure 2: Average biomarker levels observed after an experimentally induced joint bleed in a dog
Blood and urine samples were collected from 7 dogs about 1 week before a joint bleed was induced (baseline), directly afterwards (0), after 1 week, and after 13 weeks. CTX-II was measured in urine samples and corrected for creatinine levels, whereas COMP, C1,2C, and CS846 were determined in serum samples. Biomarker levels are expressed as a percentage of the baseline values before a bleeding was induced. Additionally, a combined score of 4 biomarkers is depicted as ratio of the mean baseline concentration, thereby giving each biomarker the same weight. Mean values ± SEM are depicted. P values compared to baseline as well as between time points are given.

statistically significantly after 1 week when compared to levels measured directly after the experimentally induced joint bleed (ratio of 1.17 compared to a ratio of 0.98; p=0.020), and decreased again after 13 weeks to slightly below baseline (ratio of 0.95; p=0.129).

DISCUSSION

This study demonstrated for the first time that joint damage after a joint bleed is reflected by a set of joint tissue degradation biomarkers in blood or urine samples in a small heterogeneous group of haemophilia patients, an observation corroborated by a canine *in vivo* study in which a joint haemorrhage was induced artificially.
In human haemophilia patients an increase of CTX-II, COMP, C1,2C, and CS846 was detected 3-5 days after a joint bleed. However, due to the great variation in patient characteristics and bleeding characteristics, these effects were only approaching statistical significance. More obvious results were obtained when a combined score of these four biomarkers was used. To verify the potential use of biochemical markers in detecting the harmful effect of a joint bleed in more controlled manner, a joint bleed was

artificially induced by intra-articular blood injections in dogs. Directly after the canine joint bleed COMP levels increased, while elevation of CTX-II, C1,2C, and CS846 was demonstrated after one week. Also the combined biomarker score was clearly increased one week after the canine joint bleed.

Especially after a canine joint bleed it appears that COMP levels are elevated earlier than CTX-II, C1,2C, and CS846. Also the individual patient depicted in figure 1A demonstrates this similar pattern, although not found for the average value of all patients. For this reason, because not all biomarkers show exactly the same pattern in each patient, and because the joint changes are multifactorial, the use of a combined biomarker score is recommended. Furthermore, especially the combination of CTX-II, COMP, C1,2C, and CS846 demonstrated to be associated with the extent of haemophilic arthropathy rather than the individual biomarkers[13].

The variability between the haemophilia patient characteristics in this study was large. Patients were included in this study after they reported a joint bleed. In case of a smaller joint bleed it is difficult to check whether the patients truly suffered from a joint bleed, or from arthropathy-related pain. This might cause a bias in selection of the patient group. The only exclusion criteria were HIV infection or the presence of antibodies against supplemented clotting factor (inhibitor patients). This resulted in a group of patients with inconsistency in the number of affected joints and the degree of joint damage. Since it has been previously shown that the degree of joint damage is associated with biomarker levels[13], absolute biomarker concentrations highly differ between patients. However, when expressed as a percentage of baseline level per individual patient, this variability is controlled for. On the other hand, it is not known whether biomarker levels respond in a different way to a joint bleed in time according to the degree of already existing joint damage. *In vitro* it has been shown that degenerated cartilage is at least as susceptible to blood-induced damage as healthy cartilage[19]. Therefore, it can be assumed that in patients with severe haemophilic arthropathy, biomarkers will still be detectable in serum or urine after a joint bleed, irrespective of the degree of joint damage, although supposedly to a different extent.

Two patients had two joints replaced by prostheses. It was decided to count 13 points in the Pettersson score for each joint prosthesis (as normally done in Pettersson scoring), thereby increasing the total Pettersson score. This might cause a bias when evaluating an association between total Pettersson score and biomarker level, since these joints do not actually contribute to cartilage degradation biomarkers due to lack of cartilage, whereas they are included in the Pettersson score. This could explain that for this small group no association between any clinical parameter and changes in biomarker levels was found.

The results of biomarkers over time after an experimental canine joint bleed were only slightly better than those of the human study. However, also this study consisted of a relatively small number of dogs, which might explain the lack of statistical significance. An important observation from the canine experiment was that it is demonstrated that biomarker levels 13 weeks after the joint bleed indeed drop to (and sometimes even slightly below) baseline values again, thereby validating the use of baseline values 90

days after a joint bleed in the human study. However, some of the haemophilia patients (may have) experienced recurrent joint bleeds during the follow up period of 90 days, thereby most likely disturbing baseline values. This might additionally explain why statistical significance is lacking in the human data.

For this study all human and canine samples were taken in the morning, but not at a fixed time point, nor fasted. This is because it was not feasible in practice and it was considered of inferior relevance because we hypothesized that levels would rise significantly due to the joint bleed stimulated joint damage. However, in this small study, the circadian changes in biomarker levels over the day and due to food intake[15-17] could be responsible for slightly more variance and thereby the absence of statistically significant results. This explorative study demonstrates that on average in a small heterogeneous group of haemophilia patients a single joint bleed transiently increases biochemical markers of joint tissue damage shortly after the bleed. Although this observation only approached statistical significance, it was corroborated by an animal *in vivo* study, and therefore warrants further research on the value of biochemical markers to detect the joint destructive properties of a joint bleed.

REFERENCES

1. Rodriguez-Merchan EC, Jimenez-Yuste V, Aznar JA, Hedner U, Knobe K, Lee CA, et al. Joint protection in haemophilia. Haemophilia 2011;17 Suppl 2:1-23.
2. Acharya SS. Exploration of the pathogenesis of haemophilic joint arthropathy: understanding implications for optimal clinical management. Br J Haematol 2011;156:13-23.
3. Jelbert A, Vaidya S, Fotiadis N. Imaging and staging of haemophilic arthropathy. Clin Radiol 2009;64:1119-28.
4. Lundin B, Babyn P, Doria AS, Kilcoyne R, Ljung R, Miller S, et al. Compatible scales for progressive and additive MRI assessments of haemophilic arthropathy. Haemophilia 2005;11:109-15.
5. Pettersson H, Ahlberg A, Nilsson IM. A radiologic classification of hemophilic arthropathy. Clin Orthop Relat Res 1980;149:153-9.
6. Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N Engl J Med 2007;357:535-44.
7. Silva M, Luck JV, Jr., Quon D, Young CR, Chin DM, Ebramzadeh E, et al. Inter- and intra-observer reliability of radiographic scores commonly used for the evaluation of haemophilic arthropathy. Haemophilia 2008;14:504-12.
8. van Spil WE, Jansen NW, Bijlsma JW, Reijman M, Degroot J, Welsing PM, et al. Clusters within a wide spectrum of biochemical markers for osteoarthritis: data from CHECK, a large cohort of individuals with very early symptomatic osteoarthritis. Osteoarthritis Cartilage 2012;20:745-54.
9. Hoch JM, Mattacola CG, Medina McKeon JM, Howard JS, Lattermann C. Serum cartilage oligomeric matrix protein (sCOMP) is elevated in patients with knee osteoarthritis: a systematic review and meta-analysis. Osteoarthritis Cartilage 2011;19:1396-404.
10. van Spil WE, Drossaers-Bakker KW, Lafeber FP. Associations of CTX-II with biochemical markers of bone turnover raise questions on its tissue origin: data from CHECK, a cohort study of early osteoarthritis. Ann Rheum Dis 2012.
11. Tanishi N, Yamagiwa H, Hayami T, Mera H, Koga Y, Omori G, et al. Relationship between radiological knee osteoarthritis and biochemical markers of cartilage and bone degradation (urine CTX-II and NTX-I): the Matsudai Knee Osteoarthritis Survey. J Bone Miner Metab 2009;27:605-12.
12. Garnero P, Landewe R, Boers M, Verhoeven A, Van Der Linden S, Christgau S, et al. Association of baseline levels of markers of bone and cartilage degradation with long-term progression of joint damage in patients with early rheumatoid arthritis: the COBRA study. Arthr Rheum 2002;46:2847-56.
13. Jansen NW, Roosendaal G, Lundin B, Heijnen L, Mauser-Bunschoten E, Bijlsma JW, et al. The combination of the biomarkers urinary C-terminal telopeptide of type II collagen, serum cartilage oligomeric matrix protein, and serum chondroitin sulfate 846 reflects cartilage damage in hemophilic arthropathy. Arthritis Rheum 2009;60:290-8.
14. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. J Rheumatol 1997;24:1350-4.
15. Andersson ML, Petersson IF, Karlsson KE, Jonsson EN, Mansson B, Heinegard D, et al. Diurnal variation in serum levels of cartilage oligomeric matrix protein in patients with knee osteoarthritis or rheumatoid arthritis. Ann Rheum Dis 2006;65:1490-4.
16. Karsdal MA, Byrjalsen I, Bay-Jensen AC, Henriksen K, Riis BJ, Christiansen C. Biochemical markers identify influences on bone and cartilage degradation in osteoarthritis--the effect of sex, Kellgren-Lawrence (KL) score, body mass index (BMI), oral salmon calcitonin (sCT) treatment and diurnal variation. BMC Musculoskelet Disord 2010;11:125.
17. Kong SY, Stabler TV, Criscione LG, Elliott AL, Jordan JM, Kraus VB. Diurnal variation of serum and urine biomarkers in patients with radiographic knee osteoarthritis. Arthritis Rheum 2006;54:2496-504.
18. Van Meegeren ME, Roosendaal G, Barten-van Rijbroek AD, Schutgens RE, Lafeber FP, Mastbergen SC. Coagulation aggravates blood-induced joint damage. Arthritis Rheum 2012 june 5. Epub ahead of print.
19. Jansen NW, Roosendaal G, Bijlsma JW, DeGroot J, Theobald M, Lafeber FP. Degenerated and healthy cartilage are equally vulnerable to blood-induced damage. Ann Rheum Dis 2008;67:1468-73.

Chapter 5

IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage

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ABSTRACT

Objective:

It has been reported that interleukin (IL)-10 limits blood-induced cartilage damage. Our aim was to study the effect of IL-4 alone and in combination with IL-10 on blood-induced cartilage damage.

Methods:

Healthy human full thickness cartilage explants were cultured for 4 days in the presence of 50% v/v blood. IL-4, IL-10, or a combination of both cytokines was added during blood exposure. Cartilage matrix turnover was determined after a recovery period; additionally cytokine production, chondrocyte apoptosis, and expression of the IL-4 and IL-10 receptors were analyzed directly after exposure.

Results:

Blood-induced damage to the cartilage matrix was limited by IL-4 in a dose-dependent way ($p<0.05$). Also IL-10 limited this damage, although to a lesser extent ($p<0.03$). The effect of IL-4 plus IL-10 was more pronounced and protective than IL-10 alone ($p<0.05$). Production of IL-1β and tumor necrosis factor (TNF)-α was limited by both IL-4 and IL-10 ($p<0.05$), but more strongly by IL-4. Blood-induced apoptosis of chondrocytes was limited by IL-4 and the combination, and not by IL-10 alone. No direct beneficial effect of IL-4 or IL-10 on cartilage was found, however, the chondrocyte receptor expression of both cytokine receptors was upregulated by exposure to blood.

Conclusion:

This study demonstrates that IL-4 alone and in combination with IL-10 prevents blood-induced cartilage damage. Expectedly, anti-inflammatory effects on monocytes in the blood fraction and protective effects on chondrocytes are both involved. IL-4 in combination with IL-10 might be used to prevent blood-induced joint damage as a result of trauma or surgery.

INTRODUCTION

A joint bleed can occur during joint surgery, as a result of joint trauma, and in patients suffering from a bleeding disorder such as hemophilia. Joint bleeds, for whatever cause, are expected to lead to an inflammatory response in the joint and to destruction of joint cartilage, the latter both directly as well as indirectly via inflammation[1,2].
Natural evacuation of blood from the joint cavity leads to deposition of iron in the synovial tissue, resulting in proliferation, hypertrophy, fibrosis, and neovascularization[3]. This inflammation of synovial tissue contributes to cartilage damage[4]. Recently it has been demonstrated that experimentally induced joint bleeds in hemophilic mice lead to rapid elevation of pro-inflammatory cytokines like interleukin (IL)-1β, IL-6, and keratinocyte-derived chemokine (KC; functional murine IL-8 homologue) in the synovial fluid[5], supporting the existence of an inflammatory synovial component in the pathogenesis of blood-induced joint damage. These released cytokines will have their repercussion on cartilage integrity, as it is known that especially IL-1 and tumor necrosis factor (TNF) are major players in cartilage degeneration[6].
There is also a direct harmful effect of blood on cartilage. Previous in vitro research has shown that a single blood-exposure of cartilage can lead to long-lasting, irreversible damage[7]. Monocytes/macrophages and red blood cells, as present in blood, are responsible for irreversible inhibition of matrix synthesis[8]. It is proposed that after a joint bleed, iron-containing hemoglobin is released by damage or phagocytosis of red blood cells. Small amounts of IL-1β, produced by activated monocytes/macrophages, increase production of hydrogen peroxide by chondrocytes. This, in combination with erythrocyte-derived catalytic iron, leads to formation of hydroxyl radicals in the vicinity of chondrocytes, resulting in chondrocyte apoptosis and with that irreversible inhibition of cartilage matrix synthesis[9,10].
The devastating effects of blood in a joint may be ameliorated by treatment with medication. Immunosuppressive cytokines, like IL-4 and IL-10, could be candidates to protect joints from the damaging effects of blood-exposure. IL-4 binds to the multimeric IL-4 receptor (IL-4R) consisting of a primary subunit IL-4Rα associated with a common gamma chain (γc or IL-2R) and/or IL-13Rα’ subunit (also known as IL-13Rα1)[11,12]. IL-4 inhibits production of IL-1β and TNF-α by stimulated synovial fluid mononuclear cells[13]. In animal models of inflammatory arthritis IL-4 inhibits cartilage and bone degradation as well as inflammation[14-17].
IL-10 binds to the tetrameric IL-10 receptor (IL-10R), consisting of two IL-10R1 chains and two IL-10R2 chains[18.] IL-10 is able to stimulate intrinsic immunoregulatory activity. It inhibits production of IL-1β and TNF-α by activated macrophages[19,20] and RA synovial tissue[21,22] in vitro, limits production of metalloproteinase by mononuclear cells, and stimulates production of their inhibitor (TIMP-1)[23]. In experimental arthritis models IL-10 ameliorates arthritis and joint destruction[15,24]. In addition to beneficial effects in rheumatoid- and osteoarthritis, IL-10 also protects cartilage from blood-induced damage in terms of cartilage matrix turnover in vitro. Moreover, IL-10 beneficially affects

cartilage and synovial tissue from patients with hemophilic arthropathy[25]. However, blood-induced cartilage damage is not fully prevented by IL-10[25].

IL-4 and IL-10 use different signaling pathways; IL-4 inhibits cytokine mRNA accumulation in monocytes by enhancing degradation of mRNA, while IL-10 inhibits cytokine mRNA accumulation in monocytes by inhibiting nuclear localization of NFκB[26]. Because IL-4 and IL-10 use different signaling pathways, they are able to exert different, but also potentially additive effects. It has been reported that IL-10 acts synergistically with IL-4 in suppressing macrophage cytotoxic activity[27] and production of IL-1 receptor antagonist by human neutrophils[28]. Moreover, the combination of IL-4 and IL-10 has an additive effect on inhibition of IL-1β and TNF-α production by antigen stimulated mononuclear cells from the synovial fluid and peripheral blood from patients with rheumatoid arthritis[29]. Therefore, in the present study the protective effect of IL-4 alone and in combination with IL-10 on blood-induced cartilage damage was studied.

MATERIALS AND METHODS

Cartilage culture technique

Healthy human articular cartilage tissue was obtained post mortem from humeral heads within 24 hours after death of the donor, according to the medical ethical regulations of the University Medical Center Utrecht. The donors (n=14, mean age 66.8 ± 3.1 years, 7 males and 7 females) had no known history of joint disorders. Full thickness slices of cartilage were cut aseptically from the humeral head, excluding the underlying bone, and kept in phosphate-buffered saline (PBS, pH 7.4). Within 1 hour after dissection, slices were cut in small full thickness cubic explants and weighted aseptically (range 5-15 mg, accuracy ± 0.1 mg). The explants were cultured individually in a 96-wells round-bottomed microtiter plate (at 5% CO2 in air, pH 7.4, 37°C, and 95% humidity). Culture medium (200 µL) 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 PAA), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled human male AB+ serum (Gemini Bioproducts).

Experimental design

For each experiment, fresh blood was drawn from healthy human donors (n=14, mean age 25.8 ± 0.5 years, 8 males and 6 females) in a vacutainer tube (nr. 367895; Becton Dickinson). To mimic a human joint bleed, cartilage was exposed to 50% v/v whole blood for 4 days, which is considered to be the natural evacuation time of blood from the joint cavity[30-32]. After blood-exposure, cartilage explants were washed twice under culture conditions for 45 minutes to remove all additives and were cultured for an additional 12 days. Medium was refreshed every 4 days.

In the first set of experiments, the effects of IL-4 on blood-induced cartilage damage were determined by addition of 10 µL IL-4 to the blood cartilage co-culture in a concentration of 1, 3, 10, 30, and 100 ng/mL during blood-exposure. Experiments were performed with cartilage of 5 independent donors and blood of 5 independent donors (n=5). Previously, it was shown that IL-10 limits blood-induced cartilage damage, using 10 ng/mL as an optimal concentration[25].
In the second set of experiments IL-4 was added in addition to IL-10 (both 10 µL; final concentration 10 ng/mL; Sigma) and compared to the effect of IL-10 alone on blood induced cartilage damage. The experiments were performed in n=9 with cartilage of 9 independent donors and blood of 9 independent donors.
Thirdly, in order to determine whether IL-4 and IL-10 have direct beneficial effects on cartilage, explants were cultured for 4 days in the presence of 10 ng/mL IL-10, IL-4 or a combination of the two cytokines in the absence of blood (5 independent cartilage donors; n=5).

Determination of proteoglycan turnover

As a measure of proteoglycan synthesis rate the sulphate incorporation rate into glycosaminoglycans (GAGs) was determined. At the end of each experiment 74 kBq $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) was added per well. After 4 hours of pulse labeling of the newly formed sulfated GAGs, cartilage samples were washed twice in cold PBS and stored at -20°C. Thawed samples were digested for 2 hours at 65°C with 2% papain (Sigma). 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 was measured by liquid scintillation analysis. Radioactive counts were normalized to the specific activity of the medium, labeling time, and wet weight of cartilage. Results were expressed as moles of sulfate incorporated per gram wet weight of cartilage tissue (nmol/h*g).
Proteoglycan content of each cartilage explant and release of proteoglycans into culture medium of the first 4 days were established by staining and precipitation of GAGs with Alcian Blue (Sigma). Staining was quantified by absorptiometry at 620 nm using chondroitin sulfate (Sigma) as a reference. Results were expressed as mg GAG per wet weight of cartilage tissue (mg/g) and mg GAG released during 4 days per wet weight of tissue (mg/g).
Because of focal differences in composition and bioactivity of the cartilage, proteoglycan turnover parameters were determined of 10 cartilage explants (10 replicates) of the same donor, obtained randomly and handled individually. The average of these 10 samples was taken as a representative value for that cartilage donor.

Enzyme-linked immunosorbent assay (ELISA)

To investigate whether IL-4 and IL-10 had an effect on production of pro-inflammatory cytokines, cartilage explants were cultured for 4 days in absence or presence of 50% blood, and 10 ng/mL IL-4 and/or IL-10 (8 independent donors; n=8). The concentrations

Table 1: Properties of RT-PCR primers

Receptor subunit	Primer		Melting temperature (°C)	Product (bp)
IL-4Rα	Fwd	5'-GGAAGAGGGGTATAAGCCTTT-3'	53	570
	Rev	5'-CACGGAGACAAAGTTCACGAT-3'		
IL-13Rα'	Fwd	5'-AACTCGTCGTTCAATAGAAG-3'	55	454
	Rev	5'-TGTGGAGGATCAGGTTTCACACGG-3'		
γc	Fwd	5'-CTCCTTGCCTAGTGTGGATGG-3'	60	345
	Rev	5'-CACTGTAGTCTGGCTGCAGAC-3'		
IL-10R1	Fwd	5'-CCATCTTGCTGACAACTTCC-3'	60	440
	Rev	5'-GTGTCTGATACTGTCTTGGC-3'		
β-actin	Fwd	5'-CCAAGGCCAACCGCGAGAAGATGAC-3'	60	579
	Rev	5'-AGGGTACATGGTGGTGCCGCCAGAC-3'		

of IL-1β and TNF-α were determined in singlicate in culture supernatants after 1-2 hours of culture, and after 1, 2, 3, and 4 days. Commercially available ELISA cytosets (Biosource) were used and the analyses were performed according to the manufacturer's protocol. Data are expressed in pg/mL.

Immunohistochemistry

For immunohistochemical detection of chondrocyte apoptosis anti-single strand DNA monoclonal antibody (clone F7-26; Alexis) was used. Three human cartilage explants of a single donor were exposed to 50% v/v whole blood of a single donor for 4 days with or without 10 ng/mL IL-4, IL-10 or IL-4 plus IL-10. Subsequently cartilage explants were harvested and fixed in 4% formalin at 4°C for 24 hours. Fixed samples were dehydrated with absolute ethanol, prepared with xylene and embedded in paraffin. Sections (4 µm) were cut, deparaffined, and rehydrated in PBS. Tissue was permeabilized with 0.2 mg/mL saponin (Sigma) and treated with 20 µg/mL proteinase K (Roche). Slides were 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. The amount of brown-positive stained cells and the total number of chondrocytes were counted, excluding empty lacunae. Per cartilage explant the average counted area was 1.47 mm^2. Data are expressed as percentage positive cells of the total number of chondrocytes for each of the 3 cartilage explants separately.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

To investigate whether blood exposure can alter IL-4/10 receptor expression on chondrocytes, human cartilage of 3 independent donors was cultured for 4 days with or without exposure to 50% v/v blood (of 3 independent donors; n=3). After 4 days cartilage was washed and snap-frozen. Total cellular RNA was extracted using a tissue homogenizer (ART's Miccra D-1) and Qiagen RNeasy Lipid tissue mini kit. About 100 mg

cartilage was crushed in Qiazol to maintain RNA integrity and break down cells and cell components. RNA was isolated according to manufacturer's instructions.

RT-PCR was performed using the GeneAmp PCR Core Kit (Roche) according to the manufacturer's protocol. Briefly, an equal amount of RNA per cartilage donor, varying from 44 to 90 ng, was reverse transcribed using random hexamers as primers. An extensive region was amplified from IL-4Rα, IL-13Rα', γc, IL-10R1, and β-actin cDNA, using 30 pmol of the corresponding primers. These were adopted from Guicheux et al. (IL-4Rα, IL-13Rα', γc, and β-actin)[33] or from Lysa et al. (IL-10R1)[34] (table 1). The samples of IL-4Rα, IL-13Rα', γc, and β-actin were heated for 5 min at 95°C and subjected to 35 cycles of amplification (denaturation at 94°C for 60s; annealing at 53°C, 55°C, 60°C, and 60°C, respectively, for 90s; and extension at 72°C for 60s), followed by an elongated extension step of 10 min after the last cycle. For IL-10R1, samples were heated for 5 min at 95°C and subjected to 30 cycles of amplification (denaturation at 94°C for 60s; annealing at 60°C for 90s; and extension at 72°C for 60s), followed by an elongated extension step of 10 min after the last cycle. Controls without RNA and controls in which the reverse transcription step was omitted were included. As a positive control mRNA of the mononuclear cell line U937 was included. Subsequently, the PCR products were subjected to agarose gel electrophoresis.

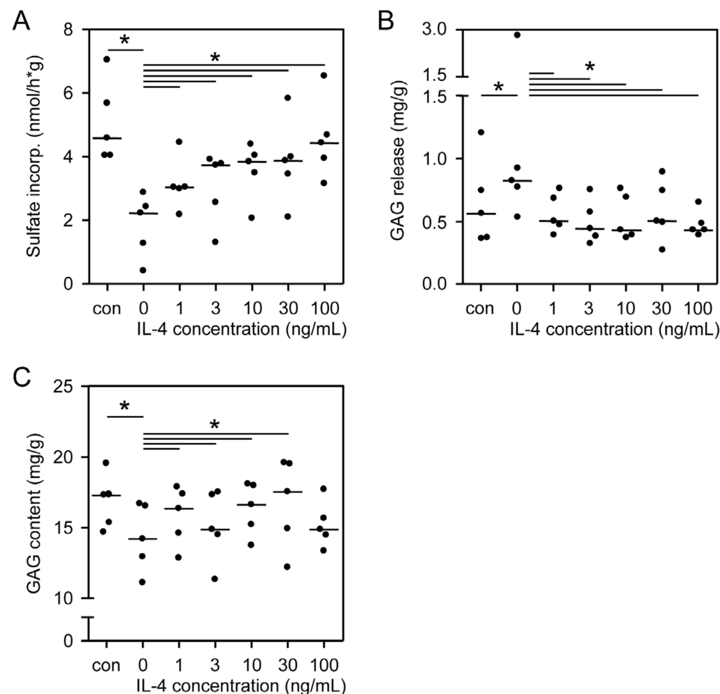

Figure 1: IL-4 prevents blood-induced cartilage damage dose-dependently

Human articular cartilage was exposed to 50% v/v whole blood for 4 days. IL-4 was added in a concentration of 1, 3, 10, 30, and 100 ng/mL during blood-exposure. Proteoglycan synthesis rate (A), -release (B), and -content (C) were determined. Each dot represents an independent cartilage and blood donor (n=5); dashes represent the median value. Asterisks indicate a statistical significant difference (p≤0.05).

5

Calculations and statistical analyses

Results are depicted as median values of individual experiments (viz. independent cartilage and blood donors), except for the immunohistochemistry experiment that was performed in triplicate with cartilage and blood of single donors. The data were analyzed using a non-parametric test for related samples (Wilcoxon signed rank test) with SPSS 15.0 software. Differences were considered to be statistically significant when $p \leq 0.05$.

RESULTS

IL-4 prevents blood-induced cartilage damage in a dose-dependent way

Exposure of cartilage explants to 50% v/v whole blood for 4 days led to an impaired turnover of proteoglycans; one of the major components of articular cartilage. After blood-exposure proteoglycan synthesis rate was decreased by 58%, proteoglycan release was increased by 73%, and total proteoglycan content was reduced by 15% (all p=0.04; first dot figure 1A, B, and C, respectively).

Addition of IL-4 in a concentration ranging from 1-100 ng/mL during the 4 day blood-exposure resulted in a dose-dependent recovery of proteoglycan synthesis rate to

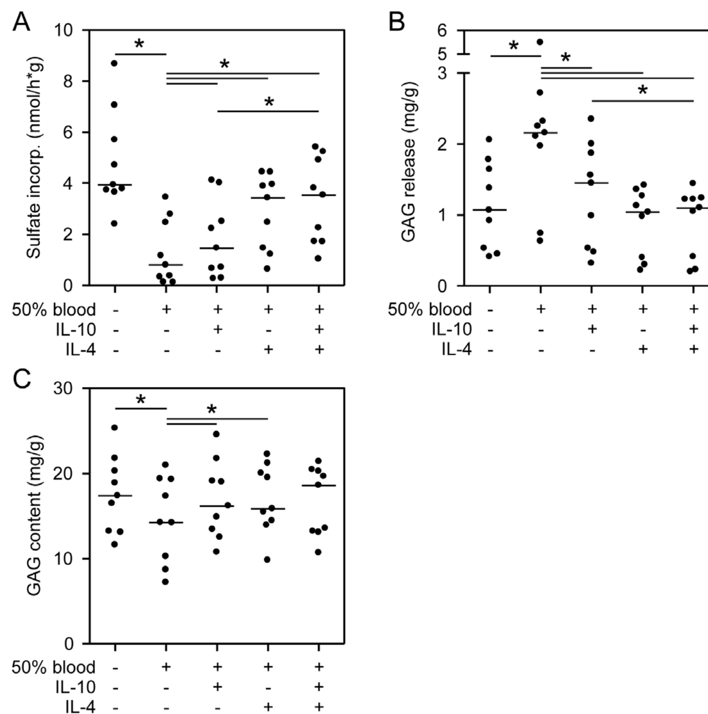

Figure 2: IL-4 and/or IL-10 prevent blood-induced cartilage damage

Human articular cartilage was exposed to 50% v/v whole blood for 4 days. IL-4 and IL-10 were added in a concentration of 10 ng/mL during blood-exposure. Proteoglycan synthesis rate (A), -release (B), and -content (C) are depicted. Each dot represents an independent cartilage and blood donor (n=9); dashes represent the median value. Asterisks indicate a statistical significant difference ($p \leq 0.05$).

complete normalization (compared to cartilage that was exposed to 50% v/v blood; at all concentrations p=0.04). Proteoglycan release demonstrated normalization already at the lowest concentration of IL-4 and this remained over the entire concentration range (at all concentrations p=0.04 compared to blood-exposed cartilage). Also proteoglycan content normalized by addition of IL-4 (from 1 up to 30 ng/mL; p=0.04 compared to blood-exposure).

IL-4 plus IL-10 is more potent than IL-10 alone

Addition of 10 ng/mL IL-10 during exposure of cartilage to 50% v/v blood for 4 days showed similar effects as reported previously[25]; it prevented downregulation of the proteoglycan synthesis rate at day 16 by 41% compared to cartilage that was exposed to blood only (p=0.03; figure 2A). Exposure of cartilage to blood in the presence of 10 ng/mL IL-4 resulted in even better recovery from blood induced inhibition of proteoglycan synthesis (125% increase compared to blood alone; p=0.01). Most importantly, IL-4 as well as the combination of IL-4 and IL-10 resulted in a normalization of proteoglycan synthesis, the effect of IL-4 in addition to IL-10 being statistically significantly better than that of IL-10 alone (p=0.01).

Proteoglycan release was diminished by 42% (p=0.01; figure 2B) upon addition of IL-10 when cartilage was cultured with 50% blood. Addition of IL-4 led to an even further decrease of proteoglycan release (59% decrease compared to blood alone, p=0.01). Moreover, IL-4 added to IL-10 reduction of proteoglycan release (p=0.01).

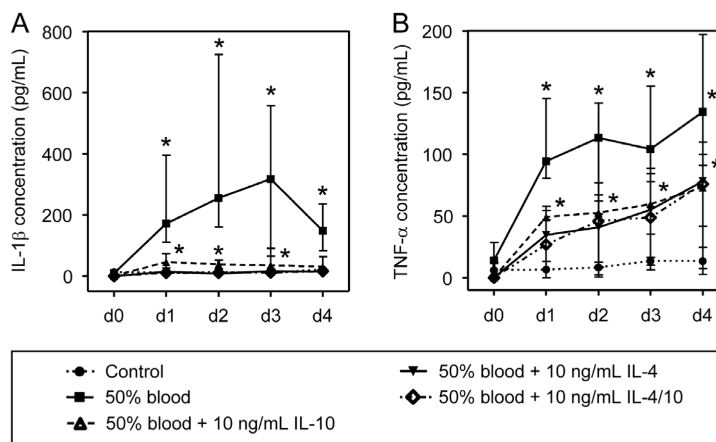

Figure 3: IL-4 and IL-10 limit production of pro-inflammatory cytokines in blood cartilage co-cultures
Cartilage in the absence and presence of 50% v/v whole blood was cultured for a period of 4 days. IL-4 and IL-10 were added in concentrations of 10 ng/mL. The concentration of IL-1β (A) and TNF-α (B) produced during culture, was measured 1-2 hours after blood collection (day 0), and after 1, 2, 3, and 4 days of culture. At day 1-4, addition of IL-4, IL-10, and the combination led to a statistically significant decrease of median IL-1β and TNF-α concentrations when compared to untreated blood cartilage co-cultures (all p≤0.05; not indicated). Asterisks indicate a difference with control values (p≤0.05, n=8 independent cartilage and blood donors); for IL-1β only present for blood without addition or blood with IL-10 addition, and for TNF-α present for all conditions.

