Blood-induced joint damage:

from mechanisms to clinical practice

Nathalie W.D. Jansen

The research described in this thesis was supported by an unrestricted grant from Baxter. Additionally, CLS Behring financially contributed to the study on biomarkers (chapter 8).

Printing of this thesis was enabled by financial support of Baxter

Cover: “Aandacht” a sculpture by Nicolette Dresselhuys

ISBN: 978-9-039-34791-1

Nathalie W.D. Jansen
University Medical Center Utrecht
Rheumatology & Clinical Immunology
Hematology and Van Creveld Clinic
F.02.127
PO Box 85500
3508 GA Utrecht
The Netherlands

Blood-induced joint damage:

from mechanisms to clinical practice

Bloed-geïnduceerde gewrichtsschade:

van mechanismen naar klinische praktijk

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, Prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 27 mei 2008 des ochtends te 10.30 uur

door

Nathalie Wilhelmina Dina Jansen

geboren op 10 januari 1980 te Apeldoorn

Promotoren:	Prof.dr. J.W.J. Bijlsma Prof.dr. M. Theobald
Co-promotoren:	Dr. F.P.J.G. Lafeber Dr. G. Roosendaal

CONTENTS

Chapter 1	Introduction	7
Section I: Mechanism		
Chapter 2	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.	25
Chapter 3	Degenerated cartilage is as vulnerable to blood-induced damage as healthy cartilage is.	45
Section II: Treatment		
Chapter 4	The non-ionic surfactant poloxamer P188 does not prevent blood-induced cartilage damage.	61
Chapter 5	Interleukin-10 protects against blood-induced joint damage.	69
Section III: Translation		
Chapter 6	Very rapid clearance after a joint bleed in the canine knee can not prevent adverse effects on cartilage and synovial tissue.	85
Chapter 7	Blood-induced cartilage damage *in vitro*: articular exposure *versus* total exposure.	101
Section IV: Clinical practice		
Chapter 8	The combination of uCTX-II, sCOMP, and sCS846 reflects cartilage damage in haemophilic arthropathy.	115
Chapter 9	Digital scoring of haemophilic arthropathy using radiographs is feasible.	131
Chapter 10	Summary and general discussion	147
	Nederlandse samenvatting	159
	Dankwoord	169
	Curriculum Vitae	177

Chapter 1

Introduction

Blood-induced joint damage evolves as a consequence of joint bleeds. These joint bleeds can occur incidentally as a result of injury or recurrently because of a coagulation defect. Of the latter, the coagulation disorder haemophilia is best known for its musculoskeletal problems due to recurrent joint bleeds, or haemarthroses.
In this thesis, studies on *blood-induced joint damage* are described, with the aim to gain more insight in various aspects of this joint damage and to gain knowledge of how these mechanisms can be translated to, and be of value for, clinical practice; *from mechanisms to clinical practice*. In this first chapter, a short general introduction to the synovial joint and blood-induced joint damage will be given, as well as some background on haemophilia, followed by the outline of this thesis.

THE SYNOVIAL JOINT

A synovial joint connects two bones and is enclosed by a capsule, composed of ligaments and on the inside synovial tissue (figure 1). This synovial tissue produces the synovial fluid that fills the synovial cavity and nourishes and lubricates articular cartilage that covers the bone ends. Together, these structures enable smooth movement of a joint and the ability to withstand the forces that are present during joint use.

Synovial tissue

Synovial tissue lines the inner surface of the joint capsule and covers all intra-articular structures, except the cartilage. Synovial tissue has three main functions: lubrication, nutrition, and cleaning of the joint. Lubrication and nutrition of the articular cartilage is realized by the production of synovial fluid. Synovial tissue does not only produce this fluid, but also regulates its volume and composition. Cleaning is done by removal of breakdown/turnover-products and debris from the joint cavity[1;2]. In order to be able to exert these functions, synovial tissue has a very specific composition.
The synovial tissue[2-4] consists of a lining layer, the synovial lining or intima, and the sublining or subintima. The synovial lining is composed of ~3 cell layers. Most of the cells in the synovial lining are macrophages (A cells) and specialized fibroblasts (B cells). The sublining of synovial tissue has both fatty and fibrous parts, dependent on the location in the joint; where the synovial tissue is attached to the joint capsule, the sublining is mostly fibrous.
Synovial tissue is highly vascularized. Blood vessels and capillaries are present in the sublining, and the density of capillaries is the highest directly below the lining. The sublining also contains fine lymphatic vessels.
Together, the structure of this tissue enables formation and maintenance of synovial fluid. The high vascularization and absence of the basal lamina in the synovial lining facilitate the formation of the basis of synovial fluid: an ultrafiltrate of plasma.