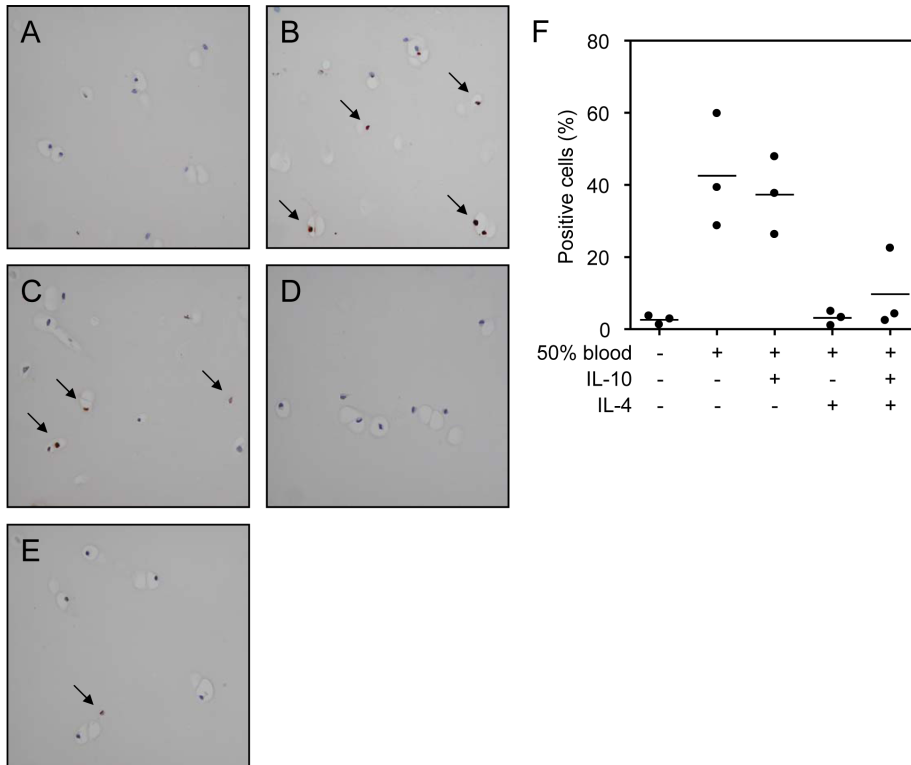

Figure 4: IL-4, but not IL-10, prevents apoptosis of chondrocytes after exposure to blood
Human articular cartilage explants were cultured in medium (A) or exposed to 50% v/v whole blood (B-E) for 4 days. IL-10 (C), IL-4 (D), or a combination of these cytokines (E) was added in a concentration of 10 ng/mL during blood-exposure. Each condition was performed in triplicate with cartilage and blood of a single donor. Apoptosis of chondrocytes was determined by immunohistochemical staining with ssDNA (brown staining indicated by arrow) and was expressed as a percentage of all stained cells (F). Slides of each of the cartilage explants were scored by 2 blinded observers. Each dot represents the average of the two observers; dashes represent the mean value. Original magnification: 40x.

Proteoglycan content significantly improved when blood-exposed cartilage was treated with IL-10 (16% increase compared to blood alone; p=0.03; figure 2C), resulting in normalization. Also addition of IL-4, as well as the combination of IL-4 and IL-10, enhanced proteoglycan content of cartilage exposed to blood (16% and 15% increase; p=0.01 and p=0.05, respectively).

Production of pro-inflammatory cytokines is limited by IL-4 and IL-10
At the start of co-culture of cartilage and blood, as well as during a 4-day cartilage culture in absence of blood, almost no IL-1β or TNF-α was detected in the culture supernatants (figure 3A and 3B; day 0 and control).
Co-culture of cartilage and 50% blood led to a statistically significant increase of IL-1β concentration when compared to control levels (figure 3A; all p=0.01 at day 1-4).

Table 2: No statistically significant direct effects of IL-4 and IL-10 on cartilage explants

	control	10 ng/mL IL-10	10 ng/mL IL-4	10 ng/mL IL-4 & IL-10
PG synthesis rate	3.9 (3.8-5.2)	5.5 (2.6-5.6)	4.4 (3.6-5.8)	4.1 (3.8-5.2)
PG release	0.9 (0.4-1.9)	1.0 (0.5-1.7)	1.0 (0.5-1.9)	1.1 (0.5-1.8)
PG content	16.5 (12.4-19.6)	16.8 (13.8-17.8)	16.8 (13.8-21.0)	19.0 (14.2-21.5)

Proteoglycan (PG) parameters (in absence of blood) are expressed as median with interquartile range (n=5 independent cartilage donors).

Production of IL-1β was suppressed by IL-10 at all time points (all p=0.01 when compared to blood), and fully prevented by IL-4 and by a combination of IL-4 plus IL-10 (all p=0.01 when compared to blood). Moreover, addition of IL-4 or IL-4 plus IL-10 decreased IL-1β levels even more when compared to IL-10 alone (all p<0.02 at day 1-4); IL-10 alone still being statistically significant elevated compared to controls without blood (p<0.02 at day 0-3).

Another important cytokine in cartilage degeneration, TNF-α, was significantly increased in cultures with 50% blood (figure 3B; all p<0.02 when compared to control at day 1-4). TNF-α production was suppressed by IL-4, IL-10, and a combination of IL-4 plus IL-10 at day 1-4 (all p<0.02 when compared to blood), although TNF-α levels were still statistically significant higher than controls (all p<0.05 at day 1-4).

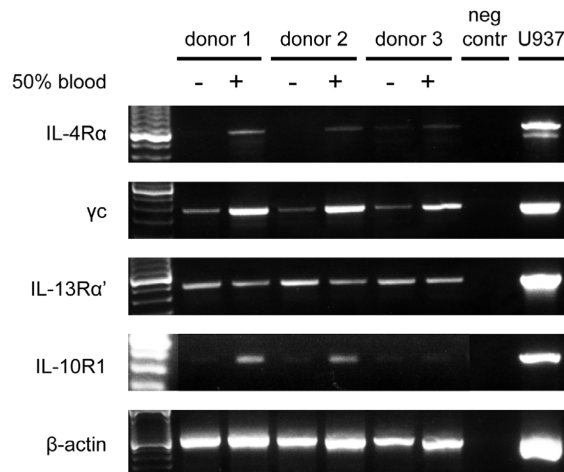

Figure 5: Cartilage upregulates mRNA expression of IL-4R and IL-10R after exposure to blood
Human articular cartilage (n=3 independent cartilage and blood donors) was exposed to 50% v/v whole blood for 4 days. Cartilage (±100 mg) was crushed and mRNA was isolated. A RT-PCR reaction was performed for subunits of the IL-4 receptor (IL-4Rα, γc, and IL-13Rα'), for the IL-10 receptor (IL-10R1), and for β-actin as a household gene. As a positive control mRNA of the mononuclear cell line U937 was included.

IL-4 and the combination of IL-4 plus IL-10 limit blood-induced apoptosis of chondrocytes

It has been shown that IL-1β in combination with lysed erythrocytes causes hydroxyl radical formation and with that apoptosis of chondrocytes[9]. Since production of IL-1β and TNF-α were limited by IL-4 and IL-10, it was tested whether this also resulted in limitation of blood-induced apoptosis of chondrocytes. Exposure of cartilage to blood for 4 days led to apoptosis of 43% of the chondrocytes compared to 3% when cartilage was cultured in medium without additives (figure 4B and A, respectively). It appeared that blood-induced apoptosis of chondrocytes was only marginally reduced by the addition of IL-10 (figure 4C; still 37% positive cells). Similar to the effects on proteoglycan turnover and IL-1β production, IL-4 was more potent than IL-10 and lowered the amount of apoptotic cells clearly (figure 4D; 3% positive cells remaining). Also the combination of the two cytokines obviously decreased the amount of blood-induced apoptotic cells (figure 4E; 10% positive cells).

No direct beneficial effects of IL-4 and/or IL-10 on healthy cartilage

Besides the beneficial effects of IL-4 and IL-10 on blood-induced cartilage damage, it was tested whether they had direct beneficial effects on healthy cartilage. It appeared that IL-4 and the combination of IL-4 plus IL-10 slightly increased proteoglycan content, although not statistically significant (table 2). In general, IL-4 and IL-10 did not have statistically significant direct beneficial effects on cartilage proteoglycan turnover in the absence of blood.

Upregulation of IL-4R and IL-10R after blood-exposure

A possible explanation for the lack of direct effects of IL-4 and IL-10 on cartilage could be lack of sufficient receptor expression. Figure 5 depicts mRNA expression of the IL-4 and IL-10 receptors and of the housekeeping gene β-actin on cartilage explants with and without 4 days exposure to blood. It appeared that mRNA expression of two subunits of the IL-4R (IL-4Rα and IL-2Rγ) was upregulated after blood-exposure in 3 different cartilage donors. Also mRNA expression of IL-10R1 was increased after exposure to blood, indicating that receptor expression for both IL-4 and IL-10 is increased after blood-exposure of cartilage. As such, this could explain the fact that IL-4 and IL-10 do not have direct beneficial effects on cartilage that is not exposed to blood.

DISCUSSION

This study shows for the first time that IL-4 is able to prevent blood-induced cartilage damage in a dose-dependent way, and more potently than it has been previously reported for IL-10[25]. At higher concentrations of IL-4, cartilage matrix turnover is normalized after blood-exposure. Moreover, it was shown that IL-4 plus IL-10 has an additional protective effect on blood-induced cartilage damage compared to IL-10 alone regarding proteoglycan synthesis rate and -release. Both IL-4 and IL-10 inhibit production

of the pro-inflammatory cytokines IL-1β and TNF-α, again IL-4 being more effective, whereas only IL-4 and the combination of IL-4 plus IL-10 prevent chondrocyte apoptosis. No long-lasting direct effects of IL-4 and IL-10 on healthy cartilage were found, which was explained by the low expression of IL-4 and IL-10 receptors on chondrocytes. After exposure of cartilage to blood, these receptors were upregulated, providing a potential secondary direct effect on cartilage.

The effect of the combination of IL-4 in addition to IL-10 was statistically significantly different from the effect of IL-10 alone, but not compared to the effect of IL-4 alone. Apparently in equal concentrations the effect of IL-4 is stronger than that of IL-10 and as such can overcome the effect of IL-10, but not the other way around. It might well be that IL-10 still adds to the effect of IL-4 in case IL-4 concentrations are lower than that of IL-10, but this needs further study. Nevertheless, the use of IL-4 plus IL-10 instead of IL-4 alone may be essential in case of clinical application. It was shown that adenoviral vector-mediated overexpression of IL-4 in collagen-induced arthritis mice leads to prevention of chondrocyte death, a reduction in cartilage erosions, and improvement of inhibition of proteoglycan synthesis. However, also synovial inflammation was enhanced because of exposure to high levels of IL-4 (approximately 400 pg/mL measured 7 days after injection of the vector)[16]. This could be explained by the fact that IL-4 is able to attract macrophages, thereby increasing an inflammatory response[35]. Sustained exposure to high levels of IL-4 can lead to accumulation of tissue macrophages and increased erythrophagocytosis in liver, spleen, and bone marrow of mice[36]. It is also known that IL-4 is crucial for full development of arthritis in K/BxN mice[37], indicating that high levels of IL-4 could be harmful to the joint. When IL-4 is combined with IL-10, a better balance between the Th1 and Th2 response is achieved[38]. This could result in less inflammation of synovial tissue, maintaining the protective effects of IL-4 on cartilage[29].

This in vitro study showed that IL-4 and IL-4 plus IL-10 can almost fully prevent blood-induced cartilage damage in a culture system without involvement of surrounding tissues like synovial tissue. However, inflamed synovial tissue contributes to joint and cartilage damage in vivo[39]. We assume that IL-4 and IL-10 have even more beneficial effects in vivo, since it is known that IL-4 and IL-10 regulate inflammation in arthritis models and rheumatoid synoviocytes[40,41]. Synovitis is characterized by an increase in proangiogenic factors and vascularity[42,43]. IL-4 is able to reduce these proangiogenic factors and to directly inhibit angiogenesis in synovial tissue of rat adjuvant-induced arthritis[44]. Furthermore, IL-4 limits VEGF production by fibroblasts derived from rheumatoid arthritis patients. Moreover, the combination of IL-4 plus IL-10 limited VEGF production even more than each of the cytokines alone[45]. As such, besides beneficial effects on cartilage upon blood exposure, IL-4 and IL-10 could additionally reduce synovitis when administered in vivo.

This study also showed that IL-4 and the combination of IL-4 plus IL-10 reduce blood-induced apoptosis of chondrocytes, while IL-10 alone does not. This is in contrast with literature demonstrating that IL-10 limits TNF-α-induced apoptosis of serum-starved human articular chondrocytes as measured by caspases activation[46]. However, in those experiments serum deprivation was needed as an additional apoptotic stimulus for

TNF-α, whereas in this study the trigger for apoptosis is exposure to blood. Another study showed that pretreatment of chondrocytes with a high concentration of IL-4 could not prevent apoptosis of chondrocytes by IL-1β and TNF-α[47]. This is also in contrast with the results of our study.

It is known that the capacity of IL-4 to act on chondrocytes depends on the differentiation status of the chondrocyte and thereby on the expression patterns of IL-4R subunits[33]. Our study shows that chondrocytes cultured in a cartilage explant for 4 days express only 2 out of 3 subunits of the IL-4R; γc and IL-13Rα'. After 4 days exposure to blood, both mRNA of the IL-4Rα and γc subunit are upregulated, while IL-13Rα' remains equally expressed. Also IL-10R1 expression is upregulated after blood-exposure. This could explain that IL-4 plus IL-10 is able to limit blood-induced cartilage damage, but does not have direct beneficial effects on healthy cartilage matrix turnover itself. The question remains how long a higher expression of IL-4R and IL-10R will last and whether it enables direct beneficial effect on cartilage after blood exposure. However, since blood exposure causes apoptosis of 43% of the chondrocytes, it is assumed that exposure of cartilage to IL-4 and IL-10 after a bleed is not capable to restore the cartilage damage. On the other hand, up-regulation of receptors on the remaining viable chondrocytes might result in sustained effects of IL-4 and IL-10.

This study showed that IL-4 and IL-10 are able to normalize production of IL-1β and TNF-α in a blood-cartilage co-culture. Culture of blood alone produces the same amounts of IL-1β and TNF-α as measured in a co-culture of blood and cartilage (data not shown), indicating that the majority of IL-1β and TNF-α in a co-culture is produced by monocytes in the blood fraction instead of by chondrocytes in the cartilage matrix. Therefore it is assumed that IL-4 and IL-10 act for a large part via monocytes in the blood fraction through inhibition of pro-inflammatory cytokines. It seems that cartilage is protected by a change of the blood culture characteristics. However, IL-4 and IL-10 probably also act via chondrocytes, because the receptors for both cytokines are upregulated after blood exposure.

In conclusion, this study shows that IL-4 alone and in addition to IL-10 protects against blood-induced cartilage damage. Expectedly, anti-inflammatory effects on monocytes in the blood fraction and protective effects on chondrocytes are both involved. As such, IL-4 in combination with IL-10 might be used to prevent and treat blood-induced joint damage as a result of trauma or surgery.

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REFERENCES

1. Jones EW. Musculoskeletal complications of hemophilia. Calif Med 1958;88:316-9.
2. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
3. Valentino LA, Hakobyan N, Enockson C. Blood-induced joint disease: the confluence of dysregulated oncogenes, inflammatory signals, and angiogenic cues. Semin Hematol 2008;45:S50-7.
4. Roosendaal G, Vianen ME, Wenting MJ, van Rinsum AC, van den Berg HM, Lafeber FP, et al. Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. J Bone Joint Surg Br 1998;80:540-5.
5. Ovlisen K, Kristensen AT, Jensen AL, Tranholm M. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from haemophilic mice with experimentally induced haemarthrosis. Haemophilia 2009;15:802-10.
6. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, et al. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum 2005;52:128-35.
7. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
8. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. J Rheumatol 1997;24:1350-4.
9. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
10. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
11. Murata T, Taguchi J, Puri RK. Interleukin-13 receptor alpha' but not alpha chain: a functional component of interleukin-4 receptors. Blood 1998;91:3884-91.
12. Murata T, Noguchi PD, Puri RK. Receptors for interleukin (IL)-4 do not associate with the common gamma chain, and IL-4 induces the phosphorylation of JAK2 tyrosine kinase in human colon carcinoma cells. J Biol Chem 1995;270:30829-36.
13. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54:836-40.
14. Joosten LA, Lubberts E, Helsen MM, Saxne T, Coenen-de Roo CJ, Heinegard D, et al. Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. Arthritis Res 1999;1:81-91.
15. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40:249-60.
16. Lubberts E, Joosten LA, van Den Bersselaar L, Helsen MM, Bakker AC, van Meurs JB, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999;163:4546-56.
17. Kageyama Y, Koide Y, Uchijima M, Nagata T, Yoshida A, Taiki A, et al. Plasmid encoding interleukin-4 in the amelioration of murine collagen-induced arthritis. Arthritis Rheum 2004;50:968-75.
18. Iannone F, De Bari C, Dell'Accio F, Covelli M, Cantatore FP, Patella V, et al. Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. Clin Exp Rheumatol 2001;19:139-45.
19. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-20.
20. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815-22.

21. Chomarat P, Vannier E, Dechanet J, Rissoan MC, Banchereau J, Dinarello CA, et al. Balance of IL-1 receptor antagonist/IL-1 beta in rheumatoid synovium and its regulation by IL-4 and IL-10. J Immunol 1995;154:1432-9.
22. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994;179:1517-27.
23. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 1995;96:2304-10.
24. Persson S, Mikulowska A, Narula S, O'Garra A, Holmdahl R. Interleukin-10 suppresses the development of collagen type II-induced arthritis and ameliorates sustained arthritis in rats. Scand J Immunol 1996;44:607-14.
25. Jansen NW, Roosendaal G, Hooiveld MJ, Bijlsma JW, van Roon JA, Theobald M, et al. Interleukin-10 protects against blood-induced joint damage. Br J Haematol 2008;142:953-961.
26. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem 1995;270:9558-63.
27. Oswald IP, Gazzinelli RT, Sher A, James SL. IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J Immunol 1992;148:3578-82.
28. Crepaldi L, Silveri L, Calzetti F, Pinardi C, Cassatella MA. Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10. Int Immunol 2002;14:1145-53.
29. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum 2001;44:3-12.
30. Niibayashi H, Shimizu K, Suzuki K, Yamamoto S, Yasuda T, Yamamuro T. Proteoglycan degradation in hemarthrosis. Intraarticular, autologous blood injection in rat knees. Acta Orthop Scand 1995;66:73-9.
31. Safran MR, Johnston-Jones K, Kabo JM, Meals RA. The effect of experimental hemarthrosis on joint stiffness and synovial histology in a rabbit model. Clin Orthop Relat Res 1994:280-8.
32. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia 2006;12 Suppl 3:117-21.
33. Guicheux J, Palmer G, Relic B, Mezin F, Caverzasio J, Apostolides P, et al. Primary human articular chondrocytes, dedifferentiated chondrocytes, and synoviocytes exhibit differential responsiveness to interleukin-4: correlation with the expression pattern of the common receptor gamma chain. J Cell Physiol 2002;192:93-101.
34. Lysa B, Tartler U, Wolf R, Arenberger P, Benninghoff B, Ruzicka T, et al. Gene expression in actinic keratoses: pharmacological modulation by imiquimod. Br J Dermatol 2004;151:1150-9.
35. Hiester AA, Metcalf DR, Campbell PA. Interleukin-4 is chemotactic for mouse macrophages. Cell Immunol 1992;139:72-80.
36. Milner JD, Orekov T, Ward JM, Cheng L, Torres-Velez F, Junttila I, et al. Sustained IL-4 exposure leads to a novel pathway for hemophagocytosis, inflammation and tissue macrophage accumulation. Blood 2010;116:2476-2483.
37. Ohmura K, Nguyen LT, Locksley RM, Mathis D, Benoist C. Interleukin-4 can be a key positive regulator of inflammatory arthritis. Arthritis Rheum 2005;52:1866-75.
38. van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996;39:829-35.
39. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 2010;6:625-35.
40. Lubberts E, Joosten LA, Helsen MM, van den Berg WB. Regulatory role of interleukin 10 in joint inflammation and cartilage destruction in murine streptococcal cell wall (SCW) arthritis. More therapeutic benefit with IL-4/IL-10 combination therapy than with IL-10 treatment alone. Cytokine 1998;10:361-9.

41. Dechanet J, Rissoan MC, Banchereau J, Miossec P. Interleukin 4, but not interleukin 10, regulates the production of inflammation mediators by rheumatoid synoviocytes. Cytokine 1995;7:176-83.
42. Acharya SS, Kaplan RN, Macdonald D, Fabiyi OT, DiMichele D, Lyden D. Neoangiogenesis contributes to the development of hemophilic synovitis. Blood 2011;117:2484-93.
43. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. Clin Orthop Relat Res 1997:6-11.
44. Haas CS, Amin MA, Allen BB, Ruth JH, Haines GK, 3rd, Woods JM, et al. Inhibition of angiogenesis by interleukin-4 gene therapy in rat adjuvant-induced arthritis. Arthritis Rheum 2006;54:2402-14.
45. Hong KH, Cho ML, Min SY, Shin YJ, Yoo SA, Choi JJ, et al. Effect of interleukin-4 on vascular endothelial growth factor production in rheumatoid synovial fibroblasts. Clin Exp Immunol 2007;147:573-9.
46. John T, Muller RD, Oberholzer A, Zreiqat H, Kohl B, Ertel W, et al. Interleukin-10 modulates pro-apoptotic effects of TNF-alpha in human articular chondrocytes in vitro. Cytokine 2007;40:226-34.
47. Schuerwegh AJ, Dombrecht EJ, Stevens WJ, Van Offel JF, Bridts CH, De Clerck LS. Influence of pro-inflammatory (IL-1 alpha, IL-6, TNF-alpha, IFN-gamma) and anti-inflammatory (IL-4) cytokines on chondrocyte function. Osteoarthritis Cartilage 2003;11:681-7.

Chapter 6

A short time window to profit from protection of blood-induced cartilage damage by IL-4 plus IL-10

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ABSTRACT

Objective:

IL-4 plus IL-10 prevent blood-induced cartilage damage. The aim of the present study was to evaluate whether cartilage damage can still be averted by addition of IL-4 plus IL-10 when added after the onset of a bleed, and whether aspiration of blood prior to addition of IL-4 plus IL-10 is of additive protective value.

Methods:

Healthy canine hip and human shoulder cartilage were exposed to whole blood for 4 days. IL-4 plus IL-10 were administered directly or after a delay of several hours up to 2 days. Furthermore, blood was aspirated after 1 or 2 days and subsequently IL-4 plus IL-10 were added. IL-1β concentration and cartilage matrix proteoglycan turnover were determined.

Results:

Exposure of canine and human cartilage to blood decreased proteoglycan synthesis rate and -content, and increased proteoglycan release. IL-4 plus IL-10 only prevented blood-induced damage of canine cartilage when directly added, and not after 4 hours or later. For human cartilage, IL-4 plus IL-10 limited blood-induced damage as well as IL-1β production when administered within 4-8 hours after the onset of a bleed, but not thereafter. Aspiration of blood within 24 hours fully prevented cartilage damage. Subsequent addition of IL-4 plus IL-10 was not of additive value.

Conclusions:

For humans, there is a short time window after onset of a joint bleed in which IL-4 plus IL-10 can limit blood-induced cartilage damage. Furthermore, aspiration of a joint to shorten blood exposure fully prevents cartilage damage. Both options can be considered in treatment of a joint hemorrhage.

INTRODUCTION

During a joint bleed in case of trauma, major surgery, or hemophilia, articular cartilage gets damaged because of direct exposure to blood[1,2]. *In vitro* research showed that single exposure of cartilage to blood leads to long-lasting, irreversible damage[3]. It was demonstrated that monocytes/macrophages within the mononuclear cell population together with red blood cells, as present in blood, are responsible for irreversible inhibition of matrix synthesis[3]. This is caused by induction of chondrocyte apoptosis through formation of hydroxyl radicals[4]. Relatively small amounts of interleukin (IL)-1β produced by activated monocytes/macrophages increase the production of hydrogen peroxide by chondrocytes. Hydroxyl radicals are subsequently formed through the Fenton reaction when hydrogen peroxide reacts with erythrocyte-derived catalytic iron in the vicinity of chondrocytes[5]. This proposed mechanism is thought to underlie the irreversible inhibition of cartilage matrix synthesis after blood exposure.
In addition to these direct effects on cartilage, the huge amount of red blood cells in the joint cavity results in synovial tissue inflammation by mechanisms involving steps of the coagulation cascade as well as formation of hemosiderin. Blood coagulation results in activation of several proteases of the coagulation cascade, leading to inflammation via activation of protease-activated receptors (PAR)[6,7]. Natural evacuation of blood from the joint cavity leads to deposition of iron (hemosiderin) in the synovial tissue. This results in proliferation and hypertrophy of the synovium, fibrosis, and neovascularization[8]. Infiltration of the synovial tissue with lymphocytes causes a more chronic inflammatory reaction, contributing to cartilage damage[9]. As such, the pathogenesis of blood-induced joint damage involves both degenerative changes in cartilage (as seen in osteoarthritis) and inflammatory changes in synovial tissue (as seen in rheumatoid arthritis)[10].
Joint bleeds can occur during joint surgery or due to major joint trauma. In animal experiments it has been demonstrated that intra-articular surgery with prevention of bleeds results in less joint damage than in case intra-articular bleeds during surgery occur[2]. Also patients with a bleeding disorder like hemophilia suffer from recurrent joint bleeds. Surprisingly there is no consensus on aspiration (arthrocentesis) of a joint after a joint bleed, neither in case of hemophilia nor after major joint trauma[11,12]. In addition to prevention of joint bleeds during surgery and quick aspiration of a joint after a bleed, the devastating effects of blood in a joint may be averted by treatment.
Modulatory/anti-inflammatory cytokines, like IL-4 and IL-10, can protect cartilage from damage in case of arthritis. IL-4 inhibits production of IL-1β and TNF-α of stimulated synovial fluid MNC[13]. In animal models of inflammatory arthritis, IL-4 inhibits cartilage and bone degradation as well as inflammation[14-17]. IL-10 inhibits production of IL-1β and TNF-α by activated macrophages *in vitro*[18] and production of metalloproteinase by mononuclear cells and stimulates production of their inhibitor (TIMP-1)[19]. It has also been shown that IL-10 protects cartilage from the damaging effects of blood exposure in terms of cartilage matrix turnover[20]. Moreover, IL-10 beneficially affects cartilage and synovial tissue *in vitro* from patients with hemophilic arthropathy[20]. Because IL-4 and IL-

10 use different signaling pathways, they are able to exert different, but also potentially additive effects. It has been reported that IL-10 acts synergistically with IL-4 in suppressing macrophage cytotoxic activity[21] and production of IL-1 receptor antagonist by human neutrophils[22]. Additionally, the combination of IL-4 and IL-10 has an additive effect on inhibition of IL-1β and TNF-α production by antigen stimulated mononuclear cells from the synovial fluid and peripheral blood from patients with rheumatoid arthritis[23].
We recently showed that a combination of IL-4 plus IL-10 protects cartilage from blood-induced damage when administered directly upon blood exposure (manuscript accepted for publication O&C). However, this is not of value for clinical practice because a patient can never be treated at the exact same time as the joint bleed starts. Therefore, in this study the clinical relevance of IL-4 plus IL-10 administration is evaluated by investigating whether IL-4 plus IL-10 are still able to protect cartilage from blood-induced damage when added after the onset of a bleed and whether aspiration of the blood prior to addition of IL-4 and IL-10 is of additive value.

MATERIALS AND METHODS

Canine in vitro experiments

First experiments were performed with canine cartilage, because the dog is an important *in vivo* model of blood-induced cartilage damage[24-29]. Healthy full thickness Beagle dog articular cartilage was obtained *post mortem* from hips (n=12 females, mean age 2.1 ± 0.1 years) within 3 hours after death of the animal. The Utrecht University Ethical Committee had given ethical approval for the animal tissue studies. Explants were cultured individually in a 96-wells round-bottomed microtiter plate containing 200 µL medium per well in a tissue incubator (5% CO_2 in air, pH 7.4, 37°C). Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with glutamine (2 mM), penicillin (100 IU/mL), streptomycin sulfate (100 µg/mL; all PAA), ascorbic acid (85 µM; Sigma), and 10% heat-inactivated pooled Beagle serum.
Cartilage of the 12 dogs was exposed to 20% v/v whole blood for 4 days, assuming the blood is cleared relatively quickly from the canine joint resulting in low blood levels within a 4 day time span[28]. For this, fresh blood was drawn from healthy dog (in total n=3, mean age 2.3 ± 0.3 years) in a heparinized vacutainer tube (catalog nr 367820, Becton Dickinson). A combination of 30 ng/mL recombinant canine IL-4 and IL-10 (10 µL; R&D systems) was added directly after addition of blood, as well as 4 and 8 hours after the start of blood exposure. Experiments demonstrated that this concentration causes optimal protection against blood-induced damage (manuscript accepted for publication O&C) and is a concentration feasible to administer intra-articularly *in vivo*[30]. After 4 days exposure to blood without and with IL-4 plus IL-10, cartilage explants were washed twice under culture conditions for 45 minutes. Subsequently, cartilage was cultured for an additional 12 days without additions, during which culture medium was refreshed every 4 days. This allowed evaluation of the prolonged effects after bleed and treatment, important for translation to *in vivo* application and outcome.

Human in vitro experiments

To evaluate whether the effects observed with canine cartilage were also obtained with human cartilage tissue and to extend different variables, a comparable set of experiments was performed with human cartilage tissue. Healthy human full thickness articular cartilage tissue was obtained *post mortem* from humeral heads within 24 hours after death of the donor, according to the medical ethical regulations of the University Medical Center Utrecht. The donors (n=16, mean age 65 ± 4 years, 10 males and 6 females) had no known history of joint disorders. Cartilage was cut aseptically and the underlying bone was excluded. Slices were kept in phosphate-buffered saline (PBS, pH 7.4). Within one hour after dissection, slices were cut in small cubes and weighted aseptically (range 5-15 mg, accuracy ± 0.1 mg).

Cartilage was cultured in the same culture medium as canine cartilage explants, except that 10% heat inactivated pooled human AB+ serum (Gemini Bioproducts) was used instead of canine serum. For each experiment, fresh blood was drawn from healthy human donors (n=16, mean age 29 ± 2 years, 5 males and 11 females) in a vacutainer tube (catalog nr 367880, Becton Dickinson). Explants were exposed to 50% v/v whole blood for 4 days, supposed to mimic the concentration and duration observed during the natural evacuation of blood from a human joint[31]. After blood exposure, cartilage explants were washed twice under culture conditions for 45 minutes and cultured for an additional 12 days. Culture medium was refreshed every 4 days without further additions.