Furthermore, the capillaries enable transfer of small molecules, such as electrolytes, glucose, and amino acids, that are important for nutrition of the cartilage.
Both cell types of the synovial lining produce the main protein components of synovial fluid: hyaluronic acid (or hyaluronan), a glycosaminoglycan (see below) and lubricin, a glycoprotein. Uptake of an excess of synovial fluid or of breakdown/ turnover-products of cartilage (or blood in case of a haemarthros) from the joint cavity is also performed by the synovial tissue. The exact route by which this is accomplished is not fully elucidated yet, but probably involves an interplay between the cells in the lining (mostly the macrophage-like type A cells), the blood vessels/capillaries, and lymphatic vessels.
Despite the fact that the presence of numerous capillaries is of great importance for the functions of the synovial tissue, these capillaries are also the source of joint bleeds.

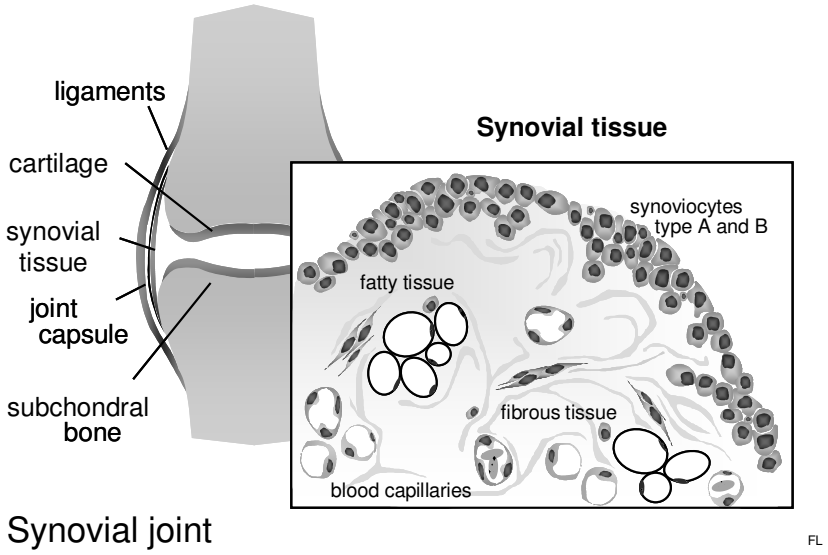

Figure 1: The synovial joint – Synovial tissue
Schematic representation of the synovial joint with a close-up of the synovial tissue. For details, see text.

Articular cartilage

Articular cartilage, which is the hyaline type of cartilage, covers the ends of bones and is linked to the subchondral bone by a layer of calcified cartilage. Articular cartilage, due to its unique composition, enables smooth movement of synovial joints, supported by the lubricating synovial fluid, and is important in resistance against compressive forces and shear stresses during joint use. Articular cartilage is avascular and aneural, depending mostly on the synovial fluid for its nutrition. There is only one cell type found in cartilage; the chondrocyte[5;6]. Chondrocytes are of mesenchymal origin and in adulthood, these cells hardly proliferate[5]. The function of chondrocytes is the production and maintenance of the extracellular matrix (ECM) cartilage is composed of (figure 2). This ECM consists of a fluid phase, with water and electrolytes, and of a solid phase, with collagens, proteoglycans, other proteins, and chondrocytes. Water forms the largest part of cartilage, being ~80% of the wet weight in adult cartilage. Collagens (~60%) and proteoglycans (~25-35%) are the major contributors to the dry weight of articular cartilage[7].

Collagen molecules are composed of three α-helixes, which twist around each other, creating a super-helix. These coiled-coils are fairly resistant to proteolytic cleavage except by specialized collagenases, like some matrix metalloproteinases (MMPs). The predominant form of collagen in cartilage is collagen type II[8-10]. Also collagen type IX and XI are found in the ECM of cartilage. Collagen type IX facilitates the interaction of collagens and proteoglycans. The collagen type II helixes are joined by interference of COMP (cartilage oligomeric matrix protein) to larger fibrils[11]. These fibrils form, in specific characteristic orientations, the collagen meshwork of articular cartilage.

In this collagen meshwork, proteoglycans are found. Proteoglycans are composed of a core protein to which glycosaminoglycans (GAGs) are attached. GAGs are unbranched polysaccharide chains of repeating disaccharide units. The GAGs found in cartilage are hyaluronan, chondroitin sulphate, keratan sulphate, and dermatan sulphate. Aggrecan is the predominant proteoglycan, composed of chondroitin sulphate and keratan sulphate. Aggrecan forms large aggregrates by attachement to hyaluronan via link protein[12]. Other proteoglycans in articular cartilage are decorin, biglycan and fibromodulin[13-15]. These leucine-rich proteoglycans are closely related in protein structure, but vary in their glycosaminoglycan composition and function. They are significantly smaller than aggrecan and are amongst others important in the interaction of proteoglycans and collagens. Several other smaller matrix molecules are found in cartilage including martrilins, fibronectin, and thrombospondins, of which COMP is a well known member.

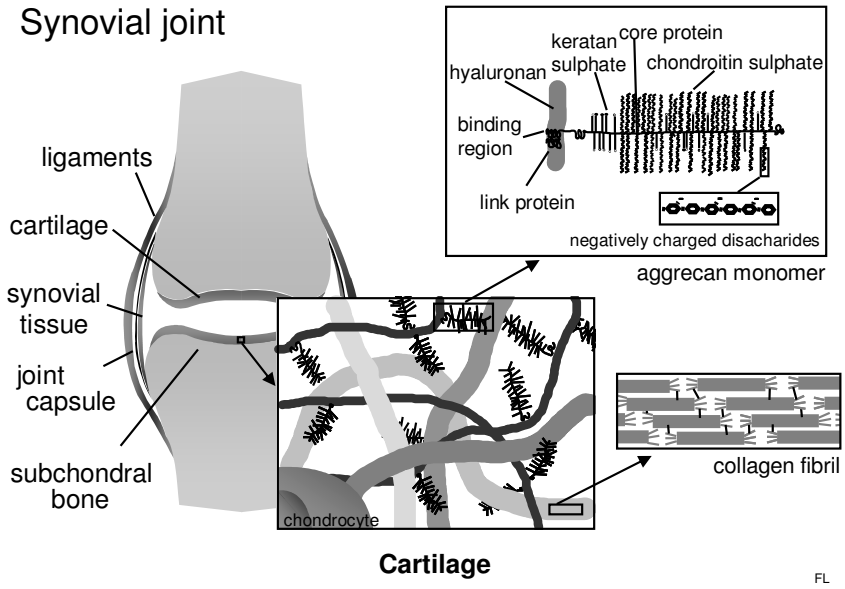

Figure 2: The synovial joint – Articular cartilage
Schematic representation of the synovial joint with a close-up of the articular cartilage and its components. For details, see text.

In general, the collagens in cartilage form a meshwork, giving the cartilage its tensile strength, in which the proteoglycans are entrapped. Since these proteoglycans are highly sulphated, they are negatively charged and thereby attract cations, creating osmotic pressure. Due to this osmotic pressure, water is attracted to the cartilage matrix, contributing to the resilience of cartilage. Cartilage works like a sponge: upon loading water is forced out of the tissue; after loading water is attracted again, but swelling is limited by the resistance of the collagen network with its tensile properties. This 'sponge-working' during loading and unloading of the tissue is of importance in nourishment of the tissue, in load distribution to protect subchondral bone against peak stress, and contributes to smooth movement under high load. Numerous reviews have been described on the composition and function of cartilage in health and disease providing more in-depth knowledge on this extraordinary tissue[7;16;17].