In a first set of experiments a combination of 30 ng/mL recombinant human IL-4 and IL-10 (10 µL; both Sigma) was added directly after addition of blood, or 2, 4, 8, 24, or 48 hours after the onset of blood exposure. In addition to proteoglycan turnover, IL-1β was determined in these experiments as a measure of pro-inflammatory and cartilage destructive cytokines and a key player in the pathway leading to chondrocyte apoptosis[5]. In a second set of experiments, the cartilage explants were put in fresh culture medium after 1 or 2 days to remove most of the blood from the cartilage (without thorough rinsing) mimicking aspiration of a joint. Subsequently, 30 ng/mL IL-4 and IL-10 was added (10 µL). At day 4 all the remaining blood and cytokines were removed, mimicking the human *in vivo* conditions as good as possible[31], and cultures proceeded for 12 days follow-up.

6

Determination of proteoglycan turnover

As a measure for proteoglycan synthesis rate, sulfate incorporation rate was determined by addition of $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont; 74 kBq per well) for 4 hours to pulse label the sulfated glycosaminoglycans (GAGs). Subsequently, the cartilage samples were washed twice in cold PBS and stored at -20°C. Thawed samples were digested for 2 hours at 65°C with 2% papain (Sigma). After dilution to the appropriate concentrations, proteoglycan synthesis rate was determined by precipitation of GAGs with 0.3M hexadecylpyridinium chloride monohydrate (CPC; Sigma). The precipitate was dissolved in 3M NaCl and the amount of radioactivity measured by liquid scintillation

counting. Sulfate incorporation was normalized to the specific activity of the medium, labeling time, and wet weight of cartilage. Results were expressed as moles of sulfate incorporated per gram wet weight of cartilage tissue (nmol/h*g).
Proteoglycan content of the papain digests of each cartilage explant and release of proteoglycan into the culture medium over 4 days were determined by staining and precipitation of GAGs with Alcian Blue (Sigma). The amount of staining was measured at 620 nm using chondroitin sulfate (Sigma) as a reference. Results were expressed as mg GAG per wet weight of cartilage tissue (mg/g) and mg GAG released during 4 days per wet weight of tissue (mg/g).

Determination of DNA content
Cellularity of cartilage tissue was established by measuring the DNA content using the fluorescent dye Hoechst (Calbiochem), with calf thymus DNA (Sigma) as a reference. Results were expressed as mg DNA per wet weight of cartilage tissue (mg/g). In all experiments the DNA content per wet weight was similar between all cartilage donors and there was no change in DNA content due to blood exposure or treatment. Therefore eventually synthesis rate, release, and content of proteoglycans are expressed per mg wet weight of cartilage.

IL-1β enzyme-linked immunosorbent assay (ELISA)
IL-1β levels were determined after 0, 2, 4, 8, and 24 hours in blood (50% v/v) cultures with or without cartilage. Subsequently, the effect of IL-4 plus IL-10 addition after 2, 4, 8, 24, and 48 hours was determined in blood cultures at day 4. Culture media were centrifuged at 1500 *g* for 10 min, and stored at -80°C until analysis. A commercially available ELISA cytoset (Biosource) was used and the analysis was performed according to the manufacturer's instructions.

Calculations and statistical analyses
Each experiment was performed with cartilage obtained from a single donor. Because joint cartilage from different locations within a joint varies in composition and cellular activity, the mean value of 10 cartilage explants per parameter per donor, obtained randomly, and handled individually, was taken as a representative value for the cartilage of that donor (10 replicates for each experiment). The n-values in this study indicate the number of cartilage donors (experiments). Results were depicted as median values ± interquartile range (IQR). The data were analyzed using a non-parametric test for related samples (Wilcoxon signed rank test) with SPSS 15.0 software. Differences were considered statistically significant when $p<0.05$.

RESULTS

Effects of IL-4 plus IL-10 on blood-induced canine cartilage damage

Proteoglycan synthesis rate of canine cartilage cultured *in vitro* decreased on average by 74% after exposure to 20% v/v blood (figure 1A; p=0.002). Administration of IL-4 plus IL-10 concomitantly with the blood resulted in a significant recovery of the proteoglycan synthesis rate (p=0.002). Synthesis rate almost normalized, not being statistically significant different from controls anymore (p=0.071). However, when the combination of IL-4 plus IL-10 was added 4 and 8 hours after onset of blood exposure, proteoglycan synthesis rate remained decreased on average by 75% and 70%, respectively, when compared to control (both p=0.002). These values were not different from blood exposure alone (p=0.455 and p=0.346, respectively).
Release of proteoglycans from the cartilage matrix was increased by 40% after blood exposure (figure 1B; p=0.005). Concomitant addition of IL-4 plus IL-10 counteracted the harmful effect of blood (p=0.037); proteoglycan release was now enhanced by only 21% and not statistically significant different from control conditions anymore (p=0.139).

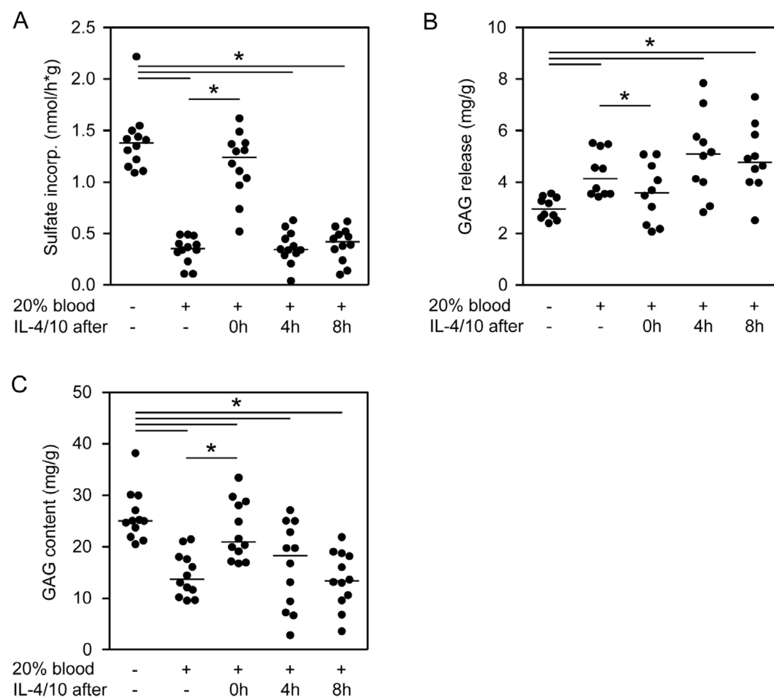

Figure 1: Effects of IL-4 plus IL-10 on blood-exposed canine cartilage
Canine hip cartilage was exposed to 20% v/v whole blood for 4 days, and canine IL-4 plus IL-10 were added in a concentration of 30 ng/mL, concomitant with the blood exposure (0) or 4, or 8 hours later. Proteoglycan synthesis rate **(A)**, -release **(B)**, and -content **(C)** are depicted. Asterisks (*) indicate statistically significant differences between groups (paired analyses; n=12; exact p-values are given in the text). Each dot represents an individual cartilage donor (for each cartilage donor 10 replicates were averaged); dashes represent median values.

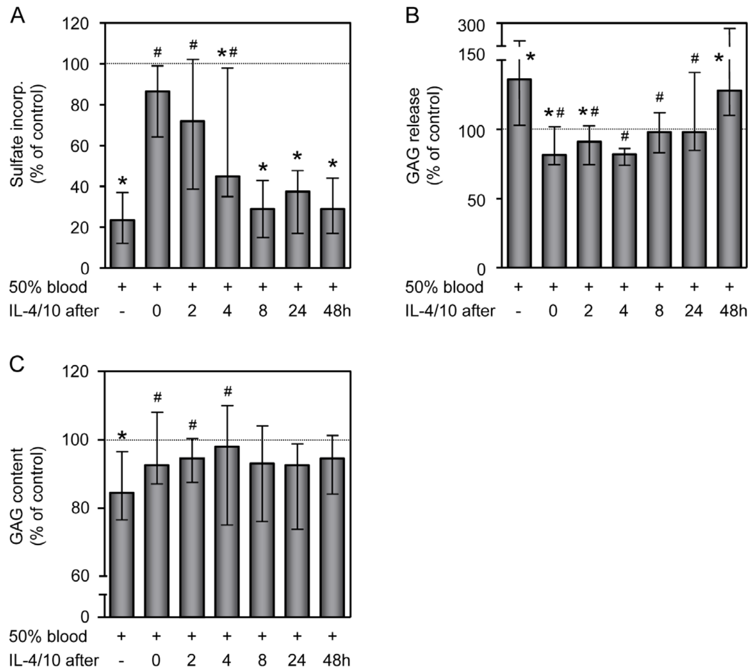

Figure 2: Effects of IL-4 plus IL-10 on human cartilage exposed to blood

Human articular cartilage was exposed to 50% v/v whole blood for 4 days. Human IL-4 plus IL-10 were added in a concentration of 30 ng/mL directly after blood exposure (0) and 2, 4, 8, 24, or 48 hours after start of blood exposure. Proteoglycan synthesis rate **(A)**, -release **(B)**, and -content **(C)** are depicted. Asterisks (*) indicate a statistically significant difference from control values, whereas hash tags (#) indicate a statistically significant difference from blood-exposed cartilage without IL-4 plus IL-10 addition ($p<0.05$). Exact p-values are given in the text. Median values ± IQR of at least 6 individual cartilage donors (10 replicates each) and blood donors are shown.

Addition of IL-4 plus IL-10 after 4 or 8 hours did not prevent an increase in proteoglycan release of 72% and 61%, respectively ($p=0.013$ and $p=0.009$, respectively, when compared to control). These values were not different from blood exposure ($p=0.285$ and $p=0.114$, respectively).

The impaired proteoglycan synthesis rate and the increased release after exposure to blood resulted in a decrease of proteoglycan content by 45% (figure 1C; $p=0.002$), 12 days after the 4-day blood exposure. Addition of IL-4 plus IL-10 together with blood exposure prevented this decrease ($p=0.005$); on average only 16% inhibition remained, although still statistically significant different from control conditions ($p=0.041$). When IL-4 plus IL-10 were added 4 or 8 hours after the start of blood exposure, proteoglycan content remained decreased by 27% and 46%, respectively, when compared to control ($p=0.003$ and $p=0.002$, respectively) and not different from blood exposure alone ($p=0.347$ and $p=0.456$, respectively).

Effects of IL-4 plus IL-10 on blood-induced human cartilage damage

Human cartilage exposed to 50% v/v whole blood showed an average decrease in proteoglycan synthesis rate by 76% (figure 2A; p<0.001). Addition of IL-4 and IL-10 immediately after addition of blood caused a significant recovery of proteoglycan synthesis rate (p<0.001 compared to blood), matching control values again. When the combination of IL-4 plus IL-10 was administered 2 or 4 hours after the start of blood exposure, proteoglycan synthesis rate still recovered significantly (p=0.028 and p=0.018, respectively, compared to blood). When added at later time points, IL-4 plus IL-10 had no effect on the inhibited decrease.

Proteoglycan release from the human cartilage matrix was increased by 36% after 4 days blood exposure (figure 2B; p=0.004). Addition of blood to cartilage immediately followed by addition of IL-4 plus IL-10 prevented the increased release of proteoglycans (p=0.001 compared to blood). The release of proteoglycans was even slightly lower than control values (reduction of 18%; p=0.008). Beneficial recovery was also observed in case IL-4 plus IL-10 was added within the first 24 hours after start of blood exposure (for 2, 4, 8, and 24 hours p=0.016, p=0.043, p=0.018, and p=0.017). When IL-4 plus IL-10 were added 48 hours after the onset of blood exposure, they were unable to change the enhanced release induced by blood exposure.

Decreased proteoglycan synthesis rate and enhanced proteoglycan release due to blood exposure of human cartilage explants eventually led to a decrease of total proteoglycan content by 15% (figure 2C; p=0.005). Treatment with IL-4 plus IL-10 immediately after addition of blood prevented blood-induced decrease of proteoglycan content (p=0.005 compared to blood); it was only decreased by 7% and was not statistically significant different from control values anymore. A delay in addition of IL-4 plus IL-10 of 8 hours

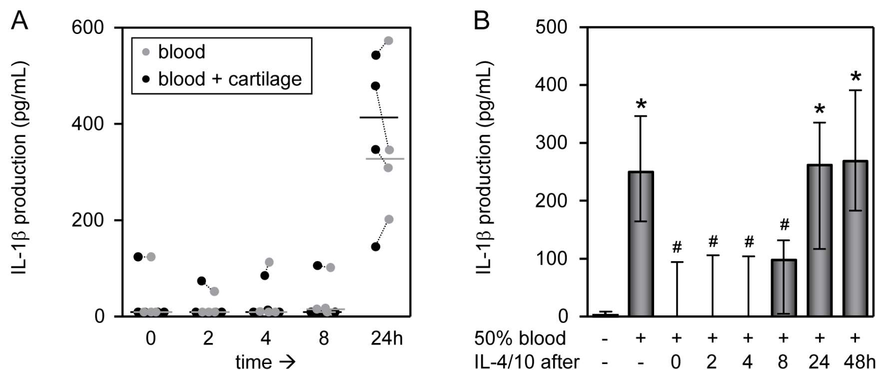

Figure 3: IL-1β production by blood cells and cartilage

During 24 hours 50% blood was cultured in presence or absence of a cartilage explant **(A)**. IL-1β concentration of the supernatants was measured at the start of culture (0) and after 2, 4, 8, and 24 hours. Median values of 4 individual blood cultures are shown. During a 4-day culture of 50% v/v blood, human IL-4 plus IL-10 was added in a concentration of 30 ng/mL directly at the start (0) and 2, 4, 8, 24, or 48 hours after onset of culture **(B)**. IL-1β concentration was measured at day 4. Asterisks indicate a statistically significant difference from control values, whereas hash tags indicate a statistically significant difference from blood-exposed cartilage without IL-4 plus IL-10 addition (p<0.05). Exact p-values are given in the text. Median values ± IQR of 6 individual cartilage and blood donors are shown.

or more was unable to recover the loss of proteoglycans. A decrease in proteoglycan content by 5-7% compared to control was measured; not statistically different from blood alone (p=0.176, p=0.400, and p=0.233 for addition after 8, 12, and 24 hours, respectively).

Effects of IL-4 plus IL-10 on IL-1β production

Figure 3A demonstrates that after 24 hours of culture IL-1β protein levels were detectable and not different between 50% blood cultures with and without cartilage. After 4 days of culture IL-1β concentration reached levels up to 250 pg/mL (figure 3B; p=0.028 compared to control). Concomitant administration of IL-4 plus IL-10, as well as 2 or 4 hours after onset, decreased IL-1β concentration to levels of control values (all p=0.028 compared to blood). This decrease in IL-1β was still observed, although less pronounced, when IL-4 plus IL-10 were administered 8 hours after start of the blood exposure (p=0.028 compared to blood). When the delay in addition of IL-4 plus IL-10 became longer (24 or 48 hours), IL-1β levels were comparable to levels observed for blood alone (262 and 269 pg/mL, respectively); statistically significant different from control (p=0.046 and p=0.028, respectively).

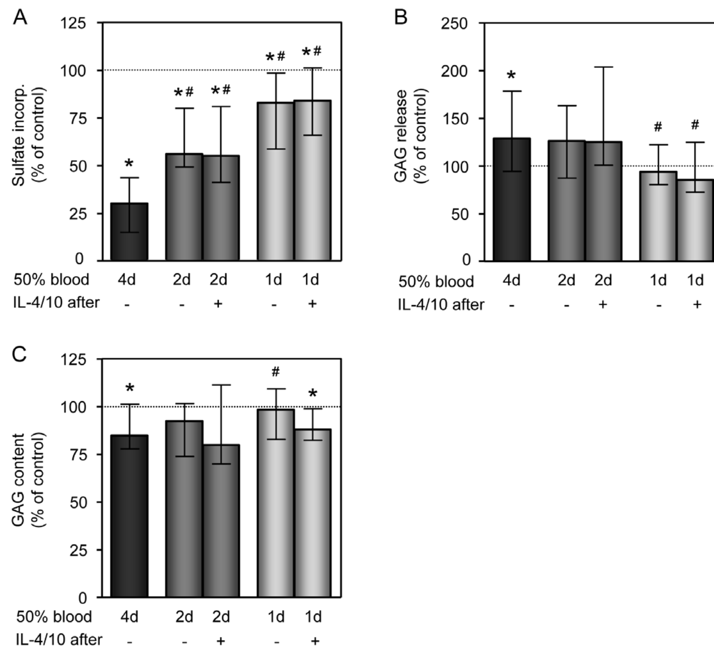

Figure 4: Effects of removal of blood combined with administration of IL-4 plus IL-10 on blood-induced cartilage damage

Human articular cartilage was exposed to 50% v/v whole blood for 4, 2 or 1 days. Subsequently, IL-4 and IL-10 were added in a concentration of 30 ng/mL. Proteoglycan synthesis rate **(A),** -release **(B),** and -content **(C)** are depicted. Asterisks (*) indicate a statistically significant difference with controls (100%), whereas hash tags (#) indicate a statistically significant difference with cartilage exposed to blood for 4 days without addition of IL-4 plus IL-10 (p<0.05; median values of at least 8 individual cartilage and blood donors ± IQR are shown). Exact p-values are given in the text.

Effects of blood aspiration on cartilage damage

In this set of experiments 4 days exposure of human cartilage to 50% blood led to an average decrease of 70% in proteoglycan synthesis rate (figure 4A; p=0.005). When blood was removed 1 or 2 days after start of exposure to cartilage, proteoglycan synthesis rate significantly increased compared to 4 days blood exposure, resulting in 17% and 44% inhibition, respectively (p=0.046 and p=0.028). Nevertheless, proteoglycan synthesis rate was still inhibited compared to controls (both p=0.028). Surprisingly, administration of IL-4 and IL-10 directly after removal at day 1 or 2 did not have an additional beneficial effect in preventing disturbance of cartilage matrix turnover compared to removal of blood alone (p=0.249 and p=0.075, respectively).

Release of proteoglycans from the cartilage was increased by 29% after 4 days of blood exposure (figure 4B; p=0.028). When blood was removed from the cartilage 1 day after start of culture, proteoglycan release was completely normalized (p=0.028 compared to 4 days exposure). Aspiration of blood from cartilage after 2 days could not prevent the enhanced release; 27% increase compared to control (p=0.075). Addition of IL-4 and IL-10 directly after aspiration at day 1 or 2 was of no additional benefit (p=0.116 and p=0.600, respectively).

A 4-day blood exposure led to a decrease of total proteoglycan content of 15% (figure 4C; p=0.047). Removal of blood from the cartilage after 1 day normalized proteoglycan content; which was statistically significant from 4 days blood exposure (p=0.028). When blood was aspirated from cartilage after 2 days, proteoglycan content was still inhibited with 7%, although not statistically significantly different anymore from control cartilage (p=0.116). Addition of IL-4 and IL-10 directly after removal of blood at day 1 or 2 did not improve proteoglycan content when compared to aspiration alone (p=0.249 and p=0.600, respectively).

DISCUSSION

This study demonstrates that IL-4 plus IL-10 can prevent blood-induced cartilage damage, but need to be administered directly or at least shortly after blood exposure to counteract the damaging effects of blood. Administration of IL-4 plus IL-10 to blood-exposed human cartilage within 4-8 hours results in normalization of proteoglycan turnover, expectedly modulated by inhibition of blood-induced IL-1β production. Canine cartilage can only be protected from blood-induced damage when IL-4 plus IL-10 is added within 4 hours after start of blood exposure. Most interestingly, removal of blood from cartilage, mimicking joint aspiration, leads to almost full recovery when performed within 1 day and a partial recovery when performed within 2 days. Subsequent addition of IL-4 plus IL-10 is of no additional value.

It takes about 24 hours in our human *in vitro* blood/cartilage culture system before protein levels of IL-1β can be detected. These levels are comparable to unstimulated whole blood culture of healthy controls during 3 days[32]. The onset of IL-1β production can be blocked within the first 4 to 8 hours by addition of IL-4 plus IL-10. Later administration

has limited effect and after 24 hours there is no prevention of IL-1β protein release anymore. IL-1β, probably produced by the blood monocytes, in combination with red blood cell derived iron results in chondrocyte apoptosis via hydroxyl radical formation[4,5]. When blood is aspirated from cartilage within 24 hours, IL-1β is removed from the culture together with red blood cell derived iron, thereby preventing cartilage damage. Even in case catalytic iron reaches the chondrocytes, protein levels of IL-1β within the first 24 hours of culture are still too low to enhance hydrogen peroxide production by chondrocytes. As such, the effect of IL-4 plus IL-10 on inhibition of IL-1β production is the main reason for the protective effect of this cytokine combination on blood-induced cartilage damage. However, it is plausible to expect additional small direct positive effects of IL-4 plus IL-10 on cartilage chondrocytes as has been suggested[33]. Upregulation of IL-4 and IL-10 receptor expression on chondrocytes in cartilage tissue upon blood exposure has been found previously (manuscript accepted for publication O&C)It was considered of relevance to study the effects of IL-4 plus IL-10 on canine blood-exposed cartilage as well, since this is a model frequently described[24-29]. Interestingly, this study shows that in order to protect against blood-induced damage, IL-4 plus IL-10 need to be administered shorter after blood exposure of canine cartilage than in case of human cartilage. An explanation for the time difference between the effects of IL-4 plus IL-10 on blood-exposed human and canine cartilage can only be speculated on. It could be that canine cartilage is more vulnerable to blood than human cartilage. However, previous research has shown that 4 days blood exposure of both human and canine cartilage causes a very similar long lasting inhibition of proteoglycan synthesis rate *in vitro* up to 10 weeks follow-up[25]. Nevertheless, in these studies a ceiling effect may have occurred as inhibition of cartilage matrix synthesis rate was almost maximal for both species. Another explanation could be that the thickness of cartilage is of influence to the degree of blood-induced cartilage damage. Cartilage thickness varies between joints and between species. Canine hip cartilage is about 1.2 mm thick[34], and cartilage of the center of the human humeral joint surface, as used in the this study, is about 2.2 mm thick[35]. Chondrocytes in thinner cartilage get probably more easily exposed to IL-1β and free iron. Irrespective of the exact mechanism, this observation has implications for potential *in vivo* evaluations of the protective effects of IL-4 and IL-10 in canine models of blood-induced damage. Such studies should be designed carefully with the above considerations taken into account.

Shortening the time of blood exposure by removal of blood from the cartilage, mimicking aspiration of a hemorrhagic joint, demonstrates to be very effective. It is the first time that aspiration is studied in a clinically relevant set-up, as only most blood is removed from the cartilage and not all. This mimics human joint aspiration where, even when a joint is washed out, blood remains will be left in the joint. The outcome of this study is confirmed by previous research indicating that very short blood exposure time does not result in irreversible damage[36]. Moreover, removal of intra-articular blood during surgery, as demonstrated in an experimental canine model of anterior cruciate ligament transaction, results in less synovitis, synovial iron deposits, and fewer changes of the

articular cartilage, like hypertrophy, chondrocyte cloning, fibrillation, and changes in tangential zone chondrocytes[2]. As such, it is proposed that in case of traumatic joint bleeds and in hemophilia aspiration of blood from the joint within 24 hours is advocated to prevent prolonged damage over time.

Upon removal of blood from the joint, addition of IL-4 plus IL-10 is of no additive value anymore. This suggests that in clinical practice administration of IL-4 plus IL-10 after aspiration is of no supplementary value. However, due to joint compartmentalization blood can remain after aspiration in clinical practice on which IL-4 plus IL-10 can have a protective effect. Moreover, it has to be kept in mind that in this study only the direct effect of blood on cartilage is studied. Besides cartilage damage, intra-articular blood also causes inflammation of synovial tissue *in vivo*[37,38]. It has been shown in arthritis *in vitro*[39] and *in vivo*[40-43] models that administration of IL-4 and IL-10 inhibit inflammation, indicating that IL-4 plus IL-10 in addition to aspiration can have beneficial effects *in vivo* on reduction of synovial inflammation.

The present study clearly demonstrates that, despite their cartilage protective properties, there is only a short time window after the onset of a joint bleed in which IL-4 plus IL-10 can limit blood-induced cartilage damage. Aspiration of a joint to shorten blood exposure time would be the most effective treatment option and needs to be advocated. The combination of aspiration with intra-articular injection of IL-4 plus IL-10 might add to counteract inflammatory activities induced by a joint bleed, and may add in prevention of direct harmful effect of blood on cartilage because of blood remains despite aspiration.

REFERENCES

1. Luck JV, Jr., Silva M, Rodriguez-Merchan EC, Ghalambor N, Zahiri CA, Finn RS. Hemophilic arthropathy. J Am Acad Orthop Surg 2004;12:234-45.
2. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
3. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
4. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
5. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
6. Cirino G, Vergnolle N. Proteinase-activated receptors (PARs): crossroads between innate immunity and coagulation. Curr Opin Pharmacol 2006;6:428-34.
7. Esmon CT. Interactions between the innate immune and blood coagulation systems. Trends Immunol 2004;25:536-42.
8. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. Clin Orthop Relat Res 1997:6-11.
9. Roosendaal G, Vianen ME, Wenting MJ, van Rinsum AC, van den Berg HM, Lafeber FP, et al. Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. J Bone Joint Surg Br 1998;80:540-5.
10. Roosendaal G, van Rinsum AC, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-53.
11. Hermans C, De Moerloose P, Fischer K, Holstein K, Klamroth R, Lambert T, et al. Management of acute haemarthrosis in haemophilia A without inhibitors: literature review, European survey and recommendations. Haemophilia 2011;17:383-92.
12. Wallman P, Carley S. Aspiration of acute traumatic knee haemarthrosis. Emerg Med J 2002;19:50.
13. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54:836-40.
14. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40:249-60.
15. Joosten LA, Lubberts E, Helsen MM, Saxne T, Coenen-de Roo CJ, Heinegard D, et al. Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. Arthritis Res 1999;1:81-91.
16. Kageyama Y, Koide Y, Uchijima M, Nagata T, Yoshida A, Taiki A, et al. Plasmid encoding interleukin-4 in the amelioration of murine collagen-induced arthritis. Arthritis Rheum 2004;50:968-75.
17. Lubberts E, Joosten LA, van Den Bersselaar L, Helsen MM, Bakker AC, van Meurs JB, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999;163:4546-56.
18. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-20.
19. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 1995;96:2304-10.
20. Jansen NW, Roosendaal G, Hooiveld MJ, Bijlsma JW, van Roon JA, Theobald M, et al. Interleukin-10 protects against blood-induced joint damage. Br J Haematol 2008;142:953-961.
21. Oswald IP, Gazzinelli RT, Sher A, James SL. IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J Immunol 1992;148:3578-82.

22. Crepaldi L, Silveri L, Calzetti F, Pinardi C, Cassatella MA. Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10. Int Immunol 2002;14:1145-53.
23. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum 2001;44:3-12.
24. Fabry G. Early changes in the ground substance of articular cartilage in experimental hemarthrosis in dogs, measured by the fixed-charge density method. Arch Orthop Trauma Surg 1989;108:76-91.
25. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. J Rheumatol 2003;30:339-44.
26. Hooiveld MJ, Roosendaal G, Jacobs KM, Vianen ME, van den Berg HM, Bijlsma JW, et al. Initiation of degenerative joint damage by experimental bleeding combined with loading of the joint: a possible mechanism of hemophilic arthropathy. Arthritis Rheum 2004;50:2024-31.
27. Hooiveld MJ, Roosendaal G, Vianen ME, van den Berg HM, Bijlsma JW, Lafeber FP. Immature articular cartilage is more susceptible to blood-induced damage than mature articular cartilage: an in vivo animal study. Arthritis Rheum 2003;48:396-403.
28. Jansen NW, Roosendaal G, Wenting MJ, Bijlsma JW, Theobald M, Hazewinkel HA, et al. Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. Osteoarthritis Cartilage 2009;17:433-40.
29. Roosendaal G, TeKoppele JM, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum 1999;42:1033-9.
30. Davis ID, Maher DW, Cebon JS, Green MD, Fox RM, McKendrick JJ, et al. A phase I and pharmacokinetic study of subcutaneously-administered recombinant human interleukin-4 (rhuIL-4) in patients with advanced cancer. Growth Factors 2000;17:287-300.
31. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia 2006;12 Suppl 3:117-21.
32. Le Meur Y, Lorgeot V, Aldigier JC, Wijdenes J, Leroux-Robert C, Praloran V. Whole blood production of monocytic cytokines (IL-1beta, IL-6, TNF-alpha, sIL-6R, IL-1Ra) in haemodialysed patients. Nephrol Dial Transplant 1999;14:2420-6.
33. van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996;39:829-35.
34. Simon WH, Friedenberg S, Richardson S. Joint congruence. A correlation of joint congruence and thickness of articular cartilage in dogs. J Bone Joint Surg Am 1973;55:1614-20.
35. Graichen H, Jakob J, von Eisenhart-Rothe R, Englmeier KH, Reiser M, Eckstein F. Validation of cartilage volume and thickness measurements in the human shoulder with quantitative magnetic resonance imaging. Osteoarthritis Cartilage 2003;11:475-82.
36. Jansen NW, Roosendaal G, Bijlsma JW, Degroot J, Lafeber FP. Exposure of human cartilage tissue to low concentrations of blood for a short period of time leads to prolonged cartilage damage: an in vitro study. Arthritis Rheum 2007;56:199-207.
37. Valentino LA, Hakobyan N, Enockson C. Blood-induced joint disease: the confluence of dysregulated oncogenes, inflammatory signals, and angiogenic cues. Semin Hematol 2008;45:S50-7.
38. Van Meegeren M. Update on pathogenesis of the bleeding joint: an interplay between inflammatory and degenerative pathways. Haemophilia 2010;16 Suppl 5:121-3.
39. Morita Y, Yamamura M, Kawashima M, Aita T, Harada S, Okamoto H, et al. Differential in vitro effects of IL-4, IL-10, and IL-13 on proinflammatory cytokine production and fibroblast proliferation in rheumatoid synovium. Rheumatol Int 2001;20:49-54.
40. Finnegan A, Kaplan CD, Cao Y, Eibel H, Glant TT, Zhang J. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. Arthritis Res Ther 2003;5:R18-24.
41. Horsfall AC, Butler DM, Marinova L, Warden PJ, Williams RO, Maini RN, et al. Suppression of collagen-induced arthritis by continuous administration of IL-4. J Immunol 1997;159:5687-96.
42. Roybal JL, Endo M, Radu A, Zoltick PW, Flake AW. Early gestational gene transfer of IL-10 by systemic administration of lentiviral vector can prevent arthritis in a murine model. Gene Ther 2011;18:719-26.

43. Woods JM, Katschke KJ, Volin MV, Ruth JH, Woodruff DC, Amin MA, et al. IL-4 adenoviral gene therapy reduces inflammation, proinflammatory cytokines, vascularization, and bony destruction in rat adjuvant-induced arthritis. J Immunol 2001;166:1214-22.

Chapter 7

A single intra-articular injection with IL-4 plus IL-10 ameliorates blood-induced cartilage degeneration, but not synovitis, in haemophilic mice

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ABSTRACT

Objective:

IL-10, and even more IL-4 or the combination of IL-4 plus IL-10, protects against blood-induced cartilage damage *in vitro*. Since the *in vitro* model only includes cartilage explants and lacks involvement of synovial tissue, it was investigated whether a single intra-articular injection of IL-4 plus IL-10 immediately after a joint bleed limits inflammation as well as cartilage damage in an *in vivo* haemophilic mouse model of blood-induced joint damage.