Subchondral bone

As mentioned above, the articular cartilage is connected to the subchondral bone via a calcified cartilage layer. The subchondral bone consist of the subchondral plate, composed of compact bone, with trabecular bone underneath. Trabecular bone is composed of rod- and plate-like elements and in this trabecular bone, blood vessels, neurons and bone marrow are present.

Bone is a very dense, specialized form of connective tissue. Its function is to move, support, and protect various organs of the body, to produce blood cells and to store minerals. The matrix of bone consists largely of a mixture of collagen type I fibers, arranged in fibrils, and calcium phosphate in hydroxyapatite crystals. The collagen fibrils enable tensile strength, and the crystals resist compression.

While cartilage has only one cell type, bone has osteoblasts, osteocytes, and osteoclasts. In adulthood, osteoblasts are present at the surface of the existing bone matrix and these cells deposit new bone matrix, which is called the osteoid, mainly consisting of collagen. Deposition of calcium phosphate crystals results in conversion into solid matrix. Some osteoblasts gradually become embedded in their own secretion[18], and thereby are unable to divide, but they keep producing small amounts of bone matrix, important for the maintenance of the matrix[19]. These embedded osteoblasts are called osteocytes. Bone is not a static organ, but undergoes continuous renewal. So besides formation of bone matrix, bone resorption also takes place. This is done by the osteoclast, a macrophage-like type of cell[20].

BLOOD-INDUCED JOINT DAMAGE

The fact that recurrent joint bleeds lead to severe arthropathy is evident from clinical practice in patients suffering from haemophilia and is frequently described[21-26]. However, joint bleeds can also occur as a result of joint trauma and during or after major joint surgery[27]. The process leading from these haemarthroses to arthropathy is multifactorial and involves changes in the synovial tissue, in cartilage, and also in the subchondral bone.

As mentioned above, synovial tissue has a function in removal of waste from the synovial cavity, and this includes blood as well. But by removal of blood, mostly in very large amounts, the synovial tissue gets triggered. This results in hypertrophy and hyperplasia of synoviocytes[28], and hypervascularization[29] of the subintima, which even increases the risk of subsequent bleeds. Synovitis, inflammation of the synovial tissue, develops[30-33], but ultimately the synovial tissue becomes fibrotic[21;29]. The breakdown product of haemoglobin, haemosiderin, accumulates in the synovial tissue, adding to the inflammatory triggering of the synovial tissue[34]. These haemosiderin depositions have been observed in cartilage also[21;35].

There is ample evidence for a role of iron (derived from red blood cells) in the synovial changes upon haemarthroses. Iron is shown to accumulate in synovial tissue[36-39], and to have a proliferative effect on synoviocytes[40;41]. Furthermore, the expression of the pro-oncogene c-myc[41] is increased by iron as well as the p53-binding protein mdm2[42], which both can be involved in the hypertrophy of the synovial tissue after joint bleeds by interference with cell proliferation and apoptosis. Haemosideritic synovial tissue has an increased production of the pro-inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNFα)[36], which have tissue-destructive properties. Altogether these processes lead to disturbance of synovial tissue and subsequent damage to the cartilage by excretion of tissue-destructive mediators such as enzymes and cytokines (figure 3). However, cartilage is not only indirectly affected by haemarthroses.

Blood also has a direct adverse effect on cartilage, independent of synovial interference, as shown in many studies[43-47]. The addition of blood to articular cartilage *in vitro* leads to disturbance of the cartilage matrix turnover. Exposure of 4 days, speculated to be the natural evacuation time of blood from a joint in humans, to 50% volume/volume (v/v) blood leads to a virtually complete inhibition of the proteoglycan synthesis rate and an increase in the release of proteoglycans from the cartilage matrix[45;46]. This inhibition in proteoglycan synthesis rate lasts for at least ten weeks after the initial exposure of 4 days to 50% v/v blood[44]. The combination of erythrocytes, or red blood cells (RBC) and mononuclear cells (MNC) appears to be responsible for this effect[46]. The production of the inflammatory cytokines IL-1 and TNFα contributes to the transient inhibition of proteoglycan synthesis, but is not responsible for the prolonged effects[45].