Methods:

Factor VIII knock-out mice with severe haemophilia A were punctured once with a needle below the patella to induce a joint haemorrhage. Subsequently IL-4 plus IL-10 (n=24) or vehicle (n=24) were injected intra-articularly. The left knee joint of each animal served as an unaffected control. After 35 days, the knee joints were macroscopically examined for the absence or presence of remains of the joint bleed and joint diameter was measured. Joints were histologically scored for inflammation and cartilage damage.

Results:

Intra-articular injection of IL-4 plus IL-10 tended to decrease the existence of bleeding remains and the joint diameter, although not statistically significant. IL-4 plus IL-10 was unable to reduce synovial inflammation, but ameliorated cartilage degeneration caused by the joint bleed.

Conclusion:

A single intra-articular injection with IL-4 and IL-10 directly after a joint bleed limits cartilage degeneration, but not synovial inflammation. More frequent intra-articular injection of IL-4 plus IL-10 will cause higher bioavailability, thereby further preventing cartilage damage and maybe also synovial inflammation.

INTRODUCTION

Blood-induced joint damage is still a major cause of morbidity amongst haemophilia patients, despite the increased use of prophylaxis[1,2]. Besides that, joint bleeding also frequently occurs after trauma like cruciate ligament rupture or during major joint surgery[3]. In the pathogenesis of blood-induced joint damage both synovial inflammation as well as inflammation-independent, direct cartilage damage is involved. Synovial inflammation is seen as hypertrophy and hyperplasia of synovial cells[4], and neovascularisation[5]. Natural evacuation of damaged and lysed red blood cells from the joint cavity leads to deposition of iron as haemosiderin in the synovial tissue[6]. It is known that iron increases expression of oncogenes like mdm2[7] and leads to proliferation of synovial cells[8], thereby contributing to synovial inflammation. Inflamed synovial tissue causes elevation of pro-inflammatory cytokines (including IL-1β) in the synovial fluid of haemophilic mice after induction of a joint bleed[9]. These pro-inflammatory cytokines are known for their direct harmful effect on cartilage integrity[10].

Besides the inflammatory component in the pathogenesis of blood-induced joint damage, it has been shown that a single blood-exposure of cartilage results in irreversible damage to the cartilage matrix, without involvement of synovial tissue[11]. The combination of red blood cells together with monocytes/macrophages is responsible for this damage[12]. Monocytes/macrophages increase their production of pro-inflammatory cytokines like IL-1β. These cytokines enhance H_2O_2 production by chondrocytes, which reacts with free iron to form hydroxyl radicals. In the vicinity of the chondrocyte, these hydroxyl radicals result in apoptosis, leading to an irreversible decrease of cartilage matrix synthesis[13,14].

IL-4 and IL-10 are modulatory cytokines and candidates to stop the progress of blood-induced joint damage. IL-4 is known to inhibit the synthesis of pro-inflammatory cytokines like IL-1β and TNFα by macrophages and monocytes[15] and to upregulate the expression of inhibitors of proinflammatory cytokines such as IL-1 receptor antagonist[16]. Furthermore, pre-incubation of chondrocytes with IL-4 diminishes IL-1 induced production of nitric oxide (NO) and enhanced chondrocyte proliferation[17]. In several in vivo models, IL-4 ameliorated cartilage and bone destruction in knee joints[18,19]. However, it also appeared that high concentrations of IL-4 may provoke synovial inflammation[20]. When IL-4 is combined with IL-10, a more favourable balance between the Th1 and Th2 response is achieved[21]. IL-10 also inhibits the release of pro-inflammatory mediators like IL-1β and TNFα from monocytes/macrophages[22,23]. Moreover, in experimental arthritis models IL-10 ameliorates arthritis and joint destruction[24,25]. Therefore the combination of IL-4 and IL-10 appears to be more optimal to control inflammation in arthritis conditions in vivo then either of them alone.

In an in vitro model of blood-induced cartilage damage IL-10 limited cartilage destruction[26]. Moreover, cartilage obtained from haemophilia patients that underwent a total knee replacement was beneficially influenced by IL-10. Also production of the pro-inflammatory cytokines IL-1β and TNFα by synovial tissue from the same patients was reduced due to administration of IL-10[26]. In another study it was shown that IL-4 and

the combination of IL-4 plus IL-10 protected even better against blood-induced cartilage damage than IL-10 alone[27], even in case when administered shortly after the onset of blood-exposure. Unfortunately these *in vitro* models only include cartilage explants and lack involvement of surrounding tissues like the synovial tissue. Since it is known that exposure of blood results in inflammation of synovial tissue and that synovitis adds to degradation of cartilage, it is important to test the combination of IL-4 plus IL-10 in an *in vivo* model of blood-induced damage. Therefore, this study investigated whether a single intra-articular injection of IL-4 plus IL-10 immediately after a joint bleed limits cartilage damage and inflammation in a haemophilic mouse model.

MATERIALS AND METHODS

Mouse model

A Factor VIII knock-out mouse model with severe haemophilia A was purchased from Jackson Lab. This mouse model was generated by gene targeting of E16 factor VIII-deficient B6;129S4-F8^{tm1Kaz}/J[28,29]. Mice were bred and kept at the animal laboratory of the Utrecht University, The Netherlands. They were housed in groups and fed a standard commercial diet with water *ad libitum*. The Utrecht Medical Ethical Committee for animals evaluated and approved the study.

Experimental design

Mice of approximately 3 months of age were anaesthetized using isoflurane. Hair was removed from both knee joints with a hair removal cream (Veet) to facilitate direct observation. The right knee joint of 48 mice was punctured once with a 30G needle below the patella to induce a haemorrhage[30]. Subsequently a 3 µL solution, containing 100 ng recombinant mouse IL-4 plus 100 ng recombinant mouse IL-10 (both R&D systems) or vehicle only (PBS containing 20 mM Tris and 100 mM NaCl, pH 7.5), was injected through a 33G needle in 24 mice for each treatment. The left knee joint of each animal served as an unaffected control. After these injections the mice received acetaminophen via their drinking water to relieve pain until the end of the experiment. Animals were killed by cervical dislocation 35 days after induction of the joint bleed. The knee joints were macroscopically examined and were scored blinded with respect to treatment group by two observers for the absence or presence of remains of the joint bleed. Additionally, joint diameter was defined as greatest diameter across the joint from lateral to medial using a calliper.

Histological inflammation

Knee joints were isolated and fixed in 4% formaldehyde containing 2% sucrose for at least 48 hours. Subsequently they were decalcified and embedded in paraffin in routine histological procedures. Coronal tissue sections of 5 µm were stained with haematoxylin-eosin (H&E) and scored blinded by two observers for tissue inflammation according to Valentino *et al.*[31]. The grading system is based on synovial hyperplasia (0-3), vascular hyperplasia (0-3),

the absence or presence of haemosiderin (0 or 1), erythrocytes in synovial tissue (0 or 1), synovial villus formation (0 or 1), and cartilage erosion with pannus formation (0 or 1). The total score is summarised with a maximal score of 10.

Histological cartilage damage

Deparaffined sections were stained with Safranin-O Fast-Green (Saf-O) staining. Cartilage damage was scored blinded by two observers in detail according to the Osteoarthritis Research Society International (OARSI) score focussing on cartilage degeneration[32]. This semi-quantitative scoring system is specifically recommended for assessment of osteoarthritis (cartilage degeneration) in the mouse by the OARSI histopathology initiative and exists of grade 0-6. Loss of Saf-O is scored as well as the amount of fibrillations, clefts, and erosions of a certain cartilage surface. The score is expressed as summed scores of these characteristics, split out for the femoral condyle and tibial plateau, as well as a mean total joint score.

Statistical analyses

A Pearson's chi-square test was used to compare the remaining presence of a macroscopic joint bleed in the punctured 'vehicle' joints versus the punctured 'IL-4 plus IL-10' joints. Joint diameter and histological scoring were analysed using a paired Student's t-test to compare data of the experimental and control knee joints within each group. An unpaired t-test was used to analyse differences between the control joints of both groups and between the experimental joints of the 'vehicle' and 'IL-4 plus IL-10' group. Differences were considered to be statistically significant when $p<0.05$. SPSS 15.0 software was used for analyses.

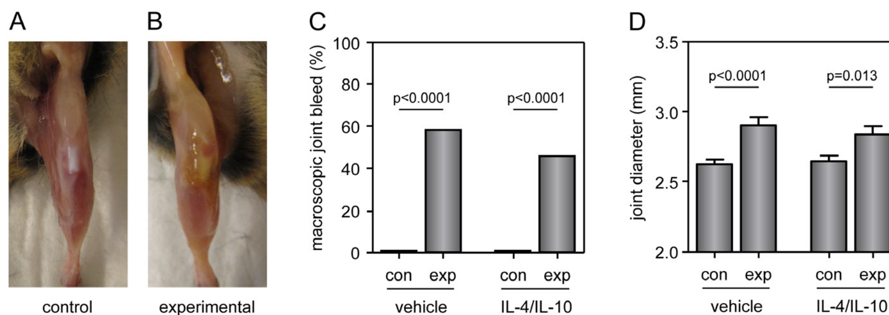

Figure 1: Macroscopic scoring of the joint

The left knee joint of each mouse served as an unaffected control (A). The right knee joint of each animal was punctured once to induce an experimental intra-articular haemorrhage (B). Subsequently IL-4 plus IL-10 or vehicle was injected; both groups containing 24 animals each. Animals were killed 35 days following induction of the joint bleed. Macroscopic examination of the knee joints was performed and the presence of remains of the joint bleed was scored. Mice having macroscopic remains of the joint bleed were expressed as a proportion of the total amount of mice (C). Joint diameter was defined as greatest diameter across the joint from lateral to medial and expressed as mean ± SEM (D).

RESULTS

Macroscopic scoring of the joint

In the control knee joints of both groups that were not punctured no remaining macroscopic joint bleed was seen as expected (figure 1A). The punctured joints often demonstrated remains of the joint bleed (figure 1B; yellowish colour). When quantified, in 58% of the joints that were punctured and received an intra-articular injection with vehicle, remains of the haemorrhage were macroscopically detectable after 35 days (figure 1C). Of the punctured joints injected with IL-4 plus IL-10, only 46% showed haemosiderin deposition as a remaining sign of a joint bleed. Although not statistically significant (p=0.386), the IL-4 plus IL-10 injected joints tended to present less remains of a joint bleed compared to the vehicle-injected joints.

In the 'vehicle' group the mean joint diameter of the control joints is 2.63 mm (figure 1D). Induction of a joint bleed and subsequent injection of the vehicle led to a (remained)

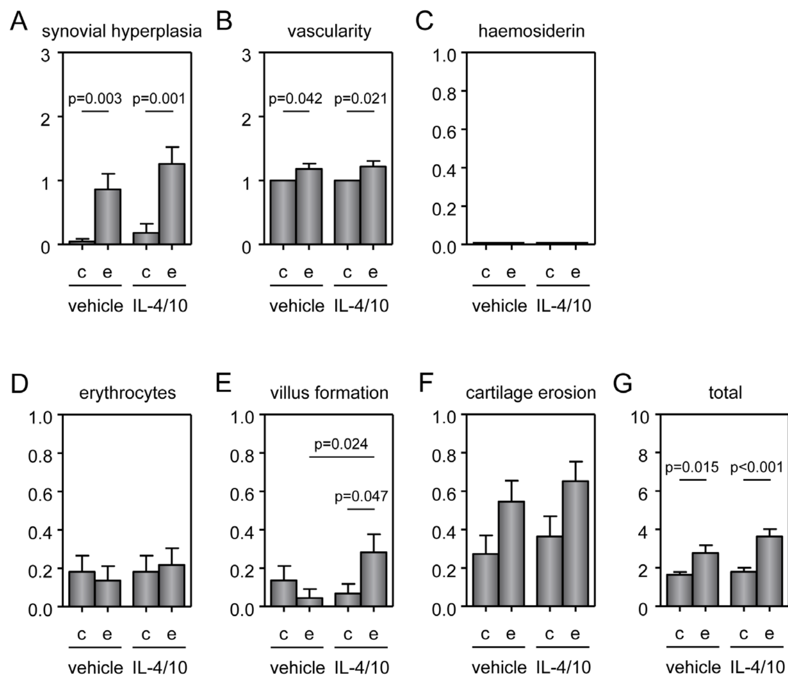

Figure 2: Histological joint damage

Joint damage on H&E slides was determined using the Valentino score based on synovial hyperplasia (A; 0-3), vascular hyperplasia (B; 0-3), the absence or presence of haemosiderin (C; 0 or 1), erythrocytes present in synovial tissue (D; 0 or 1), synovial villus formation (E; 0 or 1), and cartilage erosion (F; 0 or 1). The total score is summarised with a maximal score of 10 (G). The left knee joint of each mouse served as an unaffected control (c), while the right knee joint of each animal was punctured once to induce an experimental intra-articular haemorrhage (e). Following the joint bleed vehicle or IL-4 plus IL-10 was injected (n=24 animals per treatment group). Histological joint damage was determined 35 days following injury. Results are depicted as mean values ± SEM and differences between control and experimental joints were considered statistically significantly when p<0.05.

increased joint diameter (2.91 mm; p<0.0001). The diameter of the control joint of the 'IL-4 plus IL-10' group was 2.65 mm and not statistically significantly different from the control joint of the 'vehicle' group (p=0.605). Intra-articular injection of IL-4 plus IL-10 after induction of a joint bleed resulted in a slightly less increased joint diameter, but still different from the control joint (2.84 mm; p=0.013). The joint diameter of the punctured joints treated with IL-4 plus IL-10 tended to be smaller than that of the vehicle injected joints, although not statistically significantly different (p=0.174).

Histological inflammation

The H&E slides of the mouse knee joint were scored by use of the Valentino score (figure 2). Due to a joint bleed synovial hyperplasia was increased (p=0.003 compared to the control knee; figure 2A). This increase was also present when a combination of IL-4 and IL-10 was injected directly after the joint bleed (p=0.001 compared to control), thereby showing that a single injection with IL-4 and IL-10 did not prevent development of synovial hyperplasia because of the joint bleed as observed 35 days later. Similarly, vascularity of the synovial tissue increased due to induction of a knee joint bleed (figure 2B), both when the bleeding was followed by intra-articular vehicle injection (p=0.042) and when IL-4 plus IL-10 was injected (p=0.021). Although some of the joint bleeds were still visible macroscopically after 35 days, no clear haemosiderin depositions were found histologically in the synovial tissue (figure 2C). In contrast, whole erythrocytes were found in synovial tissue (expectedly as a result of vascular leakage during sectioning). This number was not increased after a joint bleed and did not differ between the vehicle group and the IL-4 plus IL-10 group (figure 2D). Surprisingly, formation of synovial villi was only increased after a bleeding followed by IL-4 plus IL-10 (figure 2E; p=0.047). This was statistically significantly different from joints that were intra-articularly injected with vehicle (p=0.024). Cartilage erosion according to the Valentino score increased in both groups due to the joint bleed, although not statistically significant (figure 2F). In total, slightly more signs of joint inflammation was present in joints that received a joint bleed when compared to the control joint (figure 2G; p=0.015 for the 'vehicle' group and p<0.001 for the 'IL-4/10' group). No difference between joint damage after injection with vehicle or IL-4 plus IL-10 (p=0.121) was found.

Histological cartilage damage

The above-mentioned score focuses on synovial inflammation and not primarily on the degree of cartilage damage. Therefore cartilage degradation was evaluated in more detail according to the OARSI score using Saf-O stained slides (figure 3A-D). It appeared that a joint bleed and subsequent injection of vehicle caused cartilage degeneration of the femur (figure 3E; mean OARSI score change=1.0, p=0.010 compared to the contralateral control knee). When IL-4 plus IL-10 were injected immediately after the joint bleed, femoral cartilage damage was not statistically significantly increased anymore compared to the contralateral control joints (mean OARSI score change=0.6, p=0.171). The control paws did not differ between the two groups (p=0.604). In contrast to the femoral

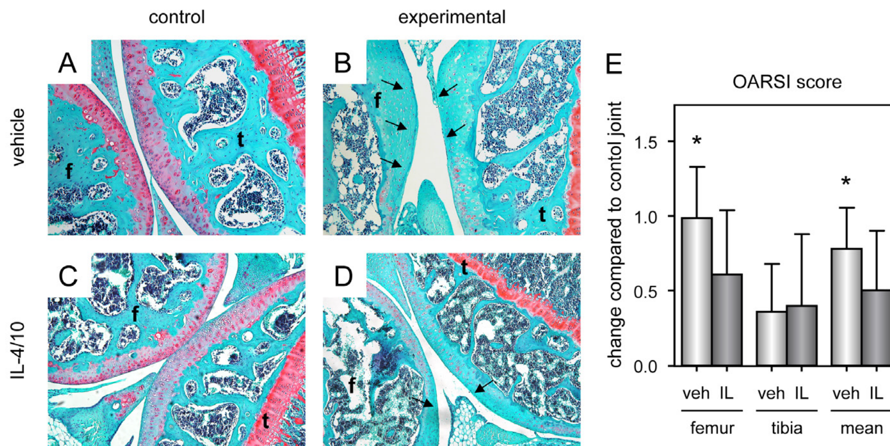

Figure 3: Histological cartilage degeneration
Histological photographs of representative sections of both control (A and C; both a mean score of 2) and experimental (B and D; mean scores are 4 and 3, respectively) joints of the vehicle (A and B) and IL-4 plus IL-10 injected animals (C and D) are depicted (original magnification 10x). Of each joint the femur condyle (f) as well as the tibial plateau (t) are shown. Arrows indicate loss of Saf-O staining and loss of chondrocytes in the experimental joints, which is more severe in the vehicle than in the IL-4 plus IL-10 injected joints. According to the OARSI histopathology initiative, loss of Saf-O in cartilage is scored as well as the amount of fibrillations, clefts, and erosions of the cartilage surface ranging from 0-6. The score is expressed as a change of the experimental joint compared to the contra-lateral control joint, and split out for the femoral condyle and tibial plateau (E). White bars represent vehicle injected mice (veh) and grey bars represent IL-4 plus IL-10 injected mice (IL; n=24 animals per treatment group). Results are depicted as mean values ± SEM and differences between control and experimental joints were considered statistically significantly when $p<0.05$.

condyle, cartilage degeneration of the tibia plateau was not increased compared to the control joint due to a joint bleed followed with vehicle or with IL-4 plus IL-10 ($p=0.269$ and $p=0.416$, respectively). The control paws did not differ between the two groups ($p=0.266$). The total OARSI joint score also demonstrated a statistically significant higher score for cartilage damage after a joint bleed followed by vehicle injection when compared to the contralateral control knee (mean OARSI score change=0.8, $p=0.012$). When the joint bleed was followed by IL-4 plus IL-10 injection, the difference with the contralateral control joints was not statistically significant anymore (mean OARSI score change=0.5, $p=0.216$)

DISCUSSION

A single intra-articular injection of IL-4 plus IL-10 immediately after induction of a joint bleed in haemophilic mice reduced cartilage destruction, as observed 35 days after the injection. Moreover, the remains of a joint bleed, based on macroscopic bleeding score and on joint diameter, tended to be less frequently visible for the IL-4 plus IL-10 injected joints than for the vehicle-injected joints. Surprisingly, injection of IL-4 plus IL-10 directly

after a joint bleed did not diminish bleeding-induced synovial inflammation.
Assuming an equal number and severity of joint bleeds in both treatment groups, a single intra-articular injection of IL-4 plus IL-10 following induction of the joint bleed showed a tendency to decrease macroscopic remains of the joint bleed as compared to injection with vehicle. This could be a result of synovial hyperplasia and haemosiderin deposition, leading to macroscopically measurable thickness of the knee and visible remains of the joint bleed. However, these microscopic parameters were not different between the experimental joints of the two treatment groups. Moreover, no haemosiderin depositions at all were seen histologically. This apparent difference in macroscopic and microscopic observation can therefore only be speculated on. It could be that haemosiderin depositions are detected more in the outer zones of the synovial capsule where they are macroscopically detectable, instead of in the inner lining layer of synovial tissue evaluated by microscopy. Furthermore, synovial effusion and even blood remains in the joint cavity that could lead to an increase in joint diameter may have been lost during histological procedures (washed out), and may as such explain part of the difference.
In contrast to findings in the haemophilia mouse model in other studies[30,31] as well as in haemophilia patients[6], no haemosiderin depositions were found in the synovial tissue. The reason for the absence of these depositions in our study could be caused by the subjective way in which small haemosiderin depositions can be scored as present or not, although the two observers who scored slides in this study were well trained and inter-observer variability was low. Since our mouse model is not significantly different from the one reported by Valentino, except for injection of vehicle or IL-4 plus IL-10, no other plausible explanations can be thought of. Besides lack of haemosiderin depositions, no clear accumulation of whole erythrocytes in the synovial tissue 35 days after a joint bleed was found. This is in line with observations showing that blood is already evacuated from the joint in mice during the first two weeks after a joint bleed[30], and even much quicker in canine joints[33].
Interestingly, and in contrast to the *in vitro* and *in vivo* studies where IL-4 plus IL-10 in combination diminishes inflammation and arthritis[18,25], in our study IL-4 plus IL-10 did not reduce inflammation induced by a single haemorrhage. On the contrary, villi formation was even increased in the IL-4 plus IL-10 treated joints when compared to the vehicle-injected joints. It might be that in this specific haemophilic haemorrhage mice model the concentration and frequency of treatment and the duration of follow-up explains the tendency of IL-4 to induce a pro-inflammatory response[20] still slightly dominating the controlling effect by IL-10. Additional *in vitro* and *in vivo* studies should be performed to further explore this effect on synovial tissue inflammation.
The total degree of inflammation after an induced joint bleed in this study is similar to a previously reported study[34]. The absence of an anti-inflammatory effect of IL-4 plus IL-10 could be due to the time point at which joints were analysed. Previously it has been described that vascular and synovial hyperplasia are especially detectable after 14 days, when blood in the articular space has been resorbed and joint space is filled

with inflammatory cells. At day 30 after joint haemorrhage cartilage and bone erosion have developed. At this time point synovial hyperplasia is still present, although in a more fibrous form[30]. It might well be that IL-4 and IL-10 partly limited the early stages of synovial inflammation, but did not fully prevent it. As a result fibrous synovial hyperplasia is seen in the same degree after treatment with IL-4 and IL-10 as after injection with vehicle. Reduction of synovial inflammation by IL-4 and IL-10 in several arthritis models was evaluated after 2-18 days[35-37]. For our study we evaluated cartilage damage in more detail and the effect that IL-4 plus IL-10 has on this cartilage damage. Therefore the joints were analysed after 35 days. Furthermore, in the arthritis models cartilage damage is a result of the synovial inflammation. In our model cartilage damage is only partly dependent on inflammation and is also, if not primarily, a direct result of blood-induced damage. This (partially) uncoupling of cartilage damage from inflammation has also been reported for the effect of IL-4[18]. In a collagen-induced arthritis (CIA) mouse model that displays several features of rheumatoid arthritis, it was demonstrated that IL-4 was clearly protective for cartilage and bone tissue, although it did not demonstrated clear anti-inflammatory properties.

This study shows that treatment with IL-4 and IL-10 ameliorates cartilage degeneration, but does not fully prevent joint damage like it has been demonstrated *in vitro*[27]. This could be due to the short half-life of cytokines *in vivo*. It has been shown that following intravenous administration, recombinant human IL-10 has a half-life of 1.9 hours[38], while subcutaneous administration results in a half-life of 2.7-4.5 hours[39]. The half-life of IL-4 is even lower: 19 minutes after intravenous injection[40]. Although it is assumed that the bioavailability of IL-4 and IL-10 is larger when directly injected in the joint, bioavailability is clearly not high enough to fully overcome the damaging effects of a joint bleed over a period of 35 days. Irrespectively, even a single injection appeared (partly) effective in reducing cartilage damage induced by a haemorrhage.

We demonstrated in concordance with human *in vitro* work, but for the first time *in vivo*, that a single intra-articular injection of IL-4 plus IL-10 following a joint haemorrhage limits blood-induced cartilage degeneration. No effect on synovial inflammation was observed. Increasing the bioavailability of IL-4 and IL-10 might improve this effect and, based on its anti-arthritic effects in other mice models, might even reduce synovial inflammation.

REFERENCES

1. Fischer K, van der Bom JG, Mauser-Bunschoten EP, Roosendaal G, Prejs R, Grobbee DE, et al. Changes in treatment strategies for severe haemophilia over the last 3 decades: effects on clotting factor consumption and arthropathy. Haemophilia 2001;7:446-52.
2. Philipp C. The aging patient with hemophilia: complications, comorbidities, and management issues. Hematology Am Soc Hematol Educ Program 2010;2010:191-6.
3. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
4. Hoots WK, Rodriguez N, Boggio L, Valentino LA. Pathogenesis of haemophilic synovitis: clinical aspects. Haemophilia 2007;13 Suppl 3:4-9.
5. Acharya SS, Kaplan RN, Macdonald D, Fabiyi OT, DiMichele D, Lyden D. Neoangiogenesis contributes to the development of hemophilic synovitis. Blood 2011;117:2484-93.
6. Roosendaal G, Vianen ME, Wenting MJ, van Rinsum AC, van den Berg HM, Lafeber FP, et al. Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. J Bone Joint Surg Br 1998;80:540-5.
7. Hakobyan N, Kazarian T, Jabbar AA, Jabbar KJ, Valentino LA. Pathobiology of hemophilic synovitis I: overexpression of mdm2 oncogene. Blood 2004;104:2060-4.
8. Wen FQ, Jabbar AA, Chen YX, Kazarian T, Patel DA, Valentino LA. c-myc proto-oncogene expression in hemophilic synovitis: in vitro studies of the effects of iron and ceramide. Blood 2002;100:912-6.
9. Ovlisen K, Kristensen AT, Jensen AL, Tranholm M. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from haemophilic mice with experimentally induced haemarthrosis. Haemophilia 2009;15:802-10.
10. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, et al. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum 2005;52:128-35.
11. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
12. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. J Rheumatol 1997;24:1350-4.
13. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
14. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
15. te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and IL-6 by human monocytes. Blood 1990;76:1392-7.
16. Vannier E, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc Natl Acad Sci U S A 1992;89:4076-80.
17. Schuerwegh AJ, Dombrecht EJ, Stevens WJ, Van Offel JF, Bridts CH, De Clerck LS. Influence of pro-inflammatory (IL-1 alpha, IL-6, TNF-alpha, IFN-gamma) and anti-inflammatory (IL-4) cytokines on chondrocyte function. Osteoarthritis Cartilage 2003;11:681-7.
18. Joosten LA, Lubberts E, Helsen MM, Saxne T, Coenen-de Roo CJ, Heinegard D, et al. Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. Arthritis Res 1999;1:81-91.
19. Yorimitsu M, Nishida K, Shimizu A, Doi H, Miyazawa S, Komiyama T, et al. Intra-articular injection of interleukin-4 decreases nitric oxide production by chondrocytes and ameliorates subsequent destruction of cartilage in instability-induced osteoarthritis in rat knee joints. Osteoarthritis Cartilage 2008;16:764-71.

20. Lubberts E, Joosten LA, van Den Bersselaar L, Helsen MM, Bakker AC, van Meurs JB, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999;163:4546-56.
21. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arth Rheum 2001;44:3-12.
22. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-20.
23. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815-22.
24. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40:249-60.
25. Persson S, Mikulowska A, Narula S, O'Garra A, Holmdahl R. Interleukin-10 suppresses the development of collagen type II-induced arthritis and ameliorates sustained arthritis in rats. Scand J Immunol 1996;44:607-14.
26. Jansen NW, Roosendaal G, Hooiveld MJ, Bijlsma JW, van Roon JA, Theobald M, et al. Interleukin-10 protects against blood-induced joint damage. Br J Haematol 2008;142:953-961.
27. van Meegeren ME, Roosendaal G, Jansen NW, Wenting MJ, van Wesel AC, van Roon JA, et al. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. Osteoarthritis Cartilage 2012;20:764-72.
28. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 1995;10:119-21.
29. Bi L, Sarkar R, Naas T, Lawler AM, Pain J, Shumaker SL, et al. Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. Blood 1996;88:3446-50.
30. Hakobyan N, Enockson C, Cole AA, Rick Sumner D, Valentino LA. Experimental haemophilic arthropathy in a mouse model of a massive haemarthrosis: gross, radiological and histological changes. Haemophilia 2008;14:804-09.
31. Valentino LA, Hakobyan N. Histological changes in murine haemophilic synovitis: a quantitative grading system to assess blood-induced synovitis. Haemophilia 2006;12:654-62.
32. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage 2010;18 Suppl 3:S17-23.
33. Jansen NW, Roosendaal G, Wenting MJ, Bijlsma JW, Theobald M, Hazewinkel HA, et al. Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. Osteoarthritis Cartilage 2009;17:433-40.
34. Valentino LA, Hakobyan N, Kazarian T, Sorensen BB, Tranholm M. Prevention of haemarthrosis in a murine model of acute joint bleeding. Haemophilia 2009;15:314-9.
35. Haas CS, Amin MA, Allen BB, Ruth JH, Haines GK, 3rd, Woods JM, et al. Inhibition of angiogenesis by interleukin-4 gene therapy in rat adjuvant-induced arthritis. Arthritis Rheum 2006;54:2402-14.
36. Horsfall AC, Butler DM, Marinova L, Warden PJ, Williams RO, Maini RN, et al. Suppression of collagen-induced arthritis by continuous administration of IL-4. J Immunol 1997;159:5687-96.
37. Lubberts E, Joosten LA, Helsen MM, van den Berg WB. Regulatory role of interleukin 10 in joint inflammation and cartilage destruction in murine streptococcal cell wall (SCW) arthritis. More therapeutic benefit with IL-4/IL-10 combination therapy than with IL-10 treatment alone. Cytokine 1998;10:361-9.
38. Radwanski E, Chakraborty A, Van Wart S, Huhn RD, Cutler DL, Affrime MB, et al. Pharmacokinetics and leukocyte responses of recombinant human interleukin-10. Pharm Res 1998;15:1895-901.
39. Huhn RD, Radwanski E, Gallo J, Affrime MB, Sabo R, Gonyo G, et al. Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. Clin Pharmacol Ther 1997;62:171-80.
40. Prendiville J, Thatcher N, Lind M, McIntosh R, Ghosh A, Stern P, et al. Recombinant human interleukin-4 (rhu IL-4) administered by the intravenous and subcutaneous routes in patients with advanced cancer--a phase I toxicity study and pharmacokinetic analysis. Eur J Cancer 1993;29A:1700-7.

Chapter 8

A fusion protein of IL-4 and IL-10 (IL4-10 synerkine) is equally effective in protecting cartilage from blood-induced damage compared to the individual components

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ABSTRACT

Objective:

It has been shown that recombinant human IL-4, IL-10, and the combination of both protect cartilage from blood-induced damage in terms of cartilage matrix turnover *in vitro*. To overcome low bioavailability of IL-4 and IL-10 *in vivo*, a fusion protein was developed (IL4-10 synerkine), having a larger molecular weight and thereby higher bioavailability. In this study it was investigated whether the IL4-10 synerkine protects against blood-induced cartilage damage similarly to the combination of the individual components.