The prolonged effects of the exposure of cartilage to blood are due to apoptosis of the chondrocyte. This has been shown by the fact that blood exposure leads to an increase in ssDNA and TUNEL-staining of chondrocytes[43]. Furthermore, inhibition of caspases, the proteases that execute the process of apoptosis, reduces the inhibition in proteoglycan synthesis upon blood exposure. As mentioned, chondrocytes are the only cell type in cartilage, and thereby fully responsible for the production and maintenance of the cartilage matrix. Since in adulthood these cells hardly proliferate, loss of chondrocytes due to apoptosis therefore leads to long-lasting disturbance in the turnover of the cartilage matrix[48].

But what causes the apoptosis of the chondrocytes? A proposal for the process leading to apoptosis has originated from various *in vitro* studies. Since oxidative stress can result in apoptosis, it was studied whether oxidative stress evokes apoptosis of chondrocytes as a result of blood exposure. The presence of N-acetylcysteine, that stimulates the production of glutathione, (a scavenger of hydrogen peroxide (H_2O_2) during the exposure of cartilage to blood partly counteracts the inhibition in proteoglycan synthesis[45]. Iron, derived from the RBC present during blood exposure, reacts with H_2O_2 to form hydroxyl radicals[49-51]. When cartilage is co-

cultured with both lysed RBC as a source of catalytic iron, and IL-1β to stimulate H_2O_2 production, a lasting inhibition in proteoglycan synthesis occurs, which is not observed with either of the two components alone[47]. Thus, H_2O_2 that is produced by chondrocytes[52] and iron appear to be involved in the effects of exposure of cartilage to blood. Evidence for the suspected involvement of hydroxyl radicals originated from studies in which the presence of a scavenger of hydroxyl radicals, dimethylsulphoxide (DMSO), during blood exposure diminishes the inhibition in the proteoglycan synthesis[47].

From these results it was hypothesized (as schematically depicted in figure 3) that IL-1β, which is produced by activated monocytes/macrophages as present in the joint cavity during a haemarthros, increases the production of H_2O_2 by the chondrocytes. This H_2O_2 can react with iron from RBC that can result in the formation of hydroxyl radicals in the vicinity of the chondrocytes, inducing chondrocyte apoptosis.

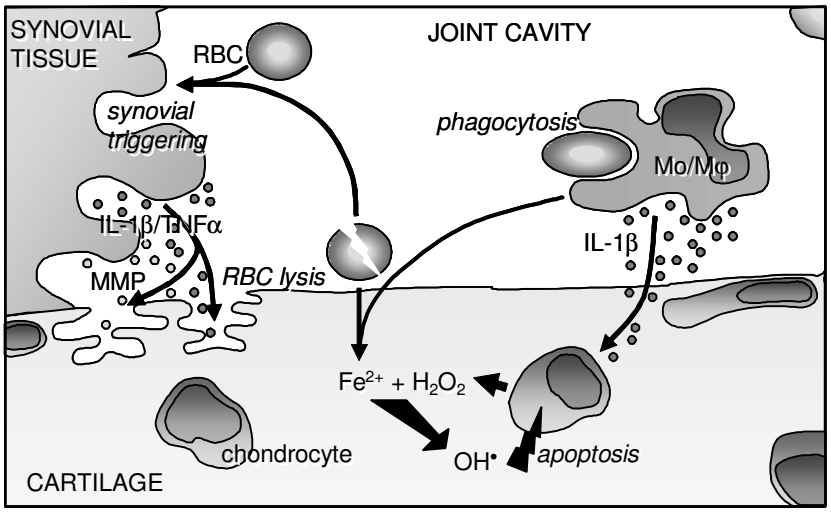

Figure 3: Proposed mechanism of blood-induced joint damage

When blood is present in de joint cavity it will be taken up by the synovial tissue leading to synovial inflammation and thereby production of inflammatory cytokines and MMPs, which have a destructive effect on the cartilage.