Methods:

Human cartilage explants were exposed for 4 days to 50% v/v blood and simultaneously to a broad concentration range of the IL4-10 synerkine. Furthermore, the effects of 10 ng/mL of the IL4-10 synerkine were compared to the same concentrations of the individual cytokines and the combination. Proteoglycan turnover was assessed after a recovery period of 12 days to determine long-term effects. Levels of IL-1β and IL-6 were determined in a 4 days 50% v/v whole blood culture without cartilage in presence or absence of the IL4-10 synerkine and its individual components to asses mechanisms of action involved in the cartilage protective effect.

Results:

A clear dose-response curve with the IL4-10 synerkine was observed, leading to full normalisation of proteoglycan synthesis rate and -release at higher concentrations. The IL4-10 synerkine had a similar effect on proteoglycan turnover as the combination of the two individual cytokines. Furthermore, the IL4-10 synerkine was able to reduce IL-1β and IL-6 production in whole blood cultures, comparable to the combination of both cytokines.

Conclusion:

The IL4-10 synerkine strongly protects against blood-induced cartilage damage *in vitro* in a similar way as a combination of the individual cytokines does. The effect seems at least in part to be dependent on the reduction of IL-1β by blood cells, with that preventing induction of cartilage damage. Considering better bioavailability of this synerkine than of the individual cytokines, testing of this molecule in an *in vivo* model of blood-induced cartilage damage is warranted.

INTRODUCTION

Exposure of cartilage to blood leads to irreversible cartilage damage, which is demonstrated both *in vitro*[1] and in experimental animal models of joint haemorrhage[2]. Cartilage can get exposed to blood in case of joint trauma, during major joint surgery, or due to a spontaneous joint bleed in case of haemophilia[3,4]. Activated blood monocytes/macrophages, as present in blood in the joint after a bleed, increase their interleukin (IL)-1 production. Although the amount of IL-1 is still limited as for example compared to arthritic conditions[5], this cytokine, and probably also other macrophage derived cytokines, stimulates hydrogen peroxide production by chondrocytes. H_2O_2 in turn reacts with haemoglobin-derived iron from damaged and phagocytosed erythrocytes to form hydroxyl radicals[6]. In the vicinity of chondrocytes, these radicals induce apoptosis resulting in irreversible cartilage damage[7]. These direct effects on cartilage will trigger synovial activity by released cartilage matrix breakdown products, leading to inflammatory and proteolytic responses adding to the primary cartilage damage. Additionally, the evacuation of the red blood cells from the joint, containing massive amounts of iron, will result in hemosiderin deposition in the synovial membrane[8]. Consequently, this will lead to an additional inflammatory response, in time adding to cartilage damage as well.

Except for prevention of joint bleeds, there is no treatment for cartilage damage due to blood exposure. It has previously been shown that IL-10 protects cartilage from blood-induced damage in terms of cartilage matrix turnover *in vitro*[9]. Additionally, IL-10 beneficially influenced cartilage obtained from patients with haemophilic arthropathy and reduced production of the pro-inflammatory cytokines IL-1β and TNFα by synovial tissue from the same patients[9]. Likewise, IL-4 prevents against blood-induced cartilage damage, and IL-4 appears to add to IL-10 regarding this protective effect[10]. These *in vitro* effects of IL-4 and IL-10 were directly on cartilage, as no inflammatory component like synovial tissue was present in the *in vitro* cultures. In an *in vivo* haemophilia mouse model we demonstrated that a single intra-articular injection of IL-4 plus IL-10 after induction of a joint bleed reduces blood-induced cartilage degeneration, while this single injection was not sufficient to limit synovial inflammation (chapter 7). The exact mechanism by which IL-4 and IL-10 and the combination are protective needs further study. We demonstrated that blood exposure leads to upregulation of the IL-4 and IL-10 receptor components on chondrocytes as present in the cartilage tissue[10]. Moreover, both cytokines can prevent and inhibit the production of IL-1 by blood monocytes/macrophages, which is an essential step in the pathway leading to chondrocyte apoptosisIn several *in vitro* and *in vivo* arthritis models, IL-4 and IL-10 alone and in combination have shown to be potent inhibitors of inflammation[11-14]. Because IL-4 and IL-10 use different signalling pathways[15], they are able to exert different, but also potentially additive effects. It has been postulated that they can act in synergy enhancing their beneficial effects and controlling their individual adverse effects[16]. An adverse effect of IL-4 is that high concentrations provoke synovial inflammation, as appeared in a mouse arthritis model[17]. However, by

the presence of IL-10 this could be controlled[16]. Therefore a combination of IL-4 and IL-10 might be the best option to administer *in vivo*.

Until now, in spite of their impressive effects in human *in vitro* and animal *in vivo* models, recombinant anti-inflammatory cytokines like IL-4 and IL-10 have not fulfilled their promising capacities in clinical studies. An important reason for this may be a suboptimal bioavailability. IL-4 and IL-10 have a molecular weight of 18-20 kD, which is below the critical size for filtration in the kidney (50-60 kD depending on charge), and therefore they are rapidly cleared from the circulation. Following intravenous administration, human IL-10 has a half-life of 1.9 hours[18]. Subcutaneous administration resulted in lower but sustained concentrations; leading to a half-life of 2.7-4.5 hours[19]. Half-life and area under the curve of concentration over time for IL-10 are increased in patients with moderate to severe renal insufficiency[20], supporting the concept of quick renal clearance in renal healthy conditions. The half-life of IL-4 is even lower than that of IL-10: 19 minutes after intravenous injection[21].

To overcome low bioavailability of IL-4 and IL-10, a fusion protein of these two cytokines has been developed, having a larger molecular weight (glycosylated dimer of ~80 kDa) and thereby higher bioavailability as a consequence of reduced renal clearance. This fusion protein, called IL4-10 synerkine, has full biologic activity of each of the individual cytokines and combines the activities of each individual cytokine, resulting in additive and potentially even synergistic activities. *In vitro*, the IL4-10 synerkine inhibits multiple pro-inflammatory mediators including IL-1β, TNFα, IL-6, and IL-8, while increasing/ maintaining production of IL-1 receptor antagonist and soluble TNFα receptor (SAY Hartgring, personal communication; manuscript in preparation). In the present study it was investigated whether the IL4-10 synerkine protects against blood-induced cartilage damage similarly to the combination of IL-4 plus IL-10 as well as compared to the individual cytokines.

MATERIALS AND METHODS

Development of the IL4-10 synerkine

The IL4-10 synerkine is a single molecule in which the N-terminus of IL-4 is connected to the C-terminus of IL-10 by a linker sequence (figure 1A). In this way the separate cytokines can still signal through their own receptors without interference in receptor interactions by steric hindrance. The IL4-10 synerkine was produced by transient transfection of HEK293 cells according to standard procedures with a vector containing a transgene[22]. After transfection, supernatant was collected and the IL4-10 synerkine was purified from the supernatant by ion-exchange-chromatography.

The IL-4 and IL-10 content in supernatant or chromatography fractions was determined by ELISA (IL-4 PeliPair ELISA Kit; Sanquin, Amsterdam, the Netherlands; Cat# M9314 or IL-10 PeliPair ELISA Kit; Sanquin; Cat# M9310) according to manufacturer's instructions. Results of these ELISAs were compared with those of reference curves of dilutions of recombinant IL-4 and IL-10 provided by the manufacturer. Also, an assay for the specific

detection of the IL4-10 synerkine was developed by use of anti-IL-10 coated plates and biotinylated anti-IL-4 monoclonal antibody for the detection (figure 1B). This assay was performed exactly the same as the ELISA for IL-4 except that anti-IL10 coated plates were used instead of anti-IL-4 coated plates. The anti-IL-10 coated plates were prepared similarly as described for the IL-10 ELISA. As there was no standard for this assay, the results are given as OD (figure 1C). The same assay was performed for detection of the IL-10 moiety by use of IL-4 coated plates. Further biochemical characterization to verify the structure and molecular mass of the protein was done by SDS-Page, Western blotting, and size exclusion chromatography. Data have thus far demonstrated that IL4-10 synerkine forms a (hetero)dimer, which in glycosylated form has a molecular size of 80kDa. These characterisation data have been filed in a provisional patent (Patent Serial No 61/556,843; Nov 8 2011).

Cartilage culture

Healthy human articular cartilage tissue was obtained post mortem from humeral heads within 24 hours after death of the donor, approved by the medical ethical regulations of the University Medical Center Utrecht. The donors (n=8; mean age 69.8 ± 8.7 years, 3 males and 5 females) had no known history of joint disorders. Full thickness slices of cartilage were cut aseptically from the humeral head, excluding the underlying bone, and kept in phosphate-buffered saline (PBS, pH 7.4). Within 1 hour after dissection, slices were cut in small full thickness cubic explants and weighted aseptically (range

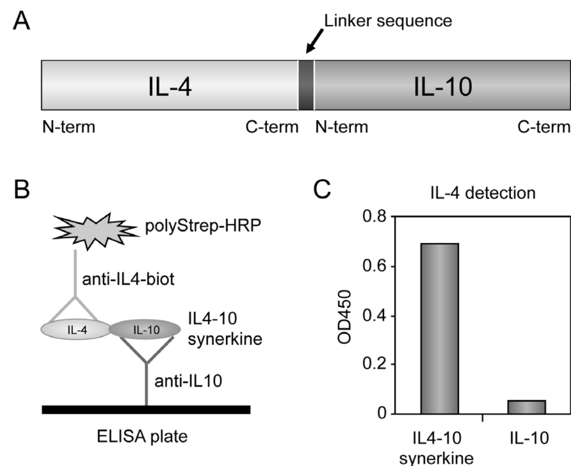

Figure 1: Development of the IL4-10 synerkine

Schematic representation of the IL4-10 synerkine (A); a fusion protein consisting of IL-4 at the N-terminal position and IL-10 at the C-terminal position connected via a linker sequence. Immunochemical identification of the IL4-10 synerkine was performed by a cross-ELISA (B). Note that IL-10 will not be detected in this assay. Supernatant of HEK293 cells transfected with a pUPE expression vector carrying the transgene for IL4-10 synerkine equivalent to 75 pg/ml IL-10 was tested in the ELISA with anti-IL10 monoclonal antibody as a capture antibody and biotinylated anti-IL4 as detecting antibody (C). IL-10 was tested as a negative control. The other way around (IL-4 coated plates with IL-10 detection) gave similar results for the IL4-10 synerkine and for IL-4 as a negative control (data not shown).

5-15 mg, accuracy ± 0.1 mg). The explants were cultured individually in a 96-wells round-bottomed microtiter plate (at 5% CO_2 in air, pH 7.4, 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 PAA), ascorbic acid (85 µM; Sigma), and 10% heat inactivated pooled human male AB+ serum (Gemini Bioproducts).
For each experiment, fresh blood was drawn from healthy human donors (n=8, mean age 28.0 ± 5.0 years, 2 males and 6 females) in a vacutainer tube (nr. 367895; Becton Dickinson). To mimic a human joint bleed, cartilage was exposed to 50% v/v whole blood for 4 days, which is considered to be the natural evacuation time of blood from the joint cavity[23-25]. After blood exposure, cartilage explants were washed twice under culture conditions for 45 minutes to remove all additives and were cultured for an additional 12 days. Medium was refreshed every 4 days. In the first experimental set-up using cartilage and blood of 5 of the 8 donors, a dose-response curve of the IL4-10 synerkine was made by adding the IL4-10 synerkine during blood exposure in a concentration of 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 ng/mL (n=5). In a separate experiment, the optimal concentration of 10 ng/mL IL4-10 synerkine was compared to a similar concentration of the combination of IL-10 plus IL-4, as well as the individual components (each 10 ng/mL, n=8).

Determination of proteoglycan turnover

As a measure of proteoglycan synthesis rate, proteoglycans being one of the major cartilage matrix components, the sulphate incorporation rate into glycosaminoglycans (GAGs) was determined. At the end of each experiment 74 kBq $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) was added per well. After 4 hours of pulse labelling of the newly formed sulphated GAGs, cartilage samples were washed twice in cold PBS and stored at -20°C. Thawed samples were digested for 2 hours at 65°C with 2% papain (Sigma). 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 was measured by liquid scintillation analysis. Radioactive counts were normalised to the specific activity of the medium, labelling time, and wet weight of cartilage. Results were expressed as nanomoles of sulphate incorporated per gram wet weight of cartilage tissue (nmol/h*g).
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 absorptiometry at 620 nm using chondroitin sulphate (Sigma) as a reference. Results were expressed as mg GAG per wet weight of cartilage tissue (mg/g) and mg GAG released during 4 days per wet weight of tissue (mg/g), for content and release, respectively.
Because of focal differences in composition and bioactivity of the cartilage, proteoglycan turnover parameters were determined of 10 cartilage explants of the same donor,

obtained randomly and handled individually. The average of these 10 samples was taken as a representative value for that cartilage donor.

Blood culture

Additionally, separate whole blood cultures were performed without cartilage. Whole blood (50% v/v) of 6 healthy donors was cultured for 4 days (n=6; mean age 26.7 ± 1.6 years, 2 males and 4 females). The IL4-10 synerkine (10 ng/mL) as well as IL-10, IL-4, or a combination of the two cytokines (both 10 ng/mL; Sigma) was added. After 4 days the samples were collected, centrifuged at 1500xg for 10 minutes, and the supernatants were stored at -80°C. The concentration of IL-1β and IL-6 was determined with commercially available cytosets (Biosource) and the analyses were performed according to the manufacturer's protocol provided.

Statistical analyses

Results are depicted as median values ± interquartile range. The data was analysed using a non-parametric test for related samples (Wilcoxon signed rank test) with SPSS 15.0 software. Differences were considered to be statistically significant when $p \leq 0.05$.

RESULTS

Dose dependent protection of blood-induced cartilage damage by the IL4-10 synerkine

Exposure of cartilage to 50% v/v blood for 4 days strongly decreased proteoglycan synthesis rate by 74% (figure 2A; p=0.043). Addition of the IL4-10 synerkine resulted in a dose dependent recovery of proteoglycan synthesis rate, providing a dose-response curve with a clear sigmoid appearance. From 0.1 ng/mL IL4-10 synerkine on a statistically significant increase in proteoglycan synthesis rate was observed when compared to blood exposure without additives (all p=0.043). From this concentration on the remaining inhibition was not statistically significant from controls anymore.

Also for the release of proteoglycans a clear sigmoid dose response curve was observed. The increase of 62% in proteoglycan release due to blood exposure could be reversed statistically significantly by addition of the IL4-10 synerkine from 0.1 ng/mL on (all p=0.043, except for 1 ng/mL, p=0.080). This resulted in normalisation of proteoglycan release that was not statistically significantly different form control cultures anymore.

Total proteoglycan content decreased by 11% when cartilage explants were exposed to blood (figure 2C; p=0.043). A clear dose-response curve was not obtained due to the limited loss of proteoglycans in such a relative short time period and variation in outcome. Irrespectively, the decrease in content by blood exposure was counteracted by addition of the IL4-10 synerkine at concentrations of 0.3 ng/mL and higher (all p=0.043, except for 1 and 100 ng/ml, both p=0.080).

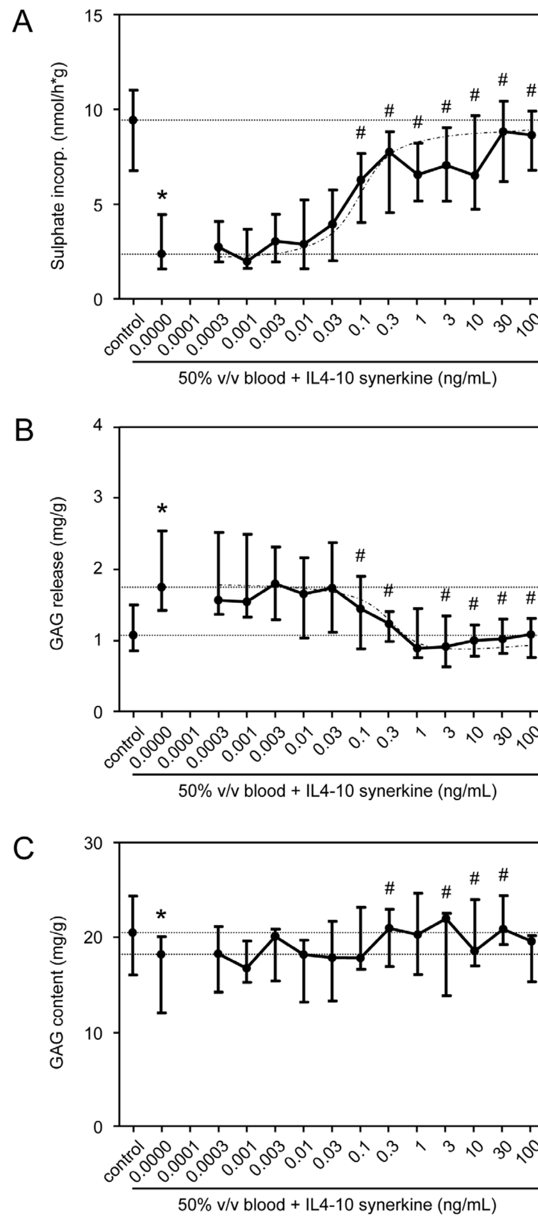

Figure 2: Dose-response effect of the IL4-10 synerkine on cartilage proteoglycan turnover
Cartilage explants of 5 donors were exposed for 4 days to 50% v/v blood of 5 donors (n=5). During blood exposure the IL4-10 synerkine was added in a concentration of 0.0001 to 100 ng/mL. Proteoglycan synthesis rate (A), -release (B), and -content (C) were determined after a recovery period of 12 days. Proteoglycan synthesis rate and -content were significantly decreased due to blood exposure when compared to control cartilage, while proteoglycan release was increased (indicated by asterisks; p<0.05). Hashtags indicate statistically significant differences compared to 50% v/v blood (p<0.05), while the dotted line in A and B emphasizes the sigmoid appearance of the dose-response. Median values ± interquartile range are depicted.

Protection of blood-induced cartilage damage by the IL4-10 synerkine equals that of the combination of IL-4 and IL-10

To compare the effect of the IL4-10 synerkine to the combination of the cytokines and the individual components, the IL4-10 synerkine, IL-4, IL-10, and the combination of both (each 10 ng/mL) were tested in the same assay. Proteoglycan synthesis rate was decreased by 76% due to blood exposure (figure 3A; p=0.012). Both IL-10 and IL-4 statistically significantly increased synthesis rate when compared to blood (p=0.017 and p=0.012, respectively). The protective effect of IL-4 was greater than the protective effect of IL-10 (p=0.036). The combination of IL-4 plus IL-10 also resulted in a statistically significant higher proteoglycan synthesis rate than IL-10 alone (p=0.025), but was not different from IL-4 alone. IL4-10 synerkine in the same concentration increased proteoglycan synthesis rate equally effective compared to the combination of the two individual cytokines (241% and 245%, respectively, compared to blood exposure). Also the effect of the IL4-10 synerkine was statistically significantly better than the effect of IL-10 alone

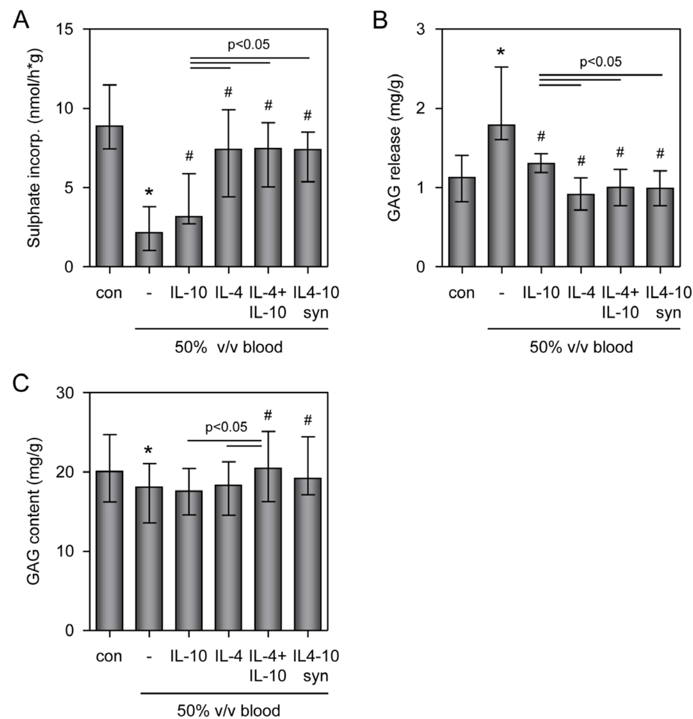

Figure 3: Effect of the IL4-10 synerkine compared to IL-4 and IL-10 on cartilage proteoglycan turnover
Cartilage explants of 8 donors were exposed for 4 days to 50% v/v blood of 8 donors (n=8). During blood exposure the IL4-10 synerkine (IL4-10 syn) as well as IL-4, IL-10, and the combination of IL-4 plus IL-10 were added (all 10 ng/mL). Proteoglycan synthesis rate (A), -release (B), and -content (C) were determined after a recovery period of 12 days. Proteoglycan synthesis rate and -content were significantly decreased due to blood exposure when compared to control (con) cartilage, while proteoglycan release was increased (indicated by asterisks; p<0.05). Hashtags indicate a statistically significant difference compared to 50% v/v blood (p<0.05). Median values ± interquartile range are depicted.

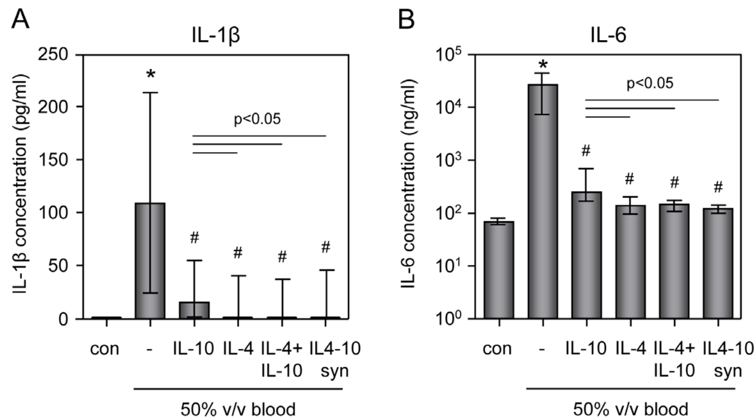

Figure 4: Effect of the IL-4/10 synerkine compared to IL-4 and IL-10 on whole blood cytokine production
50% whole blood of 6 donors (n=6) was cultured for 4 days. The IL4-10 synerkine (IL4-10 syn) as well as IL-4, IL-10, and a combination of IL-4 plus IL-10 were added (all 10 ng/mL). The production of pro-inflammatory cytokines IL-1β (A) and IL-6 (B) was measured. The concentration of both cytokines was significantly higher in 50% v/v blood when compared to control (con; indicated by asterisks; $p<0.05$). Addition of IL-4, IL-10, the combination, and IL4/10 synerkine all decreased the production of IL-1β as well as IL-6. Hashtags indicate a statistically significant difference compared to 50% v/v blood ($p<0.05$). Median values ± interquartile range are depicted.

($p=0.025$). Proteoglycan synthesis rate of the cultures with the IL4-10 synerkine, with the combination of both cytokines, and with IL-4 alone were not significantly different from controls anymore. Complete recovery from the blood-induced inhibition of proteoglycan synthesis, namely normalisation, was obtained.

Blood exposure of cartilage increased proteoglycan release with 59% (figure 3B; $p=0.017$). Addition of IL-10 reduced this enhanced release ($p=0.012$ compared to blood). IL-4, the combination of IL-4 plus IL-10, and the IL4-10 synerkine decreased the release even more when compared to blood exposure (all $p=0.012$). The IL4-10 synerkine was more potent when compared to IL-10 alone ($p=0.012$), but equally effective as the combination of the individual cytokines ($p=0.611$). Again these cultures retrieved a condition which was not different from controls anymore.

Cartilage exposed to blood showed a decrease in proteoglycan content by 10% (figure 3C; $p=0.012$). The individual cytokines IL-4 and IL-10 did not prevent this reduction in content ($p=0.093$ and $p=0.327$, respectively, when compared to blood exposure). However, the combination of IL-4 plus IL-10 statistically significantly increased proteoglycan content compared to blood exposure without additions ($p=0.012$). The IL4-10 synerkine demonstrated to have the same effect ($p=0.012$), also different from the individual components (both $p=0.05$) and equal to the combination of both ($p=0.484$). Proteoglycan content was not different from control conditions anymore.

The IL4-10 synerkine inhibits pro-inflammatory cytokine production by blood
Control culture media without any addition lacked detectable levels of IL-1β (figure 4A), whereas very small amounts of IL-6 (68 pg/mL; figure 4B) were present (medium containing 10% serum). In a 4-day 50% v/v whole blood culture 109 pg/mL IL-1β and 26814 pg/mL IL-6 was measured ($p=0.043$ and $p=0.028$ compared to controls, respectively). At a concentration of 10 ng/mL, as used for the cartilage assays, the IL4-10 synerkine, the combination of IL-4 plus IL-10, and these cytokines alone prevented IL-1β and IL-6 production by the blood culture over 4 days (all $p<0.05$). IL-10 alone performed slightly less effective as compared to IL4-10 synerkine, the combination of IL-4 plus IL-10, and IL-4 alone (all $p<0.05$). The IL4-10 synerkine prevented IL-1β and IL-6 production equally effective as the combination of IL-4 plus IL-10 ($p=0.180$ and $p=0.173$, respectively).

DISCUSSION

This study is the first to show that a fusion protein of IL-4 and IL-10, the so-called IL4-10 synerkine, is able to prevent blood-induced cartilage damage similarly to the combination of the individual components. The effect is clearly dose-dependent leading to full normalisation of both cartilage proteoglycan synthesis rate and -release. This result is at least in part due to reduction of IL-1β production by blood cells, and with that prevention of cartilage damage evoked by chondrocyte apoptosis. Moreover, at the concentration tested, the IL4-10 synerkine is equally effective as the combination of the individual cytokines, and more effective than IL-10 alone.
Previous studies demonstrate that the therapeutic effect of IL-4 and IL-10 is partly depending on the inhibition of pro-inflammatory cytokine production, like IL-1β, by blood cells[10]. Since administration of IL-4 and IL-10 prevents apoptosis of chondrocytes, it can be assumed that the reduced amount of IL-1β is not sufficient anymore to enhance hydrogen peroxide production by chondrocytes and with that hydroxyl radical formation, thereby preventing chondrocyte apoptosis6. Therefore, it can be concluded that the IL-4-10 synerkine exerts its cartilage protective effect at least in part via their effect on blood cells. Additionally, upregulation of the IL-4 and IL-10 receptors on chondrocytes upon exposure of cartilage to blood is reported10, indicating that IL-4 and IL-10 can bind to chondrocytes. This process can add to the effects of IL-4 and IL-10 on blood cells, both processes additively (directly and indirectly) preventing cartilage damage. However, additional studies to further clarify the exact mode of action of the IL4-10 synerkine need to be performed, since the larger synerkine may hinder penetration into the cartilage matrix and with that obstruct a direct effect on chondrocytes.
The concentrations used in the second part of this study were based on previously performed experiments showing optimal effects at a concentration of 10 ng/mL[9,10]. This study demonstrated an additive effect of the combination of IL-4 and IL-10 compared to administration of IL-10 alone, but not when compared to IL-4 alone[10]. These results suggest that using a combination of the two cytokines is not essential compared to IL-4 alone when used in these relatively high concentrations. However, there are several

reasons to assume that IL-4 plus IL-10 does have superior efficacy compared to each of the individual components. First, it has been shown that at suboptimal levels, IL-4 and IL-10 additively inhibit cytokine production (IL-1β and TNFα) and prevent tissue destruction by mononuclear cells from RA patients[26]. Expectedly, in our study there is a ceiling effect; IL-4 in this concentration already exerts maximal effects on proteoglycan turnover parameters and suppression of pro-inflammatory cytokine production. As such, addition of IL-10 to IL-4 can not have any supplementary effects to IL-4 alone. Therefore, it is not unreasonable to expect that lower concentrations of the two individual cytokines are capable to synergise their effects, as has been described previously[16]. With respect to prevention of blood induced cartilage damage, this has to be established.

Secondly, it has been demonstrated that an individual cytokine can act differently in diverse *in vitro* systems. For example, it was shown that synovial fluid monocytes are poor responders to IL-4 regarding inhibition of TNFα, while IL-4 is very well capable of suppression of TNFα production by peripheral blood monocytes[27]. This difference in response between monocytes/macrophages of different origin was not present after exposure to IL-10[27,28]. To obtain an optimal effect in more than one type of tissue, a combination of cytokines will be most favourable.It has been demonstrated that the individual cytokines IL-4 and IL-10 are able to limit production of pro-inflammatory cytokines by arthritic synovial tissue[26]. An adverse observation was that high concentrations of IL-4 (upon retroviral expression) can enhance inflammation while inhibiting tissue destruction[17]. Therefore, a third reason to use a combination of IL-4 plus IL-10, instead of IL-4 alone, is that pro-inflammatory properties induced by IL-4 can be controlled by IL-10.

Although the effect of IL4-10 synerkine is equal to that of the individual cytokines there is a clear advantage of the IL4-10 synerkine over the combination of the individual cytokines: namely the potentially higher bioavailability. Since IL4-10 synerkine consists of a dimer and is glycosylated, the molecule is larger (80kDa) than the individual cytokines and thereby reduces its secretion velocity by the kidney, which secretes proteins that are below ~50kDa rapidly. The half-life of the IL4-10 synerkine *in vivo* will consequently be increased, which is expected to enhance its value as a therapeutic agent. Moreover, regarding clinical application, testing a combination of two individual cytokines will be more expensive than testing a single new fusion protein. Validation of two product development processes and clinical development programmes of the separate cytokines plus the combination, instead of one are required. As such, development of IL4-10 synerkine is more advantageous and therefore *in vivo* animal experiments are necessary to study whether it indeed has higher bioavailability and with that efficacy in preventing blood-induced cartilage damage *in vivo*.

Another reason to test the IL4-10 synerkine *in vivo* is because blood-induced joint damage is evoked via two routes, one directly on cartilage and the other on inflammation and with that indirectly on cartilage. Joint bleeds lead to inflammation of synovial tissue, which is then provoked to produce several damaging mediators, including pro-inflammatory cytokines and proteases. These mediators are known to degrade cartilage. The cartilage

culture system used in the present study does not include synovial tissue, and therefore lacking the indirect inflammatory route. As such, the direct beneficial effect of the IL4-10 synerkine on cartilage damage as observed in the present study will expectedly be accompanied by an indirect beneficial effect via control of synovial inflammation. Thus, *in vivo* studies evaluating the IL4-10 synerkine on the modulation of both the direct and indirect harmful effects of blood on cartilage are necessary.

This study shows that the IL4-10 synerkine strongly protects against blood-induced cartilage damage *in vitro* in a similar way as a combination of the individual cytokines does. The effect seems at least in part to be dependent on the reduction of IL-1β by blood cells, with that preventing cartilage damage induced by chondrocyte apoptosis. Assuming better bioavailability of the synerkine than of the individual cytokines, testing of this molecule in an *in vivo* model of blood-induced cartilage damage is warranted.

REFERENCES

1. Roosendaal G, Vianen ME, Marx JJ, van den Berg HM, Lafeber FP, Bijlsma JW. Blood-induced joint damage: a human in vitro study. Arthritis Rheum 1999;42:1025-32.
2. Van Meegeren ME, Roosendaal G, Barten-van Rijbroek AD, Schutgens RE, Lafeber FP, Mastbergen SC. Coagulation aggravates blood-induced joint damage. Arthritis Rheum 2012 june 5. Epub ahead of print.
3. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33:1406-15.
4. Luck JV, Jr., Silva M, Rodriguez-Merchan EC, Ghalambor N, Zahiri CA, Finn RS. Hemophilic arthropathy. J Am Acad Orthop Surg 2004;12:234-45.
5. Roosendaal G, van Rinsum AC, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-53.
6. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
7. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
8. Roosendaal G, Vianen ME, Wenting MJ, van Rinsum AC, van den Berg HM, Lafeber FP, et al. Iron deposits and catabolic properties of synovial tissue from patients with haemophilia. J Bone Joint Surg Br 1998;80:540-5.
9. Jansen NW, Roosendaal G, Hooiveld MJ, Bijlsma JW, van Roon JA, Theobald M, et al. Interleukin-10 protects against blood-induced joint damage. Br J Haematol 2008;142:953-961.
10. van Meegeren ME, Roosendaal G, Jansen NW, Wenting MJ, van Wesel AC, van Roon JA, et al. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. Osteoarthritis Cartilage 2012;20:764-72.
11. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40:249-60.
12. Chomarat P, Vannier E, Dechanet J, Rissoan MC, Banchereau J, Dinarello CA, et al. Balance of IL-1 receptor antagonist/IL-1 beta in rheumatoid synovium and its regulation by IL-4 and IL-10. J Immunol 1995;154:1432-9.
13. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994;179:1517-27.
14. Persson S, Mikulowska A, Narula S, O'Garra A, Holmdahl R. Interleukin-10 suppresses the development of collagen type II-induced arthritis and ameliorates sustained arthritis in rats. Scand J Immunol 1996;44:607-14.
15. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem 1995;270:9558-63.
16. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum 2001;44:3-12.
17. Lubberts E, Joosten LA, van Den Bersselaar L, Helsen MM, Bakker AC, van Meurs JB, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999;163:4546-56.
18. Radwanski E, Chakraborty A, Van Wart S, Huhn RD, Cutler DL, Affrime MB, et al. Pharmacokinetics and leukocyte responses of recombinant human interleukin-10. Pharm Res 1998;15:1895-901.
19. Huhn RD, Radwanski E, Gallo J, Affrime MB, Sabo R, Gonyo G, et al. Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. Clin Pharmacol Ther 1997;62:171-80.

20. Andersen SR, Lambrecht LJ, Swan SK, Cutler DL, Radwanski E, Affrime MB, et al. Disposition of recombinant human interleukin-10 in subjects with various degrees of renal function. J Clin Pharmacol 1999;39:1015-20.
21. Prendiville J, Thatcher N, Lind M, McIntosh R, Ghosh A, Stern P, et al. Recombinant human interleukin-4 (rhu IL-4) administered by the intravenous and subcutaneous routes in patients with advanced cancer--a phase I toxicity study and pharmacokinetic analysis. Eur J Cancer 1993;29A:1700-7.
22. Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res 2002;30:E9.
23. Niibayashi H, Shimizu K, Suzuki K, Yamamoto S, Yasuda T, Yamamuro T. Proteoglycan degradation in hemarthrosis. Intraarticular, autologous blood injection in rat knees. Acta Orthop Scand 1995;66:73-9.
24. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia 2006;12 Suppl 3:117-21.
25. Safran MR, Johnston-Jones K, Kabo JM, Meals RA. The effect of experimental hemarthrosis on joint stiffness and synovial histology in a rabbit model. Clin Orthop Relat Res 1994:280-8.
26. van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996;39:829-35.
27. Hart PH, Ahern MJ, Jones CA, Jones KL, Smith MD, Finlay-Jones JJ. Synovial fluid macrophages and blood monocytes differ in their response to IL-4. J Immunol 1993;151:3370-80.
28. Hart PH, Ahern MJ, Smith MD, Finlay-Jones JJ. Comparison of the suppressive effects of interleukin-10 and interleukin-4 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. Immunology 1995;84:536-42.

Chapter 9

Joint distraction results in clinical and structural improvement of haemophilic ankle arthropathy; a series of three cases

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ABSTRACT

Objective:

The incidence of haemophilic arthropathy in multiple joints decreased due to treatment with clotting factor. Nowadays patients are enabled to live a rather normal life, resulting in more (sports) trauma-induced arthropathy in isolated joints like the ankle. As surgical treatment options fusion of the tibiotalar joint and total ankle replacement are available. Both standard treatments have complications, and therefore an alternative treatment is desired. In this study treatment of haemophilic ankle arthropathy with joint distraction was explored.

Methods:

Three patients with haemophilic ankle arthropathy were treated with joint distraction using an Ilizarov external fixator. Clinical outcomes like function, participation, and pain were evaluated in retrospect with 3 different questionnaires: Haemophilia Activities List (HAL), Impact on Participation and Autonomy (IPA), and the Van Valburg questionnaire. Structural changes were assessed blinded on X-ray by the Pettersson score and AIDA, and by an MRI score.

Results:

All 3 patients were very satisfied with the clinical outcome of the procedure. They reported a clear improvement for self-perceived functional health, participation in society and autonomy, and pain. Partial ankle joint mobility was preserved in the 3 patients. The Pettersson score remained the same in 1 patient and slightly improved in the 2 other patients, while joint space width measured by AIDA and the MRI score demonstrated improvement for all 3 patients after ankle distraction.

Conclusion:

This study suggests that joint distraction is a promising treatment for individual cases of haemophilic ankle arthropathy, without additional risk of bleedings during treatment.

INTRODUCTION

Replacement therapy using clotting factor has decreased the frequency of bleeding episodes in persons with haemophilia (PWH), resulting in clear reduction of severe arthropathy[1]. Due to this treatment, PWH are enabled to live a rather normal life, including participation in sports. As a consequence, PWH nowadays suffer more often from (sports) trauma-induced bleeds[2,3]. This leads to secondary blood-induced arthropathy in isolated joints, instead of arthropathy in multiple joints as a result of recurrent non-traumatic intra-articular bleeds. Amongst PWH playing soccer, a popular sport in Western Europe, traumatic ankle bleeds are frequently observed. These deteriorate the joint and lead to ankle arthropathy, causing chronic pain, loss of range of motion, and result in less activity and therefore participation in society[4].

In case of advanced stages of ankle arthropathy of the tibiotalar joint, 2 surgical treatment options are available: fusion (arthrodesis) and total ankle replacement (TAR)[5]. The standard surgical treatment is arthrodesis of the ankle joint, which is effective in pain relief, but at the expense of joint motion. Loss of joint motion has an increased risk for overloading of adjacent joints like the subtalar joint, leading to osteoarthritic processes in those joints[6,7]. The past few years TAR has been performed in different haemophilia treatment centres in Europe with good results[8-10]. However, there are no clinical follow-up data on the long term yet. It has been reported that in case of osteoarthritis, 23% of the patients receiving TAR had a major revision surgery within 5 years[11]. Because of the complications of arthrodesis and TAR, and the occurrence of haemophilic arthropathy at a relatively young age, an alternative treatment is desired to postpone arthrodesis or TAR to an older age.

Joint distraction using an Ilizarov external fixator could be such an alternative treatment. It has been successfully performed (pain relief and reduction of physical and functional impairment) in hundreds of patients with severe ankle osteoarthritis that were considered for arthrodesis[12-15]. By fixing 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. During the traction period, loading and unloading of the ankle joint leads to intra-articular intermittent fluid pressure changes[14] facilitating nutrition of chondrocytes. Bone turnover decreases and results in lower subchondral bone density[12], which normalizes after distraction[16]. Thereby it provides a rich source of growth factors involved in cartilage growth and repair[17]. Moreover, a canine model showed an altered chondrocyte metabolism indicating normalization of chondrocyte function due to joint distraction[18]. At present several orthopaedic surgeons worldwide apply this technique. However, fusion of the ankle joint still prevails, ignoring the consequences of creating a stiff joint, because the technique is easier to perform than ankle distraction.

Ankle distraction has never been applied so far for haemophilic arthropathy. At first it was not considered possible, since pins are drilled into soft tissue and bone, resulting in a higher risk of bleeding. Because of the clear benefits demonstrated by treatment of ankle osteoarthritis, its application for haemophilic ankle arthropathy has been

Table 1: Patient characteristics

case	type	factor level (VII/IX; %)	clotting factor treatment	affected ankle joint	cause of complaints	age at distraction (years)	duration of distraction (weeks)	follow-up (months)
1	A	< 1	prophylactic	left	trauma	18	15	48
2	B	2	on demand	left	trauma	33	10	32
3	A	10	on demand	left	multiple bleeds	29	10	26

explored. This report describes 3 PWH with severe ankle degeneration due to (trauma-induced) intra-articular bleeding episodes. These patients received treatment with ankle distraction and were clinically evaluated in retrospect.

PATIENTS AND METHODS

Patients

Characteristics of 3 PWH that were treated with ankle distraction are summarized in table 1.

Case 1: An 18 year old male with severe haemophilia A had complaints in his left ankle. These complaints were caused by a mountain biking accident 1.5 years prior to surgery and were very progressive. Before this trauma, no significant ankle complaints existed.

Case 2: A 33 year old patient with moderate haemophilia B suffered from a trauma to his left ankle 4 years before surgery. At that time there was no clinical sign of bleeding and the patient reported never to have had a clinical joint bleed. Since the trauma, the patient complained about mild pain in his left ankle that became progressive over time.

Case 3: A 29 year old male with mild haemophilia A suffered from arthropathy in his left ankle after several traumas in the past. The complaints were treated following a conservative policy, using manual traction, exercises, shoe adjustment, and a brace. This treatment offered little relief in pain and restriction of activities adequate to his age remained. In time the ankle became stiffer and pain increased.

Operative technique

Before surgery, a bolus injection of factor VIII or factor IX concentrate was given to raise the clotting factor level to approximately 80% of the average level of a healthy population. After achieving the appropriate level of clotting factor activity, the external fixation frame was applied as described before[14] and depicted by figure 1.

Following the procedure, the FVIII or FIX activity level was maintained at through levels of at least 60%

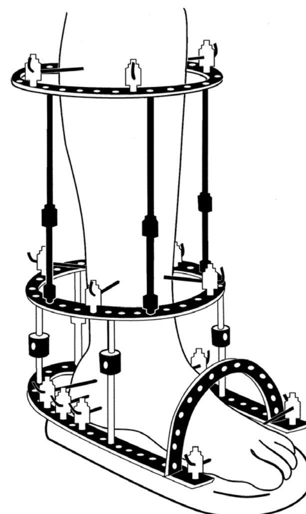

Figure 1: Schematic picture of an external frame for ankle distraction

during the first week and at through levels of 30% during the second week. After these 2 weeks, normal treatment protocol was resumed. After 10 to 15 weeks, the factor VIII or factor IX level was raised to approximately 80% of normal again and the frame was surgically removed.

Clinical outcome: questionnaires

Three different questionnaires were used to evaluate the clinical efficacy of the treatment: the Haemophilia Activities List (HAL)[19], Impact on Participation and Autonomy (IPA)[20,21], and the Van Valburg score[12]. Pre-operative status was obtained in retrospect only. The time between surgery and post-operative status was not equal for the 3 cases; case 1 completed the questionnaire 3.4 years after surgery, case 2 after 2.3 years, and case 3 after 1.7 years.

The HAL assesses self-perceived functional health of the patients consisting of 42 items in 7 domains. Response options are on a 5-point Likert scale, complemented with the option ‘impossible’; chosen by patients who felt unable to perform the activity. Three component scores were calculated: activities involving the upper extremity, basic activities involving lower extremity, and complex activities involving the lower extremity. Additionally, an overall summarised score was determined. Scores were normalized to a 100% scale from 0 to 100, where 0 represents the worst possible functional status and 100 the best.

The IPA is a generic questionnaire evaluating the perceived personal impact of chronic disability on participation and autonomy. The questionnaire contains 32 items in 5 domains: autonomy indoors, family role, autonomy outdoors, social relations, and work and education. The perceived participation is graded on a 5-point Likert scale, ranging from 0 (no limitation) to 4 (very poor participation/autonomy).

The third questionnaire was developed to measure function and pain during ankle distraction in osteoarthritis patients by Van Valburg et al.[12,15]. For this study we used the pain score by means of a box-scale, with 10 being the worst, and expressed as a percentage of the maximum score.

Ankle joint mobility

Post-surgery ankle joint mobility was measured as the range of motion in degrees between maximum dorsal and plantar flexion.

Structural changes by imaging: X-ray and MRI

As part of standard care, weight-bearing ankle radiographs were taken pre-operative in all cases (6 months, 11 months, and 14 months before surgery, respectively) and postoperative (3.4 years, 2.3 years, and 1.7 years, respectively). Pettersson score, with a maximum score of 13, was assessed by a blinded observer[22]. Radiographs were digitized and analysed by the ankle images digital analysis (AIDA) method as described previously[23] by a blinded observer. Joint space width (JSW) was defined as mean inter-bone distance of the tibiotalar joint and was normalized to the width of the ankle joint. Bone density

could not be evaluated due to the absence of a reference wedge.

MRIs were made before treatment (4 months, 14 months, and 14 months, respectively) and after treatment (4.0 years, 2.8 years, and 2.2 years, respectively). All ankle joints were imaged using magnetic resonance (MR) equipment (Philips) with field strength of 1.5T. T1-weighted (SE; time to repeat (TR) = 500 ms, time to echo (TE) = 20 ms) coronal and sagittal images were taken with a section thickness of 3 mm, because they provide the best anatomical overview. The MRI score followed the additive consensus score of the International Prophylaxis Study Group (IPSG) with a maximum score of 20[24].

Statistical analysis

Questionnaire data are depicted for each patient individually and as a mean value ± SD. Although this is purely a descriptive evaluation of 3 cases, some exploratory statistics on the mean values were performed. Paired t-test was used to compare pre- and post-treatment data. P-values less than 0.05 were considered statistically significant.

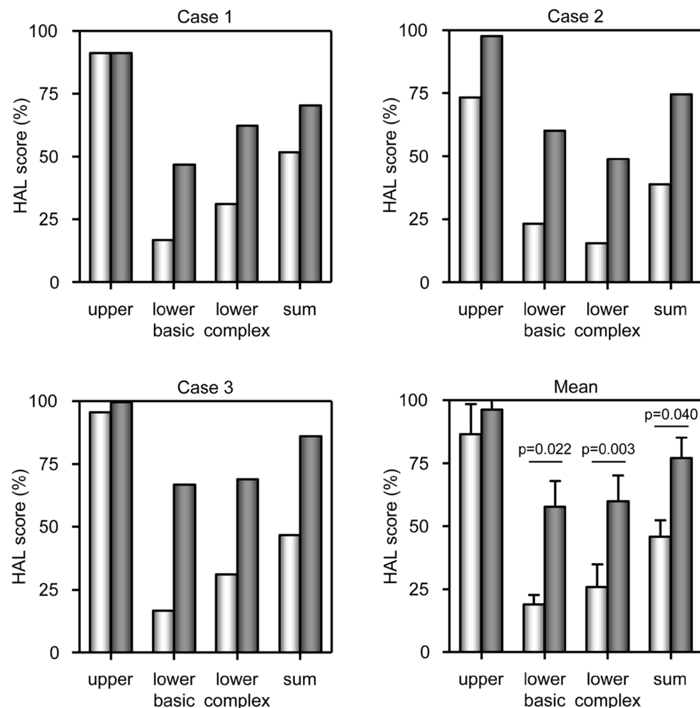

Figure 2: Functional health increased after ankle distraction

For the individual 3 cases the HAL component scores (activities involving the upper extremity, basic activities involving the lower extremity, and complex activities involving the lower extremity) and the overall summarised score were calculated. All scores were calculated before (retrospectively; white bars) and after (grey bars) ankle distraction. Scores of the 3 cases were averaged and depicted as mean ± SD. The HAL score ranges from 0 to 100, where 0 represents the worst and 100 the best possible functional status. Differences between the HAL score before and after ankle distraction were considered statistically significant when $p<0.05$.

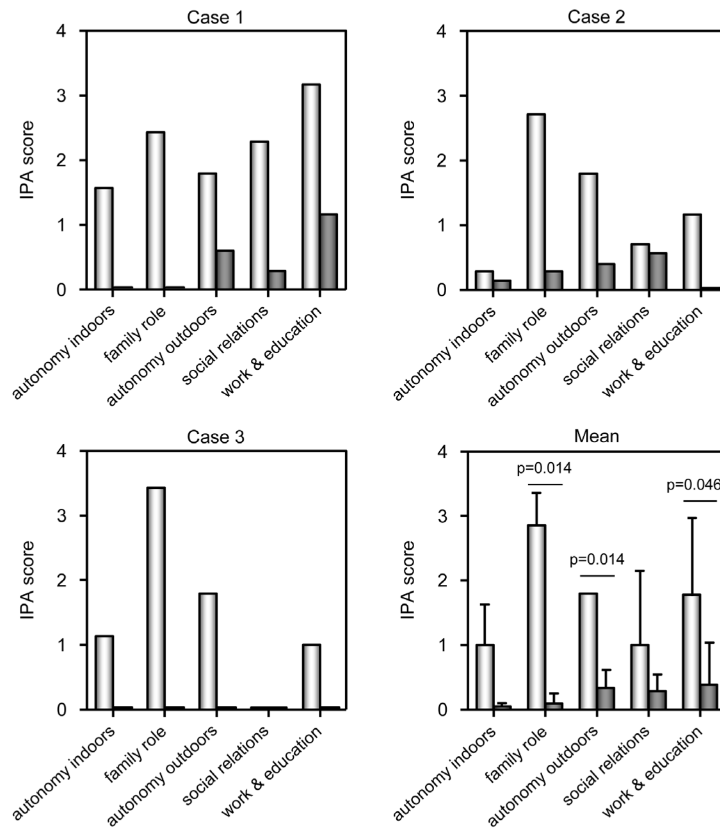

Figure 3: Less limitations in participation after ankle distraction
For each case the perceived participation score was calculated for 5 different domains: autonomy indoors, family role, autonomy outdoors, social life and relationships, and work and education. These scores were calculated before (retrospectively; white bars) and after (grey bars) ankle distraction (note that in some cases the values were 0; there were no missing data). Scores of the 3 individual cases were averaged and depicted as mean ± SD. The IPA score ranges from 0-4, where higher scores mean more limitations in participation and autonomy. Differences between the IPA score before and after ankle distraction were considered statistically significant when p<0.05.

RESULTS

Functional health

All 3 patients had a higher HAL score after ankle distraction for basic as well as complex activities involving the lower extremities (figure 2; case 1-3). The mean score for these 2 HAL component scores statistically significant increased after ankle distraction (figure 2; mean; p=0.022 and p=0.003, respectively). In all 3 cases patients hardly experienced functional restrictions of the upper extremities before treatment, and ankle distraction minimally improved this component score (p=0.329). The increase in overall summarised score was higher for all 3 cases (p=0.040); indicating an improvement of functional status after ankle distraction.

Participation and autonomy

All individual cases experienced improvement in perceived participation after ankle distraction (figure 3; case 1-3). For several domains after treatment zero was scored by some cases indicating that patients experienced no limitation (anymore) of their chronic disability on participation and autonomy. Moreover, the mean scores for 'family role', 'autonomy outdoors', and 'work and education' were statistically significant enhanced when compared to the situation before treatment (figure 3; mean; p=0.014, p=0.014, and p=0.046, respectively), whereas a non statistically significant improvement existed for 'autonomy indoors' (p=0.153) and 'social life and relationships' (p=0.383). However, the patients already experienced less limitation before ankle distraction concerning the latter 2 subscores.

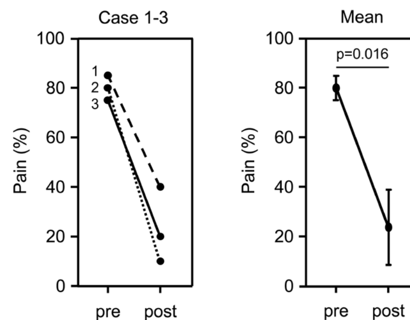

Figure 4: Pain decreased after treatment with ankle distraction
The individual 3 cases scored pain before (pre; retrospectively) and after ankle distraction (post) on a scale of 0-100%, where 100% is the worst. Scores of the 3 cases were averaged and depicted as mean ± SD. Differences between the Van Valburg pain score before (pre) and after (post) ankle distraction were considered statistically significant when p<0.05.

Pain

The patients experienced less ankle pain after the surgery when compared to the situation before distraction (figure 4). Case 1 reported a decrease in pain with 45%, case 2 with 70%, and case 3 with 55%. The mean pain score before distraction was 80% and statistically significant different from the pain score of 23% after distraction (p=0.016).

Ankle joint mobility

Ankle joint mobility is listed in table 2. There was less ankle mobility in the treated ankles of all 3 cases when compared to the contralateral ankle. The range of motion of the untreated ankle of case 1 was 70° (50° plantar flexion (pf) - 20° dorsal flexion (df))

Table 2: Clinical results of treatment with ankle distraction

	ankle joint mobility (°)				Pettersson score		AIDA score		MRI score	
	contralateral ankle*		treated ankle				joint space width (mm)			
case	plantar flexion	dorsal flexion	plantar flexion	dorsal flexion	before	after	before	after	before	after
1	50	20	32	10	3	3	3.5	4.4	10	5
2	50	16	32	8	7	6	0.3	2.0	16	13
3	56	6	33	6	4	3	3.2	3.9	10	6

* Normative values for males of this age are considered 13° for ankle dorsiflexion and 55° for plantar flexion[28].

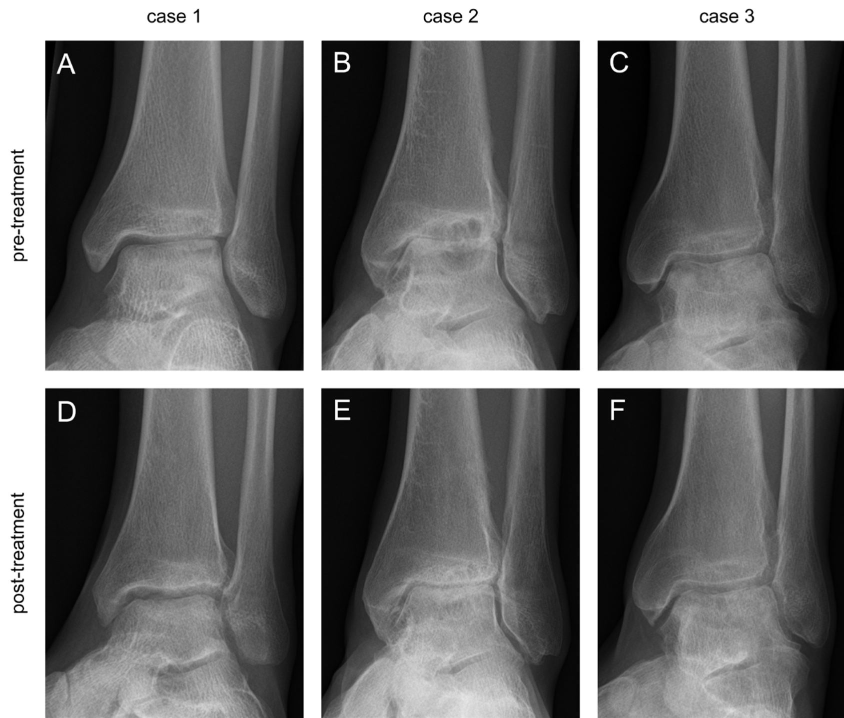

Figure 5: Structural improvement after ankle distraction on X-ray
Pre-treatment (A-C) and post-treatment (D-F) radiographs of all 3 cases are depicted. Case 1 had a slightly narrowed JSW before treatment (A), which increased due to ankle distraction (D). Radiological examination of case 2 shows osteochondral lesions, osteophyte formation and a severely narrowed joint space (B). Post-surgery an increase of JSW is shown and a decrease of subchondral cysts (E). In the ankle of case 3 especially osteophyte formation and osteochondral defects were seen (C). The JSW slightly increased due to treatment (F).

compared to 42° (32° pf - 10° df) of the ankle that received treatment with distraction. The range of motion for case 2 was 66° (50° pf - 16° df) for the contralateral ankle and 40° (32° pf - 8° df) for the treated ankle joint. Also case 3 had a larger range of motion of 62° (56° pf - 6° df) in the untreated ankle compared to the treated ankle (range of motion = 39°; 33 pf - 6° df).

Structural changes

Radiographs of the ankles of the 3 cases before and after treatment are depicted in figure 5A-F. Table 2 shows that the Pettersson score for the ankle of case 1 was 3 before treatment with ankle distraction and remained the same when scored post-surgery. The Pettersson score of case 2 decreased from 7 to 6 and the Pettersson score of case 3 was reduced from 4 to 3. The mean JSW, as measured by AIDA, increased in all patients after ankle distraction. The JSW of case 1 was relatively large before ankle distraction, 3.5 mm, and increased to 4.4 mm after treatment. Case 2 had a very small JSW of 0.3 mm prior

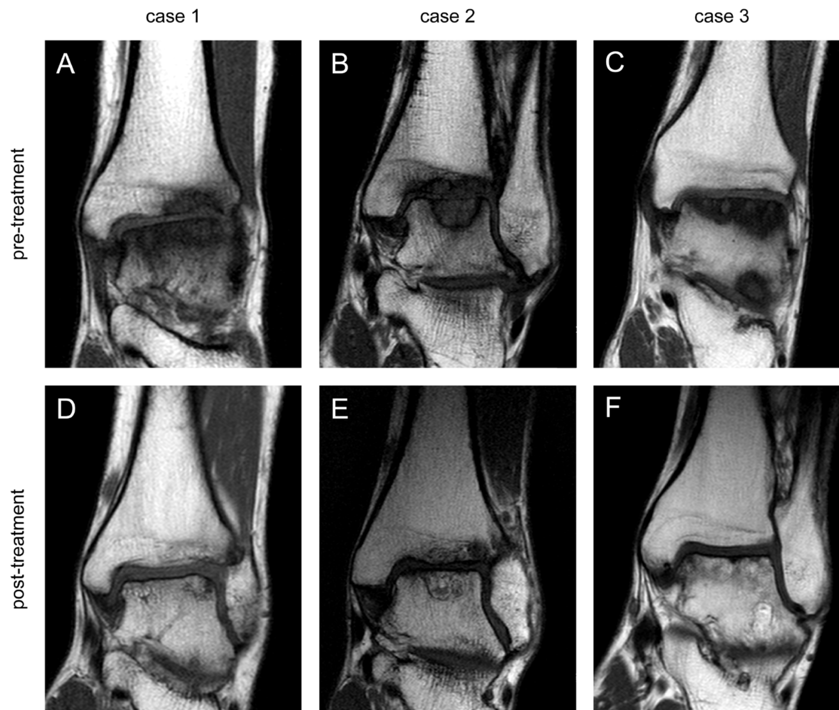

Figure 6: Structural improvement after ankle distraction on MRI
Pre-treatment (A-C) and post-treatment (D-F) T1-weighted MRI images of all 3 cases are depicted. Case 1 had osteochondral lesions with strong degeneration of surrounding cartilage pre-surgery (A). Almost all bone oedema and most subchondral cysts disappeared after distraction, and cartilage seems slightly thicker (D). The pre-treatment MRI of case 2 shows loss of cartilage, widespread cysts, bone erosions and oedema, and hemosiderin deposition in synovial tissue (B). A clear decrease of bone oedema and the volume of the large subchondral cysts was seen, as well as a slightly larger JSW (E). Case 3 had a narrowed JSW, oedema, and multiple subchondral cysts before ankle distraction (C). Treatment caused almost complete disappearance of bone oedema and of some of the subchondral cysts, but the cartilage remained thin (F).

to treatment, which improved to 2.0 mm after treatment. The JSW of case 3 increased from 3.2 mm to 3.9 mm.

Pre- and post-surgery MRIs are shown in figure 6A-F. Almost all bone oedema in the ankle of case 1 disappeared after distraction, but some subchondral cysts were still present (figure 6D). Cartilage seemed slightly thicker. In case 2 a clear decrease of bone oedema was seen as well as a slightly larger JSW (figure 6E). The number and size of subchondral cysts was significantly reduced. Case 3 showed almost complete disappearance of bone oedema and of some of the subchondral cysts (figure 6F). The cartilage remained thin. Table 2 shows decreased MRI scores for all 3 patients after ankle distraction; from 10 to 5 for case 1, from 16 to 13 for case 2, and from 10 to 6 for case 3.

DISCUSSION

The present study in retrospect is the first to describe treatment of haemophilic ankle arthropathy with joint distraction. Three PWH report clear improvement in clinical status: enhancement in self-perceived functional health, reduction of the impact of their chronic disability on participation and autonomy, and diminished ankle pain. They are able to perform all daily activities again including sports, except for running. Also, structural improvement of the ankle joints is seen on X-ray and MRI. Taken together, the patients are very satisfied with the outcome of the operation and the originally indicated arthrodesis is postponed up to this point; at least 2.2 years for the last patient treated up to 4 years for the first patient treated. According to the good results it is expected that an arthrodesis can be postponed for even longer periods of time as is observed in case of distraction treatment in case of osteoarthritis[13].

The results of this study are very comparable to the clinical benefit obtained after distraction in severe ankle osteoarthritis. Significant improvement was obtained in 73% of the treated osteoarthritis patients and maintained for at least 7 years[13]. Osteoarthritis is a degenerative disease with subchondral bone changes as a distinctive feature. It has been demonstrated that joint distraction initiates a process of bone remodelling which is associated with clinical improvement[16]. Based on histology, haemophilic arthropathy shares several characteristics with the degenerative disease osteoarthritis[25]. For this reason we believe that alteration of the bone remodelling process can also take place in PWH, thereby causing a similar clinical improvement after ankle distraction compared to results obtained in osteoarthritis patients[16].

Another technique to unload a joint has been used in 3 paediatric PWH with progressed ankle arthropathy[26]. They were treated with arthroscopic synovectomy followed by a patella tendon-bearing brace for 1 year. The patients showed improvement in pain, function, and alignment. This corroborates the results of our study, although the 3 paediatric PWH described in the Japanese study had more severe ankle complaints compared to the 3 adult patients in our study (Pettersson score of 9-10 and 3-7, respectively). Whether synovectomy or unloading had the highest impact in the Japanese study can only be speculated on. Irrespectively, these results support that unloading a joint may contribute to clinical benefit.