RBC are taken up by the macrophages, which upon this stimulation produce IL-1β. IL-1β activates the chondrocyte and thereby increases the production of hydrogen peroxide by these cells. The hydrogen peroxide can react with iron, released from the RBC, forming hydroxyl radicals, that induce apoptosis of the chondrocyte and thereby disturbance of cartilage matrix turnover.

Besides these *in vitro* studies on the direct effects of blood on cartilage, also from canine *in vivo* studies the effects of joint bleeds became evident[44;53;54]. Injection of blood in canine knees results in a direct decrease in proteoglycan synthesis rate and an increase in proteoglycan release. Also collagen damage is observed[54]. Also when

healthy canine cartilage is co-cultured with culture supernatants of synovial tissue from these blood-exposed joints, the cartilage matrix turnover is disturbed. The direct effects on cartilage precede these indirect effects via the synovial tissue. It appears that the younger the animals, i.e. the cartilage, the more susceptible to blood-induced damage[53;55], suggesting that for humans, specifically young children are at risk for joint damage after a joint haemorrhage.
Interestingly, the observed effects *in vivo* in the dog are not as aggressive as observed *in vitro*. However, when experimentally induced haemarthroses are combined with loading of the affected joint, degenerative changes become more outspoken[56], suggesting that restrictions in joint loading after a joint bleed is advisable. The mechanisms explaining the difference in degree of cartilage damage evoked by blood *in vitro* compared to *in vivo* in the dog, still have to be established. This thesis will address some of the issues involved.
Haemarthroses also have their impact on bone. Recurrent joint bleeds in children lead to enlargement of the epiphysis and growth disturbance. Other subchondral changes that are observed after frequent joint bleeding are osteoporosis, subchondral cyst formation and both erosions and osteophyte formation[57]. Whether such changes are a consequence of cartilage damage, coincide or even may precede cartilage damage is not clear yet.
Our knowledge thus far on blood-induced joint damage has been described in more detail in recent reviews on this subject[24;25;31;58].
Taken together, haemarthroses lead to inflammatory changes in the synovial tissue and to degenerative changes in the cartilage. And although the inflammatory process in the synovial tissue affects the cartilage and, *vice versa*, cartilage degradation can induce synovial (inflammatory) changes, these processes probably do not depend on each other and will occur in parallel.

HAEMOPHILIA

Haemophilia is a coagulation disorder caused by a deficiency or functional defect of either coagulation factor VIII in haemophilia A or IX in haemophilia B[59-62]. Haemophilia B is also called the Christmas disease after the first patient described with this disease, which was published in the December issue of the British Medical Journal[63]. Haemophilia A and B are clinically similar and can only be distinguished by assays of factor VIII and IX activity. The worldwide prevalence is estimated to be 1 in 10,000 males for haemophilia A and 1 in 25,000 for haemophilia B and the total number of affected individuals is estimated to be around 400,000. Haemophilia is an X-linked recessive disease, and therefore, in principle only males are affected.
Affected individuals develop a variable phenotype of haemorrhages into joints and muscles, easy bruising, and prolonged bleeding from wounds. The severity and