In this study all data on the patients' situation before surgery were acquired retrospectively. A study comparing retrospectively and prospectively obtained Van Valburg scores, demonstrated that retrospective data obtained after 7 years or more could be considered reliable[13]. Eight osteoarthritis patients that underwent ankle distraction scored slightly more pain when obtained retrospectively and compared to the score obtained prospectively, although not statistically significant. Retrospective function was scored statistically significant lower compared to the data obtained prospectively. Nevertheless, this difference was negligible in comparison to improvement in function achieved by distraction. In general, it is known that a 'response shift' can occur when orthopaedic patients report their clinical and functional pre-operative status

retrospectively[27]. Patients judge themselves to be worse retrospectively regarding pain and physical function. As such, the improvement in clinical status and pain in our study might be slightly overestimated.
Two out of the 3 cases reported improvement in function of the upper extremities; a component especially based on lifting or carrying heavy objects. Since treatment with distraction was focussed only on the ankle joint, this effect might seem implausible. However, functional improvement of the upper extremities can be explained by the way heavy objects are lifted. For lifting a heavy object, bending of the knees and ankles is necessary. Distraction improved the function of the ankle and made it possible to bend the ankle further without experiencing pain. As a consequence, bending the knees and ankles to pick up a heavy object became easier and less painful, resulting in a higher functional score of the upper extremities.
The key advantage of ankle distraction over arthrodesis is that joint mobility is maintained[13]. Unfortunately, in this study we could not compare the ROM before and after treatment. Mean dorsiflexion (post-surgery) of the 3 treated ankle joints in this study was 8° and mean plantar flexion 32°. Normative values for ankle dorsiflexion are 13° for males of 20-44 years, and 55° for plantar flexion[28], indicating that joint mobility of the 3 PWH is almost within the normal range. All cases also had a reasonable range of motion after treatment with ankle distraction (39-42°) when compared to a range of motion of the contralateral ankle (62-70°). Because no accurate pre-treatment values were available, it remains speculative whether this is an increase, stabilisation, or decrease of ankle mobility. In osteoarthritis patients mobility increased after ankle distraction in 6 out of 16 patients with an average of 101%, decreased in 6 patients with 17%, and remained unchanged in 1 patient[13]. It is still possible to participate in daily life with a limited range of motion of the ankle, because most activities require only a partial range of motion. For example, walking on even ground requires about 29° range of motion of the ankle joint, walking upstairs about 42°, and walking downstairs about 61°, indicating that the latter activity might still be difficult for the patients described in our study[29].
In conclusion, although this is a descriptive study on 3 cases with retrospectively gathered clinical data, results suggest that joint distraction may be a promising treatment for haemophilic ankle arthropathy. Even in case of severe damage, ankle distraction is a good alternative for arthrodesis and TAR, specifically in young active patients.

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REFERENCES

1. Fischer K, van der Bom JG, Mauser-Bunschoten EP, Roosendaal G, Prejs R, Grobbee DE, et al. Changes in treatment strategies for severe haemophilia over the last 3 decades: effects on clotting factor consumption and arthropathy. Haemophilia 2001;7:446-52.
2. Buzzard BM. Sports and hemophilia: antagonist or protagonist. Clin Orthop Relat Res 1996;328:25-30.
3. Tiktinsky R, Kenet G, Dvir Z, Falk B, Heim M, Martinowitz U, et al. Physical activity participation and bleeding characteristics in young patients with severe haemophilia. Haemophilia 2009;15:695-700.
4. Ribbans WJ, Phillips AM. Hemophilic ankle arthropathy. Clin Orthop Relat Res 1996;328:39-45.
5. Pasta G, Forsyth A, Merchan CR, Mortazavi SM, Silva M, Mulder K, et al. Orthopaedic management of haemophilia arthropathy of the ankle. Haemophilia 2008;14 Suppl 3:170-6.
6. Coester LM, Saltzman CL, Leupold J, Pontarelli W. Long-term results following ankle arthrodesis for post-traumatic arthritis. J Bone Joint Surg Am 2001;83-A:219-28.
7. Tsailas PG, Wiedel JD. Arthrodesis of the ankle and subtalar joints in patients with haemophilic arthropathy. Haemophilia 2010;16:822-31.
8. Barg A, Elsner A, Hefti D, Hintermann B. Haemophilic arthropathy of the ankle treated by total ankle replacement: a case series. Haemophilia 2010;16:647-55.
9. Radossi P, Bisson R, Munari F, Risato R, Tassinari C, Sartori R, et al. Total ankle replacement for end-stage arthropathy in patients with haemophilia. Haemophilia 2008;14:658-60.
10. van der Heide HJ, Novakova I, de Waal Malefijt MC. The feasibility of total ankle prosthesis for severe arthropathy in haemophilia and prothrombin deficiency. Haemophilia 2006;12:679-82.
11. SooHoo NF, Zingmond DS, Ko CY. Comparison of reoperation rates following ankle arthrodesis and total ankle arthroplasty. J Bone Joint Surg Am 2007;89:2143-9.
12. Marijnissen AC, Van Roermund PM, Van Melkebeek J, Schenk W, Verbout AJ, Bijlsma JW, et al. Clinical benefit of joint distraction in the treatment of severe osteoarthritis of the ankle: proof of concept in an open prospective study and in a randomized controlled study. Arthritis Rheum 2002;46:2893-902.
13. Ploegmakers JJ, van Roermund PM, van Melkebeek J, Lammens J, Bijlsma JW, Lafeber FP, et al. Prolonged clinical benefit from joint distraction in the treatment of ankle osteoarthritis. Osteoarthritis Cartilage 2005;13:582-8.
14. van Valburg AA, van Roermund PM, Lammens J, van Melkebeek J, Verbout AJ, Lafeber EP, et al. Can Ilizarov joint distraction delay the need for an arthrodesis of the ankle? A preliminary report. J Bone Joint Surg Br 1995;77:720-5.
15. van Valburg AA, van Roermund PM, Marijnissen AC, van Melkebeek J, Lammens J, Verbout AJ, et al. Joint distraction in treatment of osteoarthritis: a two-year follow-up of the ankle. Osteoarthritis Cartilage 1999;7:474-9.
16. Intema F, Thomas TP, Anderson DD, Elkins JM, Brown TD, Amendola A, et al. Subchondral bone remodeling is related to clinical improvement after joint distraction in the treatment of ankle osteoarthritis. Osteoarthritis Cartilage 2011;19:668-75.
17. Lafeber FP, Intema F, Van Roermund PM, Marijnissen AC. Unloading joints to treat osteoarthritis, including joint distraction. Curr Opin Rheumatol 2006;18:519-25.
18. van Valburg AA, van Roermund PM, Marijnissen AC, Wenting MJ, Verbout AJ, Lafeber FP, et al. Joint distraction in treatment of osteoarthritis (II): effects on cartilage in a canine model. Osteoarthritis Cartilage 2000;8:1-8.
19. van Genderen FR, Westers P, Heijnen L, de Kleijn P, van den Berg HM, Helders PJ, et al. Measuring patients' perceptions on their functional abilities: validation of the Haemophilia Activities List. Haemophilia 2006;12:36-46.
20. Cardol M, de Haan RJ, de Jong BA, van den Bos GA, de Groot IJ. Psychometric properties of the Impact on Participation and Autonomy Questionnaire. Arch Phys Med Rehabil 2001;82:210-6.
21. Sibley A, Kersten P, Ward CD, White B, Mehta R, George S. Measuring autonomy in disabled people: Validation of a new scale in a UK population. Clin Rehabil 2006;20:793-803.
22. Pettersson H, Ahlberg A, Nilsson IM. A radiologic classification of hemophilic arthropathy. Clin Orthop Relat Res 1980;149:153-9.

23. Marijnissen AC, Vincken KL, Viergever MA, van Roy HL, Van Roermund PM, Lafeber FP, et al. Ankle images digital analysis (AIDA): digital measurement of joint space width and subchondral sclerosis on standard radiographs. Osteoarthritis Cartilage 2001;9:264-72.
24. Lundin B, Babyn P, Doria AS, Kilcoyne R, Ljung R, Miller S, et al. Compatible scales for progressive and additive MRI assessments of haemophilic arthropathy. Haemophilia 2005;11:109-15.
25. Roosendaal G, van Rinsum AC, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-53.
26. Tanaka Y, Shinohara Y, Narikawa K, Kumai T, Takakura Y, Sakurai Y, et al. Arthroscopic synovectomies combined with reduced weight-bearing using patella tendon-bearing braces were very effective for progressed haemophilic ankle arthropathy in three paediatric patients. Haemophilia 2009;15:833-6.
27. Razmjou H, Yee A, Ford M, Finkelstein JA. Response shift in outcome assessment in patients undergoing total knee arthroplasty. J Bone Joint Surg Am 2006;88:2590-5.
28. Soucie JM, Wang C, Forsyth A, Funk S, Denny M, Roach KE, et al. Range of motion measurements: reference values and a database for comparison studies. Haemophilia 2011;17:500-7.
29. Sinitski EH, Hansen AH, Wilken JM. Biomechanics of the ankle-foot system during stair ambulation: Implications for design of advanced ankle-foot prostheses. J Biomech 2011;45:588-94.

Chapter 10

Summary and general discussion

SUMMARY

The aims of this thesis were to further unravel the pathogenesis of blood-induced joint damage and to develop new strategies to prevent or treat this joint damage. To achieve these aims several *in vitro* and *in vivo* models were used. The results of these studies are summarised in this chapter, followed by an integrated discussion to place them in a broader perspective.

In the first part of this thesis the mechanism of blood-induced joint damage is further clarified using *in vivo* models. Until now only transient joint damage was demonstrated in a canine model following intra-articular blood injections[1]. In **chapter 2** it was investigated whether two more intensive continuous blood loads within a 4-week time period lead to more prolonged cartilage damage in a canine model than the same blood load equally distributed over a similar time period (intermittent exposure). It appeared that loss of proteoglycans was significantly increased in the continuous blood-exposed joints, but not in the intermittent blood-exposed joints. Furthermore, proteoglycan content was more decreased in the continuous blood-exposed knees compared to the knees of animals with intermittent exposure. Mild synovial inflammation was observed in both groups and not different between the two groups. This indicates that in contrast to intermittent exposure, a 4-day continuous blood exposure twice in 4 weeks leads to prolonged cartilage damage. This prolonged damage is independent of the level of synovial inflammation. It appears that there is a threshold to induce irreversible joint damage, which is only reached by a more intensive continuous blood load. The question is what happens in humans during a joint haemorrhage due to (sports) trauma, major joint surgery, or haemophilia. Is there a similar threshold as observed in the canine model and when is this threshold reached? Clinical practice demonstrates that in at least a part of the haemophilia patients a single joint bleed already leads to long term joint damage, as shown on radiographs several years later (Roosendaal, personal observation). But does this also hold true for a joint bleed during trauma or major joint surgery? And does the damaging effect depend on the degree of blood coagulation? A difference between a haemophilic joint bleed and a joint bleed during trauma or major joint surgery is that haemophilic blood is not able to coagulate to the same extent as blood of an individual without a coagulation disorder. This raises the question whether coagulating blood is as harmful to a joint as anticoagulated blood.

To investigate whether there is a difference in the effects of coagulating blood (as seen in trauma and surgery) or anticoagulated blood (to mimic haemophilic blood) on joint damage, canine knees were injected with coagulating and anticoagulated blood. Saline or the anticoagulation agent heparin was used as a control. In **chapter 3** it was demonstrated that canine knees injected with coagulating blood showed a more disturbed proteoglycan turnover than knees injected with anticoagulated blood. Moreover, synovial inflammation was only present after intra-articular injections with

coagulating blood and not after injections with anticoagulated blood. This aggravation of joint damage by coagulation was also seen *in vitro* where coagulated blood resulted in more cartilage damage compared to anticoagulated blood. This difference is at least partly explained by a reduced amount of thrombin in anticoagulated blood, since thrombin is directly damaging to cartilage and a mediator of inflammation[2,3]. Surprisingly, exposure of cartilage to plasma (with factors of the coagulation cascade) or serum (without these factors) did not alter the cartilage matrix turnover, indicating that long-lasting damage does not primarily depend on the soluble mediators of the coagulation cascade, but presumably on the mechanism of chondrocyte apoptosis in which catalytic iron from red blood cells is a prerequisite[4,5]. The damaging effects of a joint bleed and thereby the urge to prevent such joint bleeds is a well appreciated concept in haemophilia patients. However, in patients having joint trauma or major joint surgery, prevention or treatment of joint bleeds is in general not considered essential. Now that it has been shown that coagulation of blood after a bleed even aggravates joint damage, prevention or treatment of blood-exposure should be considered of major importance after joint surgery and trauma. Aspiration of blood or a lavage during surgery can prevent, or at least limit, the destructive effects.

Nowadays joint damage is only visible on radiographs several years after a joint bleed and not directly after the bleed. If the consequences of blood-exposure could be visualised immediately or very shortly after a joint bleed, instead of only after years, it would allow prevention or treatment at a more individual patient level. Moreover, it would stress the joint damaging effect of a joint bleed under *in vivo* conditions. A possibility to monitor joint damage immediately after a haemorrhage without an invasive procedure is by measuring biochemical markers representing cartilage, bone, and synovial tissue turnover in blood or urine. In a first attempt to study the harmful effects of blood in a clinical situation, the levels of several biomarkers were measured after a joint bleed. **Chapter 4** demonstrates that on average a single joint bleed did increase biochemical markers of joint tissue damage (CTXII, COMP, C1,2C, and CS846) in a small group of patients. However, due to the great variation in patient and bleeding characteristics, these effects were only approaching statistical significance. To verify the potential use of biochemical markers detecting the harmful effect of a joint bleed, a joint bleed was artificially induced in dogs by intra-articular blood injections. In this canine study, the same biochemical markers were increased shortly after the joint haemorrhage, normalising in the weeks afterwards. As such, these canine *in vivo* data corroborate the results of the human study. A combined score of the four evaluated biochemical markers demonstrated to increase statistically significantly upon the single bleeding episode both in the human study as well as upon the experimentally induced joint haemorrhage in dogs. This explorative study warrants further research on the value of biochemical markers to detect the joint destructive properties of a (even a single) joint bleed.

The second part of this thesis focuses on potential treatment modalities for blood-induced joint damage. IL-4 and IL-10 are possible candidates, since these cytokines are not only anti-inflammatory, but are also supposed to have cartilage protective properties[6]. Because IL-4 and IL-10 exert different pathways, these effects can be synergistic. Therefore these cytokines were tested on blood-induced cartilage damage as individual cytokines and as a combination. **Chapter 5** shows that blood-induced damage to the cartilage matrix was limited by IL-4 in a dose-dependent way. Clearly the effect of IL-4 was more potent than the effect of IL-10. Moreover, adding IL-4 to IL-10 leads to a more pronounced protective effect than IL-10 alone, suggesting that a combination of the two cytokines might have additional value. Besides the effects on proteoglycan turnover also IL-4, and to a lesser extent IL-10, limited production of pro-inflammatory cytokines by blood monocytes as well as apoptosis of chondrocytes. This demonstrates that irreversible cartilage damage is inhibited, probably by limitation of pro-inflammatory cytokine production and thereby limitation of hydroxyl radicals. In this study there were no clear direct effects of IL-4 or IL-10 on cartilage, but the expression of the receptors of both cytokines on chondrocytes was upregulated because of exposure to blood. These results suggest that anti-inflammatory effects on monocytes in the blood fraction and direct protective effects on chondrocytes are both involved.

In the previous study a prevention approach for the effect of IL-4 plus IL-10 on blood-induced cartilage damage was chosen. However, this set-up will not be feasible in clinical practice. It will take at least hours, if not more, before a (sports) trauma-induced joint bleed or a haemophilic bleed can be treated. This is in contrast to a major joint surgery-induced joint bleed, which can be treated during surgery at the same time as the bleed starts. In case of trauma and haemophilia, it would clinically be more relevant if IL-4 and IL-10 can be used to treat blood-induced cartilage damage instead of only preventing it. To investigate a more relevant situation in case of trauma and haemophilia, **chapter 6** studied the potency of delayed combined IL-4 and IL-10 administration after start of blood-exposure *in vitro*. It was demonstrated that there is only a short time window after onset of the joint bleed in which IL-4 plus IL-10 can still limit blood-induced cartilage damage. For human cartilage, IL-4 plus IL-10 limited disturbance of the cartilage matrix turnover as well as production of pro-inflammatory cytokines when administered within 4-8 hours after the onset of a bleed. In addition to treatment with IL-4 plus IL-10, it was shown that shortening the time span of blood exposure by aspirating the blood within 24 hours almost fully prevented cartilage damage. This suggests that blood-induced joint damage in a clinical situation can be prevented when a joint is aspirated relatively quickly after a joint bleed. In the experimental set-up of this study, subsequent addition of IL-4 plus IL-10 to aspiration was not of additive value. However, in this *in vitro* model no synovial tissue is involved. Because there will always be intra-articular blood remains *in vivo* due to compartmentalisation[7] and IL-4 and IL-10 are known to limit synovial inflammation[8,9], these cytokines probably still have potential additive value to aspiration of a joint in clinical practice.

So far, treatment with a combination of IL-4 plus IL-10 has only been tested in several *in vitro* models. Joint tissues like synovium, cartilage, and bone influence each other. Therefore it is important to investigate the effects of IL-4 plus IL-10 on a complete joint *in vivo*. An internationally accepted *in vivo* model to explore joint bleeds and possible treatment is a haemophilia mice model. Due to knockout of the factor VIII gene, these mice suffer from a clotting disorder similar to human haemophilia A. After needle puncture of the knee joint, a joint bleed will occur. In **chapter 7** the combined treatment with IL-4 plus IL-10 was tested in mice having a joint bleed for the first time. It was demonstrated that a single intra-articular injection of IL-4 plus IL-10 directly after the joint bleed ameliorated cartilage degeneration. Moreover, the remains of a joint bleed, based on macroscopic bleeding score and on joint diameter, tended to be less frequently visible for IL-4 plus IL-10 injected joints than for vehicle-injected joints. Surprisingly, injection of IL-4 plus IL-10 directly after a joint bleed did not diminish bleeding-induced synovial inflammation. The amelioration of cartilage damage by IL-4 plus IL-10, although to a limited extend as observed in these *in vivo* mice experiments, is in line with the *in vitro* results on human cartilage as described in chapter 5 and 6. The reason for a partial effect on cartilage damage and the absence of an effect on inflammation could be due to low bioavailability of the cytokines or to the time point of evaluation of the joint after the joint bleed. With respect to the first, more significant effects on prevention of cartilage damage, and perhaps also limitation of synovial inflammation, are expected in case of greater bioavailability.

To tackle low bioavailability of IL-4 and IL-10 *in vivo*, a fusion protein combining IL-4 and IL-10, called the 'IL4-10 synerkine', was designed and tested in several assays for bioactivity. Having a larger molecular mass it will have a longer half-life *in vivo*, because of restrained renal clearance. In **chapter 8** the effects of this IL4-10 synerkine were tested on blood-induced cartilage damage *in vitro*, and compared to the combination of the individual components. It was demonstrated that the IL4-10 synerkine prevents blood-induced cartilage damage to the same extent as the combination of the individual recombinant cytokines IL-4 and IL-10 does. Also production of pro-inflammatory cytokines was limited to the same degree. Moreover, a clear dose-dependent response for the IL4-10 synerkine was shown. This proofs that the beneficial effects of the IL4-10 synerkine are similar to the combination of the individual cytokines *in vitro*. Pharmacological studies still need to demonstrate whether the half-life of the IL4-10 synerkine is indeed larger than that of the individual cytokines. Furthermore, intra-articular injections with the IL4-10 synerkine after an induced joint bleed in a haemophilia mouse model or a canine model should demonstrate the chondroprotective effect and anti-inflammatory properties *in vivo*.

Despite protection and treatment, joint damage will still occur in certain patients. For patients with heavily damaged joints, surgical treatment can be an option to improve their daily functioning and to decrease pain. Joint distraction is such a technique that has been successfully used as an alternative to surgical treatments like ankle arthrodesis or total joint replacements in osteoarthritis patients. In **chapter 9** for the first time ankle joint distraction was performed in three haemophilia patients. These patients were very satisfied with the clinical outcome of the procedure. They reported a clear improvement for self-perceived functional health, participation in society and autonomy, and pain. More remarkably, treatment with ankle distraction resulted in evident structural improvement of the joint. Joint space width increased and subchondral bone normalised after treatment as seen on X-ray and MRI. Although this is a first step and a descriptive study, this chapter clearly shows that joint distraction is a promising novel treatment for individual cases of haemophilic ankle arthropathy, without additional risk of bleedings during treatment. For the three patients of this study, an arthrodesis, and with that a stiff ankle joint, was postponed for at least 2 to 4 years. Longer follow-up is needed to determine how long the effects of ankle distraction last. Irrespectively, a specific group of young haemophilia patients having a severely degenerated joint can benefit from this treatment, which enables them to keep living a more normal active life.

DISCUSSION

This thesis clearly demonstrates, as described in chapter 3, that prevention of blood-induced joint damage is also required in case of joint trauma or major joint surgery, and not only in case of joint haemorrhage due to haemophilia. In case of a joint bleed due to knee trauma, rupture of the anterior cruciate ligament (ACL) is most common[10]. The reported population incidence of ACL injury ranges from 30 per 100 000 in a study in Denmark[11] to 81 per 100 000 in a Swedish study[12]. The majority of these patients with an acute ACL tear is younger than 30 years at the time of injury and it has been suggested that more than 50% will develop osteoarthritis at the long-term[13]. Clearly a major driving factor in this joint damage is joint instability. However, it has been shown that joint damage cannot significantly be prevented by ligament reconstruction, which stabilizes the joint again[14-16]. It has also been reported that shortly after the trauma, intra-articular levels of catabolic mediators, including IL-1β, rise[17]. It might well be that the catabolic cytokines are not only synovial tissue derived, but are also originating from the blood cells most often accompanying such a major trauma. Irrespectively, these cytokines, whether or not blood derived, may together with the iron from the red blood cells result in chondrocyte apoptosis as described[4,5], leading to long-term cartilage damage.
In case of an intra-articular fracture, blood will also enter the joint cavity, but has a clearly different source when compared to a ligament rupture caused by a trauma or compared to a joint bleed in haemophilia patients originating from the synovial tissue. It is not known whether the type of trauma and thereby the source of blood is of influence to joint damage. However, blood derived from the bone marrow in case of an intra-articular

fracture presumably contains specific factors of bone marrow as well as mesenchymal stem cells (MSC). Whether these factors and MSC can add to cartilage regeneration by release of trophic factors or by differentiating into chondrocytes to (partly) overcome the devastating effects of blood, is a fast moving field of research, still not providing a clear answer[18-20]. Irrespectively, in addition to prevention of blood-induced joint damage, optimal restoration of biomechanics of the joint is presumably most important to prevent focal undesired stresses on the cartilage and thereby joint degeneration.
Future studies for blood-induced joint damage need a sensitive method to detect small changes or differences in joint damage. Biochemical markers, as described in chapter 4, could become such a way of detection. However, this study for the effectiveness of biomarker use in blood-induced joint damage needs to be expanded to a larger group of patients before a solid conclusion can be drawn.

Joint aspiration

Since patients with joint trauma or haemophilia suffer from joint damage at a relatively young age, appropriate treatment can have huge influence on their daily lives. Chapters 2 and 6 demonstrate that by reducing the blood load in a joint (for example by aspiration) the degree of joint damage was limited. Surprisingly, there is no consensus on aspiration (arthrocentesis) of a joint after a joint bleed in haemophilia patients[21], nor after acute trauma[22]. Although there is no clear evidence, sceptics are afraid of the risk of intra-articular infections. A major drawback is the risk of a recurrent joint bleed upon needle aspiration of the joint. In case of haemophilia, the use of clotting factor replacement therapy, being standard after a bleeding episode, will prevent the additional bleeds upon aspiration. After a major joint bleed due to trauma, joint aspiration is also not generally indicated, although performed more frequently. Main indications for aspiration are fixed flexion deformity or the need of pain relief because of distension of the joint capsule. It has been reported that re-accumulation of intra-articular blood after aspiration in case of a post-traumatic joint bleed is common[23]. However, no studies have been published yet on whether aspiration after an acute traumatic joint bleed improves the quality of the joint in the long term[22]. Such studies are warranted, especially because it mostly concerns young patients. Moreover, it should be studied in more detail whether needle aspiration of a joint in case of haemophilia or after trauma results in an additional bleed due to the needle puncture of the synovial tissue; and with that whether aspiration of the large amounts of blood due to the primary bleeding is of more relevance than the potentially adverse effect of the expectedly minor bleeds induced by aspiration, specifically in the light of the threshold of blood load needed to provoke long lasting cartilage damage as described in chapter 3.
The best way to prevent joint damage is to prevent exposure of joint tissues to blood by aspiration. In addition to that, or as an alternative treatment in an early phase of blood-induced joint damage, treatment with IL-4 and IL-10 is feasible. Chapters 5 to 8 clearly demonstrate that a combination of IL-4 and IL-10 is a good candidate to prevent

and possibly treat the early features of blood-induced joint damage. However, more knowledge is needed on the effect of IL-4 plus IL-10 on direct as well as indirect, (synovial) inflammation-induced cartilage damage. Additionally, the question that remains is what is the best way is to administer IL-4 and IL-10 to patients having a joint bleed providing optimal bioavailability of these small molecules with a limited biological half-life?

Clinical use of IL-4 and IL-10

A major disadvantage of IL-4 and IL-10 is that their half-lives in a (clinical) *in vivo* situation are short. Following intravenous administration, recombinant human IL-10 has a half-life of 1.9 hours[24], while subcutaneous administration results in a half-life of 2.7-4.5 hours[25]. The half-life of IL-4 is even shorter: 19 minutes after intravenous injection[26]. In case of treatment for blood-induced joint damage, the bioavailability of IL-4 and IL-10 will probably be larger when directly injected in the joint, even though part of it will be absorbed into the systemic circulation[27]. However, an intra-articular antibody against TNF-α in an arthritis rat model demonstrates relatively slow absorption into the circulation from the joint cavity[28], indicating that bioavailability in a joint of an intra-articularly injected protein like IL-4 or IL-10, although smaller than an antibody, might be sufficient to exert its function. Chapter 7 indeed demonstrates that a single intra-articular injection with IL-4 and IL-10 in mice limits blood-induced cartilage damage. Synovial inflammation, on the other hand, could not be prevented. More frequent intra-articular administration of IL-4 and IL-10, or other ways of delivery (with longer bioavailability) are needed to potentially improve efficacy. Moreover, in case of single joint bleed as a result of trauma, synovial inflammation might be of lesser importance. In these patients prevention of the direct effect of the blood-induced damage by a single administration of IL-4 plus IL-10 might be sufficient to prevent apoptosis and with that irreversible cartilage damage over time.

Molecular modulation of IL-4 and IL-10 may add to increase their half-lives. For example, the molecular size could be enhanced by glycosylation or by fusing the cytokines with the Fc tail of an antibody[29], both without losing bioactivity. Also gene therapy with prolonged deliverance of the cytokine is an attempted approach. IL-4 and IL-10 gene therapy has been investigated in a mouse diabetes model. Delivery of IL-4 and IL-10 using a non-viral vector based on polymers prevents development of type 1A diabetes in mice up to 6 weeks[30]. Ideally the gene used in gene therapy, for example the gene for IL-4 or IL-10, is only activated in presence of disease activity and stays at rest in a naïve situation. As an indicator for joint disease activity one can think of IL-1 as an important mediator of joint inflammation and cartilage destruction. In an arthritis mouse model it has been shown that IL-4 gene therapy integrated with a hybrid IL-1/IL-6 promoter, results in therapeutically effective IL-4 levels under arthritic conditions, but low levels under naïve conditions[31]. By regulating expression of intra-articular IL-4 and IL-10, possible systemically adverse effects of IL-4 and IL-10 treatment in clinical practice can be counteracted. Nevertheless, it is also clear that such techniques have their drawbacks

in clinical practice.
A different approach is to combine both molecules in one fusion protein, thereby increasing the molecular size and with that bioavailability. *In vitro* studies with such a novel designed fusion protein of IL-4 and IL-10, called the IL4-10 synerkine, demonstrated bioactivity of both IL-10 and IL-4. Testing the IL4-10 synerkine on its effect on blood-induced joint damage *in vitro* (chapter 8) provided bioactivity identical to the combination of the individual cytokines. This, in combination with the amelioration of cartilage damage upon a single injection of IL-4 plus IL-10 in an *in vivo* mouse model of blood-induced joint damage, is very promising regarding the *in vivo* effect of the IL4-10 synerkine. Clearly, in future experiments, administration of IL-4 and IL-10 using these new strategies has to be tested in *in vivo* models of blood-induced joint damage.

IL-1 as a therapeutic target

Chapters 5 and 6 of this thesis demonstrate that the therapeutic effect of IL-4 and IL-10 is expected to be for a significant part due to inhibition of IL-1β production by the blood cells. This effect will prevent apoptosis of chondrocytes. On the other hand, we also observed an upregulation of the IL-4 and IL-10 receptor components on chondrocytes after blood exposure. How this adds to cartilage repair needs further study. It has been demonstrated that IL-1β is a major player in the pathogenesis of blood-induced damage. Instead of using IL-4 and IL-10 to limit production of IL-1β, one can also use agents that directly block IL-1β.
There are several possibilities to prevent the damaging effects of IL-1 on different levels[32]. The first option is the natural occurring antagonist IL-1Ra (Anakinra). In several clinical trials in RA patients there was only little improvement due to IL-1Ra administration. However, in a randomized controlled pilot trial amongst patients with an acute anterior cruciate ligament rupture, intra-articular injected IL-1Ra reduced pain and improved function on a KOOS scale up to a period of 2 weeks[33]. It is a relatively safe therapy with injection-site reactions as the only side effect reported. Two longer acting IL-1 agents are a monoclonal IL-1β antibody (Canakinumab) and a dimeric fusion protein consisting of the ligand-binding domain of IL-1R1 and its accessory protein bound to human IgG1 (Rilonacept). Canakinumab lowers the disease activity in RA[34] and juvenile arthritis patients[35]. Until now, Rilonacept has only been used as treatment for gouty arthritis, for which it showed improvements[36]. Since different therapeutic strategies to limit IL-1 are already tested in clinical safety studies, it is interesting to investigate whether inhibition of IL-1 via these agents can also prevent blood-induced joint damage.
However, it should be kept in mind that this approach, although seemingly targeting the same pathway, is quite different. The concept of exclusively inhibition of IL-1 originates from the idea that IL-1 is a driving factor in H_2O_2 production by the chondrocytes that in combination with RBC derived iron results in chondrocyte apoptosis. Nevertheless, it may well be that also other inflammatory cytokines will be released from the activated blood monocytes/macrophages upon a joint bleed and will be able to evoke a similar

effect on the chondrocytes as IL-1 does. This has never been tested properly. In that case inhibition of only IL-1 will be insufficient and inhibition of the activation of the blood monocytes/macrophages by use of IL-4 plus IL-10 will be much more effective. This should be considered in addition to the direct effect of IL-4 plus IL-10 on chondrocytes and the controlling effect on synovial tissue inflammation.

Joint distraction

Even despite prevention and potential future treatment of joint bleeds, degeneration of the joint due to blood-induced damage will occur. Especially having the knowledge that a joint bleed after acute traumatic knee injury usually develops within 2-6 hours, but may take up to 24 hours to appear as a clinical problem that needs treatment[23]. In that case administration of IL-4 plus IL-10 might be too late to counteract joint damage caused by blood.

In more advanced stages of joint damage surgical treatment is considered as treatment option. In case of osteoarthritis, potentially caused by a major trauma years before, joint distraction appears to be very helpful[37-39]. However, this treatment is never tested in case of haemophilic arthropathy. In chapter 9 it was demonstrated for the first time that ankle distraction in case of severe haemophilic arthropathy is very successful. The 3 patients that have been treated thus far reported a clear improvement for self-perceived functional health, participation in society and autonomy, and pain. Moreover, treatment with ankle distraction resulted in evident structural improvement of the joint; joint space width increased and subchondral bone normalised as seen on X-ray and MRI. The results of this study are very similar to the clinical and structural benefit obtained after distraction in severe ankle osteoarthritis. Significant improvement was obtained in 73% of the treated ankle osteoarthritis patients and maintained for at least 7 years[38]. During the 3-months distraction period, loading and unloading of the ankle joint leads to intra-articular intermittent fluid pressure changes[40], facilitating nutrition of chondrocytes. Bone turnover decreases and results in lower subchondral bone density[41], which normalizes after distraction[42]. Thereby it provides a rich source of growth factors involved in cartilage growth and repair[43]. Moreover, a canine model showed an altered chondrocyte metabolism, indicating normalisation of chondrocyte function due to joint distraction[44]. Currently, joint distraction is also applied in knee osteoarthritis patients with prolonged clinical and structural beneficial changes[39]. For the first time intrinsic cartilage repair was demonstrated in end-stage OA patients giving opportunity to study the mechanisms behind this regeneration. In parallel, this might also be applicable to severe blood-induced cartilage damage as indicated by this first explorative study. In fact, results were rather surprising as it was considered that this treatment would be ineffective in case of an intra-articular inflammatory component (personal communication). This idea has to be reconsidered and studied in more detail as patients with sever haemophilic arthropathy have a clear inflamed synovial tissue when compared to OA patients, although significantly less than in RA patients[45].

Irrespectively, at present it means that even when more advanced blood-induced joint damage is present, repair of joint tissues can still take place, and as such can improve the quality of life of haemophilia patients.

OVERALL CONCLUSION

The overall conclusion of this thesis is that irreversible joint damage only arises after blood-exposure above a certain threshold. Both coagulating and anti-coagulated blood are harmful to the joint, although the damage is more severe when compounds of the blood coagulation cascade are involved. If blood is removed quickly from the joint by aspiration, damage is prevented. Biochemical markers in blood and/or urine might detect small changes in joint tissue turnover due to a joint bleed directly after the bleed. In addition, IL-4 and IL-10 prevent joint damage, especially when administered shortly after the onset of the joint bleed. A fusion protein of both (IL4-10 synerkine) has in this respect potential for therapy. When advanced joint damage is already present, joint distraction can significantly improve joint function and structure.

REFERENCES

1. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. J Rheumatol 2003;30:339-44.
2. Furmaniak-Kazmierczak E, Cooke TD, Manuel R, Scudamore A, Hoogendorn H, Giles AR, et al. Studies of thrombin-induced proteoglycan release in the degradation of human and bovine cartilage. J Clin Invest 1994;94:472-80.
3. Varisco PA, Peclat V, van Ness K, Bischof-Delaloye A, So A, Busso N. Effect of thrombin inhibition on synovial inflammation in antigen induced arthritis. Ann Rheum Dis 2000;59:781-7.
4. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am J Pathol 2003;162:943-51.
5. Hooiveld MJ, Roosendaal G, van den Berg HM, Bijlsma JW, Lafeber FP. Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study. Rheumatology (Oxford) 2003;42:784-90.
6. van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996;39:829-35.
7. Knight AD, Levick JR. Physiological compartmentation of fluid within the synovial cavity of the rabbit knee. J Physiol 1982;331:1-15.
8. Dechanet J, Rissoan MC, Banchereau J, Miossec P. Interleukin 4, but not interleukin 10, regulates the production of inflammation mediators by rheumatoid synoviocytes. Cytokine 1995;7:176-83.
9. Morita Y, Yamamura M, Kawashima M, Aita T, Harada S, Okamoto H, et al. Differential in vitro effects of IL-4, IL-10, and IL-13 on proinflammatory cytokine production and fibroblast proliferation in rheumatoid synovium. Rheumatol Int 2001;20:49-54.
10. Hardaker WT, Jr., Garrett WE, Jr., Bassett FH, 3rd. Evaluation of acute traumatic hemarthrosis of the knee joint. South Med J 1990;83:640-4.
11. Nielsen AB, Yde J. Epidemiology of acute knee injuries: a prospective hospital investigation. J Trauma 1991;31:1644-8.
12. Frobell RB, Lohmander LS, Roos HP. Acute rotational trauma to the knee: poor agreement between clinical assessment and magnetic resonance imaging findings. Scand J Med Sci Sports 2007;17:109-14.
13. Lohmander LS, Englund PM, Dahl LL, Roos EM. The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis. Am J Sports Med 2007;35:1756-69.
14. Frobell RB, Roos EM, Roos HP, Ranstam J, Lohmander LS. A randomized trial of treatment for acute anterior cruciate ligament tears. N Engl J Med 2010;363:331-42.
15. Lohmander LS, Ostenberg A, Englund M, Roos H. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. Arthritis Rheum 2004;50:3145-52.
16. von Porat A, Roos EM, Roos H. High prevalence of osteoarthritis 14 years after an anterior cruciate ligament tear in male soccer players: a study of radiographic and patient relevant outcomes. Ann Rheum Dis 2004;63:269-73.
17. Catterall JB, Stabler TV, Flannery CR, Kraus VB. Changes in serum and synovial fluid biomarkers after acute injury (NCT00332254). Arthritis Res Ther 2010;12:R229.
18. Bouffi C, Djouad F, Mathieu M, Noel D, Jorgensen C. Multipotent mesenchymal stromal cells and rheumatoid arthritis: risk or benefit? Rheumatology (Oxford) 2009;48:1185-9.
19. Caplan AI. Why are MSCs therapeutic? New data: new insight. J Pathol 2009;217:318-24.
20. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98:1076-84.
21. Hermans C, De Moerloose P, Fischer K, Holstein K, Klamroth R, Lambert T, et al. Management of acute haemarthrosis in haemophilia A without inhibitors: literature review, European survey and recommendations. Haemophilia 2011;17:383-92.
22. Wallman P, Carley S. Aspiration of acute traumatic knee haemarthrosis. Emerg Med J 2002;19:50.
23. Shaerf D, Banerjee A. Assessment and management of posttraumatic haemarthrosis of the knee. Br J Hosp Med (Lond) 2008;69:459-60, 462-3.

24. Radwanski E, Chakraborty A, Van Wart S, Huhn RD, Cutler DL, Affrime MB, et al. Pharmacokinetics and leukocyte responses of recombinant human interleukin-10. Pharm Res 1998;15:1895-901.
25. Huhn RD, Radwanski E, Gallo J, Affrime MB, Sabo R, Gonyo G, et al. Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. Clin Pharmacol Ther 1997;62:171-80.
26. Prendiville J, Thatcher N, Lind M, McIntosh R, Ghosh A, Stern P, et al. Recombinant human interleukin-4 (rhu IL-4) administered by the intravenous and subcutaneous routes in patients with advanced cancer--a phase I toxicity study and pharmacokinetic analysis. Eur J Cancer 1993;29A:1700-7.
27. Bird HA, Ring EF, Daniel R, Bacon PA. Comparison of intra-articular methotrexate with intra-articular triamcinolone hexacetonide by thermography. Curr Med Res Opin 1977;5:141-6.
28. Urech DM, Feige U, Ewert S, Schlosser V, Ottiger M, Polzer K, et al. Anti-inflammatory and cartilage-protecting effects of an intra-articularly injected anti-TNF{alpha} single-chain Fv antibody (ESBA105) designed for local therapeutic use. Ann Rheum Dis 2009;69:443-9.
29. Schwager K, Kaspar M, Bootz F, Marcolongo R, Paresce E, Neri D, et al. Preclinical characterization of DEKAVIL (F8-IL10), a novel clinical-stage immunocytokine which inhibits the progression of collagen-induced arthritis. Arthritis Res Ther 2009;11:R142.
30. Mandke R, Singh J. Cationic nanomicelles for delivery of plasmids encoding interleukin-4 and interleukin-10 for prevention of autoimmune diabetes in mice. Pharm Res 2012;29:883-97.
31. Geurts J, Arntz OJ, Bennink MB, Joosten LA, van den Berg WB, van de Loo FA. Application of a disease-regulated promoter is a safer mode of local IL-4 gene therapy for arthritis. Gene Ther 2007;14:1632-8.
32. Geyer M, Muller-Ladner U. Actual status of antiinterleukin-1 therapies in rheumatic diseases. Curr Opin Rheumatol 2010;22:246-51.
33. Kraus VB, Birmingham J, Stabler TV, Feng S, Taylor DC, Moorman CT, 3rd, et al. Effects of intraarticular IL1-Ra for acute anterior cruciate ligament knee injury: a randomized controlled pilot trial (NCT00332254). Osteoarthritis Cartilage 2012;20:271-8.
34. Alten R, Gomez-Reino J, Durez P, Beaulieu A, Sebba A, Krammer G, et al. Efficacy and safety of the human anti-IL-1beta monoclonal antibody canakinumab in rheumatoid arthritis: results of a 12-week, Phase II, dose-finding study. BMC Musculoskelet Disord 2011;12:153.
35. Ruperto N, Quartier P, Wulffraat N, Woo P, Ravelli A, Mouy R, et al. A phase II, multicenter, open-label study evaluating dosing and preliminary safety and efficacy of canakinumab in systemic juvenile idiopathic arthritis with active systemic features. Arthritis Rheum 2012;64:557-67.
36. Terkeltaub R, Sundy JS, Schumacher HR, Murphy F, Bookbinder S, Biedermann S, et al. The interleukin 1 inhibitor rilonacept in treatment of chronic gouty arthritis: results of a placebo-controlled, monosequence crossover, non-randomised, single-blind pilot study. Ann Rheum Dis 2009;68:1613-7.
37. Amendola A, Hillis S, Stolley MP, Saltzman CL. Prospective randomized controlled trial of motion vs fixed distraction in treatment of ankle osteoarthritis. J Bone Joint Surg Am 2011.
38. Ploegmakers JJ, van Roermund PM, van Melkebeek J, Lammens J, Bijlsma JW, Lafeber FP, et al. Prolonged clinical benefit from joint distraction in the treatment of ankle osteoarthritis. Osteoarthritis Cartilage 2005;13:582-8.
39. Intema F, Van Roermund PM, Marijnissen AC, Cotofana S, Eckstein F, Castelein RM, et al. Tissue structure modification in knee osteoarthritis by use of joint distraction: an open 1-year pilot study. Ann Rheum Dis 2011;70:1441-6.
40. van Valburg AA, van Roermund PM, Lammens J, van Melkebeek J, Verbout AJ, Lafeber EP, et al. Can Ilizarov joint distraction delay the need for an arthrodesis of the ankle? A preliminary report. J Bone Joint Surg Br 1995;77:720-5.
41. Marijnissen AC, Van Roermund PM, Van Melkebeek J, Schenk W, Verbout AJ, Bijlsma JW, et al. Clinical benefit of joint distraction in the treatment of severe osteoarthritis of the ankle: proof of concept in an open prospective study and in a randomized controlled study. Arthritis Rheum 2002;46:2893-902.
42. Intema F, Thomas TP, Anderson DD, Elkins JM, Brown TD, Amendola A, et al. Subchondral bone remodeling is related to clinical improvement after joint distraction in the treatment of ankle osteoarthritis. Osteoarthritis Cartilage 2011;19:668-75.
43. Lafeber FP, Intema F, Van Roermund PM, Marijnissen AC. Unloading joints to treat osteoarthritis, including joint distraction. Curr Opin Rheumatol 2006;18:519-25.

44. van Valburg AA, van Roermund PM, Marijnissen AC, Wenting MJ, Verbout AJ, Lafeber FP, et al. Joint distraction in treatment of osteoarthritis (II): effects on cartilage in a canine model. Osteoarthritis Cartilage 2000;8:1-8.
45. Roosendaal G, van Rinsum AC, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-53.

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NEDERLANDSE SAMENVATTING

Een bloeding in een gewricht kan leiden tot blijvende gewrichtsschade. Er zijn verschillende oorzaken voor een gewrichtsbloeding, zoals een (sport) letsel of een grote chirurgische ingreep. Daarnaast komen gewrichtsbloedingen vaak voor bij hemofilie patiënten. Hemofilie is een erfelijke stoornis in de bloedstolling die vrijwel uitsluitend bij mannen voorkomt, omdat het aangetaste gen op het X-chromosoom ligt. Er zijn twee typen hemofilie. Bij hemofilie A heeft de patiënt onvoldoende stollingsfactor VIII en bij hemofilie B heeft de patiënt te weinig stollingsfactor IX. Ongeveer 1 op 10.000 mannen wereldwijd heeft hemofilie A en 1 op de 60.000 heeft hemofilie B. Omdat hemofilie patiënten niet genoeg stollingsfactor (VIII of IX) aanmaken, kunnen er spontane bloedingen optreden. Ongeveer 90% van deze bloedingen vindt plaats in de grote gewrichten (ellebogen, knieën, enkels, heupen en schouders). Onafhankelijk van hoe een gewrichtsbloeding ontstaat (door letsel, operatie of hemofilie), kan een dergelijke bloeding leiden tot ernstige schade van de verschillende componenten van een gewricht, zoals synoviaal weefsel, kraakbeen en subchondraal bot.

Het synovium is de zachte binnenbekleding van de gewrichtsholte en ligt tegen het gewrichtskapsel aan. Een belangrijke functie is het produceren van synoviale vloeistof. Dit zorgt voor een soepele beweging van het gewricht en bevat voedingsstoffen voor het kraakbeen. Om synoviale vloeistof te kunnen maken, moet het synoviale weefsel goed doorbloed zijn. Een nadeel is dat deze bloedvaatjes in het synovium ook de bron voor gewrichtsbloedingen zijn.
Kraakbeen bedekt de uiteinden van de beide botten die het gewricht vormen. Het is niet doorbloed en daardoor volledig afhankelijk van de synoviale vloeistof voor voedingsstoffen. Het is opgebouwd uit extracellulaire matrix en cellen (chondrocyten). De chondrocyten vormen slechts 2% van het totale volume van het kraakbeen en zijn verantwoordelijk voor de opbouw en het onderhoud van de extracellulaire matrix. De matrix bestaat voornamelijk uit collageen type II (voor de vormvastheid en trekkrachten) en proteoglycanen (voornamelijk aggrecan). Doordat proteoglycanen sterk negatief geladen zijn, wordt water de kraakbeenmatrix ingetrokken via osmose. Bij belasting van het gewricht wordt dit water de matrix uitgeperst door de drukkrachten. Bij ontlasting zal er weer water worden aangetrokken. Op deze manier worden de krachten gedempt die op het gewricht komen tijdens belasting.

Bloed-geïnduceerde gewrichtsschade ontstaat zowel door ontsteking van het synovium als door directe schade aan het kraakbeen. Na een gewrichtsbloeding wordt het bloed door het synoviale weefsel uit het gewricht verwijderd, waarbij rode bloedcellen beschadigen en afgebroken worden. Hierbij komt ijzer vrij, dat in het synovium opgeslagen wordt in de vorm van hemosiderine. Daardoor raakt het synovium ontstoken en worden nieuwe bloedvaatjes gevormd. Er worden ook ontstekingsbevorderende stoffen aangemaakt zoals het cytokine IL-1β (zet de chondrocyten aan tot het afbreken van de kraakbeen matrix) en proteasen (breken het kraakbeen direct af).

Bloed kan ook rechtstreeks het kraakbeen beschadigen zonder dat ontsteking van het synovium daarbij betrokken is. Het is al eerder aangetoond dat wanneer kraakbeen tijdens een in vitro kweek gedurende 4 dagen blootgesteld wordt aan 50% bloed, er een verandering in de kraakbeen matrix turnover plaatsvindt. De aanmaak van matrix bestanddelen, zoals proteoglycanen, vermindert en de afbraak van deze bestanddelen neemt toe. Deze effecten houden minimaal 10 weken aan na de eenmalige bloedblootstelling. Er is aangetoond dat de combinatie van ontstekingscellen (mononucleaire cellen) en rode bloedcellen in het bloed verantwoordelijk is voor de schade aan het kraakbeen. De mononucleaire cellen raken geactiveerd en produceren onder andere IL-1β. Dat zet de chondrocyten aan tot een verhoging van de productie van waterstofperoxide. Dit vormt samen met ijzer, dat is vrijgekomen uit de beschadigde rode bloedcellen, hydroxyl radicalen. Als deze in de buurt van chondrocyten gevormd worden, veroorzaken ze celdood van de chondrocyt. Hierdoor kan dan geen nieuwe extracellulaire matrix meer aangemaakt worden.

De doelstellingen van dit proefschrift zijn het verder uitzoeken van het mechanisme van bloed-geïnduceerde gewrichtsschade en nieuwe methoden ontwikkelen om deze schade tegen te gaan.

In het eerste gedeelte van dit proefschrift is het mechanisme verder opgehelderd door gebruik te maken van een diermodel voor bloed-geïnduceerde gewrichtsschade. Hoofdstuk 2 laat zien dat twee intensieve 4-daagse bloedingen in een periode van 4 weken tijd (zoals acute bloedingen in de klinische praktijk) tot meer kraakbeenschade leidden dan wanneer dezelfde hoeveelheid bloed verdeeld werd over de gehele periode van 4 weken (zoals subklinische microbloedingen). Het synovium was in beide groepen in dezelfde mate licht ontstoken. Dit suggereert dat het verschil in kraakbeenschade onafhankelijk is van de mate van synoviale ontsteking. Twee grote bloedingen leiden dus wel tot kraakbeenschade, in tegenstelling tot een minimale chronische bloeding. Waarschijnlijk is er een drempelwaarde voor de hoeveelheid van de bloedblootstelling nodig voordat deze onherstelbare schade aan het kraakbeen kan veroorzaken.

In hoofdstuk 3 is in hetzelfde diermodel onderzocht of er een verschil is tussen stollend bloed (zoals in geval van gewrichtsletsel en operatie) en ontstold bloed (om een gewrichtsbloeding bij hemofilie patiënten te imiteren). Hieruit bleek dat er in beide gevallen onomkeerbare schade aan het kraakbeen ontstond. De gewrichten die blootgesteld waren aan stollend bloed hadden echter meer schade (verstoorde proteoglycaan turnover) aan het kraakbeen dan de gewrichten die blootgesteld waren aan ontstold bloed. Daarnaast was het synovium alleen ontstoken in de gewrichten die geïnjecteerd waren met stollend bloed. Er werd ook meer kraakbeenschade gezien wanneer humaan kraakbeen in vitro gekweekt werd met stollend bloed dan wanneer het gekweekt werd met ontstold bloed. Het verschil in deze effecten kan mogelijk deels verklaard worden doordat thrombine alleen in stollend bloed aanwezig is en niet in ontstold bloed. Thrombine is een component van de stollingscascade en kan directe schade aan het kraakbeen veroorzaken. Dit is echter niet de enige reden voor

het verschil, omdat in vitro blootstelling van kraakbeen aan plasma (bevat wel componenten van de stollingscascade maar geen rode bloedcellen) of serum (bevat geen rode bloedcellen en geen stollingsfactoren) geen onomkeerbare schade aan het kraakbeen veroorzaken. Het is bij hemofilie inmiddels goed bekend dat gewrichtsbloedingen op de lange termijn schade veroorzaken aan gewrichten. Dit hoofdstuk laat zien dat het minstens zo belangrijk is om ook na een letsel of operatie gewrichtsbloedingen te voorkomen. Aspiratie van bloed uit een gewricht kan de schade beperken.

Gewrichtsschade kan pas jaren na een gewrichtsbloeding aangetoond worden op röntgenfoto's. Om schade te beperken of om een behandeling te geven die meer toegesneden is op de individuele patiënt, zouden de consequenties van een gewrichtsbloeding veel eerder aantoonbaar moeten zijn. Door middel van biochemische markers (biomarkers) kan direct na een gewrichtsbloeding schade aangetoond worden. Deze biomarkers zijn te meten in bloed of urine en zijn afbraakproducten van kraakbeen en bot. In hoofdstuk 4 is beschreven dat in een kleine groep hemofilie patiënten de biomarkers van gewrichtsschade (CTXII, COMP, C1,2C en CS846) direct na een enkele gewrichtsbloeding toegenomen waren. Door de grote variatie in patiënt- en bloedingseigenschappen, waren deze resultaten echter niet statistisch significant. De resultaten konden wel bevestigd worden in een dierexperimentele studie. Het is dus relevant om biochemische markers verder te exploreren als voorspellers van gewrichtsschade.

Het tweede deel van dit proefschrift richt zich op behandelingsstrategieën voor bloed-geïnduceerde gewrichtsschade. Cytokines, zoals interleukine (IL)-4 en IL-10, zijn kleine eiwitmoleculen die mogelijk gebruikt kunnen worden om bloed-geïnduceerde schade te voorkomen. Het is bekend dat IL-4 en IL-10 niet alleen ontsteking tegengaan, maar ook kraakbeen kunnen beschermen tegen schade in andere gewrichtsziekten zoals reuma. Hoofdstuk 5 laat zien dat bloed-geïnduceerde kraakbeenschade in vitro beperkt werd wanneer een combinatie van IL-4 en IL-10 aan de bloed-kraakbeen kweek werd toegevoegd. IL-4 beschermde het kraakbeen sterker tegen deze schade dan IL-10. De combinatie van de twee cytokines had een toegevoegde waarde ten opzichte van IL-10 alleen, maar niet ten opzichte van IL-4 alleen. De proteoglycaan turnover werd genormaliseerd, er was minder celdood en er werden minder ontstekingsmediatoren (pro-inflammatoire cytokines) geproduceerd door de bloedcellen. In deze studie hadden IL-4 en IL-10 geen direct stimulerend effect op kraakbeen dat niet blootgesteld was aan bloed. De receptoren voor IL-4 en IL-10 op kraakbeen werden wel verhoogd wanneer kraakbeen blootgesteld werd aan bloed. Dit suggereert dat IL-4 en IL-10 toch direct op kraakbeen cellen (chondrocyten) kunnen aangrijpen via de receptoren. Daarnaast grijpen ze ook aan op de ontstekingsremming van bloedcellen.

In het vorige hoofdstuk is aangetoond dat IL-4 en IL-10 beschermen tegen bloed-geïnduceerde kraakbeenschade wanneer ze tegelijkertijd met de bloedblootstelling toegediend worden. In de klinische praktijk zal dit echter moeilijk toepasbaar zijn aangezien de behandeling van

een gewrichtsbloeding na een trauma en in een hemofilie patiënt niet op exact hetzelfde moment gestart kan worden als de bloeding begint. Daarom is er in hoofdstuk 6 onderzocht of een combinatie van IL-4 en IL-10 nog steeds beschermt wanneer het toegediend wordt enkele uren na het begin van de bloedblootstelling. Het bleek dat behandeling met IL-4 en IL-10 gestart moet worden binnen 4 tot 8 uur na start van de bloeding om nog steeds tegen de schadelijke effecten te kunnen beschermen. In aanvulling hierop is aangetoond dat de kraakbeenschade volledig tegengegaan werd door de duur van bloedblootstelling te verkorten tot 24 uur. In een klinische situatie zou het bloed dus bij voorkeur uit het gewricht verwijderd moeten worden binnen 24 uur na de bloeding. Het toevoegen van IL-4 en IL-10 na aspiratie heeft geen extra effect op preventie van de schade in de in vitro experimenten. De verwachting is echter dat het in de klinische setting wel gunstige effecten zou kunnen hebben, omdat IL-4 en IL-10 tevens synoviale ontsteking kunnen verminderen.

Tot dusver is de combinatie van IL-4 en IL-10 alleen getest met in vitro kraakbeen kweek modellen. Gewrichtsweefsels zoals synovium, kraakbeen en bot beïnvloeden elkaar, terwijl in het in vitro model alleen de effecten op kraakbeen worden bestudeerd. Om de effecten van een behandeling op het complete gewricht te analyseren, is in hoofdstuk 7 gebruik gemaakt van een in vivo hemofilie muis model. Het bleek dat een injectie met IL-4 en IL-10 direct na de gewrichtsbloeding resulteerde in minder kraakbeenschade. Daarnaast waren er macroscopisch minder overblijfselen van de bloeding zichtbaar en was de gewrichtsdiameter kleiner na behandeling met IL-4 en IL-10. De ontsteking van het synovium was, tegen de verwachting in, echter niet geremd. Dit zou veroorzaakt kunnen worden doordat IL-4 en IL-10 na een enkele toediening in vivo zeer snel geklaard worden uit het lichaam. We verwachten grotere effecten wanneer de biologische beschikbaarheid groter is.

Om ervoor te zorgen dat IL-4 en IL-10 in vivo langer beschikbaar zijn, is er een fusie eiwit (genaamd IL4-10 synerkine) ontwikkeld en getest met verschillende assays. Omdat het een grotere molecuulmassa heeft, zal het IL4-10 synerkine ook een langere halfwaardetijd hebben door verminderde klaring door de nieren. In hoofdstuk 8 zijn de effecten van het IL4-10 synerkine getest in het in vitro kraakbeen kweek model. In dit hoofdstuk is aangetoond dat het IL4-10 synerkine in dezelfde mate beschermt tegen bloed-geïnduceerde kraakbeenschade als de losse componenten. Ook de productie van pro-inflammatoire cytokines werd even veel geremd. Daarnaast hebben we een duidelijk dosis respons curve van het IL4-10 synerkine laten zien. In vitro gedraagt het IL4-10 synerkine zich dus hetzelfde als de combinatie van de losse componenten. Farmacologische studies moeten uitwijzen of de halfwaardetijd van het IL4-10 synerkine inderdaad hoger is. Wanneer dat het geval is, kan het IL4-10 synerkine getest worden in een in vivo hemofilie muis model.

Ondanks beschermende maatregelen en behandelingen zal er in sommige patiënten nog steeds bloed-geïnduceerde gewrichtsschade voorkomen. Voor patiënten met ernstig aangedane gewrichten kan operatieve behandeling een optie zijn om het dagelijks functioneren te verbeteren en pijn te verminderen. Gewrichtsdistractie is zo'n techniek die al

succesvol is toegepast bij artrose patiënten. Hoofdstuk 9 beschrijft drie hemofilie patiënten waarbij voor het eerst enkeldistractie is gedaan. Deze patiënten waren zeer tevreden met de klinische uitkomst van de procedure. Ze rapporteerden een toegenomen functie van de enkel, meer participatie in de samenleving en verminderde pijn. Daarnaast waren er 2 tot 4 jaar na behandeling duidelijke structurele veranderingen van het gewricht zichtbaar; de gewrichtsspleet was verwijd en het subchondrale bot was genormaliseerd. Dit onderzoek is een beschrijvende studie en slechts een eerste stap in de toepassing van enkeldistractie in hemofilie patiënten. Desalniettemin laat dit hoofdstuk zien dat gewrichtsdistractie een veelbelovende nieuwe behandeling is voor individuele hemofilie patiënten met beschadigde gewrichten.

In hoofdstuk 10 is het onderzoek van dit proefschrift samengevat en bediscussieerd. Het is duidelijk geworden dat het ook in het geval van een gewrichtsletsel of operatie zeer belangrijk is om bloed-geïnduceerde gewrichtsschade te voorkomen. Deze schade kan mogelijk voorspeld worden met behulp van biomarkers. Om schade te beperken moet als eerste de bloedblootstelling verminderd worden door bijvoorbeeld het gewricht te aspireren. Dit moet binnen 24 uur na bloedblootstelling worden gerealiseerd. Als aanvullende behandeling kunnen IL-4 en IL-10 de kraakbeenschade en de ontsteking van het synoviale weefsel verminderen. Mogelijk dat voor klinische toepassing het IL4-10 synerkine gebruikt kan gaan worden. Voor individuele patiënten met vergevorderde gewrichtsschade kan gewrichtsdistractie een heel goede en langdurige uitkomst bieden.

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LIST OF PUBLICATIONS

van Meegeren ME, Mastbergen SC, van Veghel K, Jansen NW, Lafeber FP, Roosendaal G. Blood-induced joint damage: the devastating effects of acute joint bleeds versus micro-bleeds. [submitted]

van Meegeren ME, Roosendaal G, Coeleveld K, Nieuwenhuizen L, Mastbergen SC, Lafeber FP. A single intra-articular injection with IL-4 plus IL-10 ameliorates blood-induced cartilage degeneration, but not synovitis, in haemophilic mice. [Br J Haematol, in revision]

van Meegeren ME, Roosendaal G, van Veghel K, Mastbergen SC, Lafeber FP. A short time window to profit from protection of blood-induced cartilage damage by IL-4 plus IL-10. [Rheumatology (Oxford), in revision]

van Meegeren ME*, van Vulpen LF*, Roosendaal G, Lafeber FP, Mastbergen SC. Joint distraction: a treatment to consider for haemophilic arthropathy. Haemophilia, 2012. [in press] *these authors contributed equally

Forsyth AL, Rivard GÉ, Valentino LA, Zourikian N, Hoffmann M, Monahan PE, **van Meegeren ME**, Forriol F. Consequences of intra-articular bleeding in haemophilia: science to clinical practice and beyond. Haemophilia. 2012 Jul;18 Suppl 4:112-9.

van Meegeren ME, Roosendaal G, Barten-van Rijbroek AD, Schutgens RE, Lafeber FP, Mastbergen SC. Coagulation aggravates blood-induced joint damage. Arthritis Rheum. 2012 Jun 5. [Epub ahead of print]

van Meegeren ME, van Veghel K, de Kleijn P, van Roermund PM, Biesma DH, Lafeber FP, Roosendaal G. Joint distraction results in clinical and structural improvement of haemophilic ankle arthropathy: a series of three cases. Haemophilia. 2012 Sep;18(5): 810-7.

van Meegeren ME, Roosendaal G, Jansen NW, Wenting MJ, van Wesel AC, van Roon JA, Lafeber FP. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. Osteoarthritis Cartilage. 2012 Jul;20(7):764-72.

van Meegeren M. Update on pathogenesis of the bleeding joint: an interplay between inflammatory and degenerative pathways. Haemophilia 2010 Jul;16 Suppl 5:121-3.

Shekalaghe S, Drakeley C, Gosling R, Ndaro A, **van Meegeren M**, Enevold A, Alifrangis M, Mosha F, Sauerwein R, Bousema T. Primaquine clears submicroscopic *Plasmodium falciparum* gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. PLoS ONE 2007; 2(10): e1023.

CURRICULUM VITAE

Monique van Meegeren was born on April 24th 1983 in Heerlen, the Netherlands. In July 2001 she graduated secondary school at the Bernardinuscollege in Heerlen.

In the same year she started the study Natural Sciences in Nijmegen. After one year she switched to Biomedical Sciences at the Radboud University Nijmegen, because this study focussed more on translational research. In December 2007 she obtained her Master of Science (MSc) degree.

For her bachelor's degree Monique did a research internship at the department of Pharmacology and Toxicology at the Nijmegen Centre of Molecular Sciences. Under supervision of prof.dr. FGM Russel and dr. FM van de Water, the effects of siRNA on renal Mrp2 and Mrp4 function and expression in rats were investigated.

A master research internship was performed at the department of Pathology at the Radboud University Nijmegen Medical Centre under supervision of dr. LCLT van Kempen and dr. WP Leenders. For this research project the expression regulation and function of NFAT5 were studied in human melanoma.

Monique did an additional research internship at the Kilimanjaro Christian Medical Centre (KCMC) in Moshi, Tanzania under supervision of dr. SA Shekalaghe and dr. JT Bousema. She investigated the effect of primaquine on Plasmodium falciparum gametocyte carriage in Tanzanian children.

In August 2008 she started as a PhD student at the departments of Rheumatology & Clinical Immunology and the Van Creveld Clinic at the University Medical Center Utrecht under direct supervision of prof.dr. FPJG Lafeber, dr. SC Mastbergen, and dr. G Roosendaal. In 2011 she received a poster price at the congress of the European Association for Haemophilia and Allied Disorders (EAHAD) in Switzerland and the Horoszowski Award for best presentation at the Musculoskeletal Congress (MSK) of the World Federation of Haemophilia in Dubai.