frequency of bleeding in haemophilia is inversely related to the amount of residual factor VIII or IX. Patients with less than 1% of clotting factor compared to normal values, suffer from the severe form of the disease and can suffer from spontaneous bleeds, which are mostly joint bleeds. Haemophilia is called moderate when the clotting factor activity is between 2 and 5% and patients with this moderate haemophilia often are less affected than the 'severe' patients. Patients with mild haemophilia have levels above 5% and only have problems after major trauma.
From the above, it is clear that those that suffer from the severe type of this disease, which is approximately half of the patients, have most bleeding problems. The majority of these bleeding episodes occur in the joints. These haemarthroses predominantly affect the knees, elbows and ankles[26], although bleeding in the shoulders and hips also occurs. Smaller joints exceptionally bleed. The cause of this distribution of bleeding is not known, though it is plausible that mechanical factors contribute to this characteristic of haemophilia. A known phenomenon is the development of a so-called target joint. A target joint is a joint that has been affected repeatedly by bleeds and as a result has become more susceptible to subsequent bleeds. Ultimately, orthopaedic procedures such as synovectomy, arthrodesis, and arthroplasty can be required at relatively young age[24;64].
There is no cure for haemophilia and treatment of haemophilia nowadays consists of intravenous substitution of the missing clotting factor. This form of treatment became available in the sixties, when isolating clotting factor from blood became possible[65]. This development increased the life expectancy of severe haemophilia patients. However, a problem of these blood products is the transmission of viruses such as the Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV). Significant numbers of patients have been infected in the eighties, leading to a delay of the increase in life expectancy gained by use of clotting products[66]. The development of production of recombinant factor VIII and IX in the nineties, together with viral inactivation steps in the production process of clotting factor from blood, decreased the risk of infection.
Another complication of the administration of clotting factors is the development of neutralizing antibodies (inhibitors) against the administered clotting factor. This inhibitor development challenges proper treatment. Although bypassing agents, amongst which activated prothrombin complex concentrates, activated recombinant factor VII, and porcine FVIII/IX are available, none of these and other available products are as effective as standard factor replacement.

Despite the availability of clotting factor, haemophilic arthropathy is still an issue of great concern due to several reasons. Generally, in the western world clotting factor is fairly good available, but this is not the case worldwide. It is estimated that approximately 75% of patients does not receive proper treatment. One of the reasons for this is the high cost of these products as recently discussed[67]. At the same time,

when available, development of inhibitory antibodies to the administered clotting factor hampers the prevention of joint bleeds. Furthermore, recently it was shown in a prospective study that in some cases joint damage is seen on MRI and radiographs, without clinical evident joint bleeds in the past, suggesting presence of subclinical haemarthroses leading to degenerative changes in the joint[68]. Clearly, based on this knowledge, joint destruction due to recurrent haemarthroses is and will remain the main cause of morbidity[69] in haemophilia and therefore blood-induced arthropathy will remain a problem for the coming decades.

OUTLINE OF THIS THESIS

The aim of this thesis is to gain more insight in various aspects of *blood-induced joint damage*, and to gain knowledge of how these mechanisms can be translated to clinical practice (*from mechanisms to clinical practice*). Several human *in vitro* and animal *in vivo* studies, as well as studies involving patients with haemophilia have been performed in light of this aim.

Section I: Mechanism

The first issue addressed in this thesis **(chapter 2)**, is the exposure time- and blood load dependency of blood-induced cartilage damage. In light of the discussion whether arthrocentesis (aspiration of fluid from the joint) after a joint bleed is to be considered and within what time span this is still worthwhile, the *in vitro* threshold that leads to long-lasting cartilage damage was determined.

Previous *in vitro* studies have always been performed with healthy cartilage. This mimics a first joint bleed, but in case of haemophilia, it is likely that multiple bleeding episodes occur in the same joint. Degenerated cartilage, either due to previous joint bleeds, trauma or other degenerative processes, e.g. as seen in osteoarthritis (OA), has a disturbed proteoglycan turnover[70], damaged collagen[71], and is associated with chondrocyte apoptosis[72-75]. Because of these features, degenerated cartilage is likely to have limited repair capacity[76;77] and is as such possibly more susceptible to blood-induced damage. Therefore, in **chapter 3** the susceptibility of degenerated cartilage to blood exposure was compared to that of healthy cartilage.

Section II: Treatment

With increasing knowledge of the processes involved during development of blood-induced joint damage, a search of possible treatment modalities to prevent joint damage after haemarthroses was initiated. From the poloxamer P188, also called pluronic, it was suggested that it could prevent chondrocyte death[78-80]. In **chapter 4**, a short survey was made whether this compound could be of use in prevention of blood-induced cartilage damage as well.

Blood-induced joint damage involves both synovial inflammation-driven cartilage destruction as well as intrinsic cartilage degenerative process. IL-10 is known for its regulatory activity in inflammation[81;82] and has previously been described to have favorable effects on cartilage matrix synthesis[83;84]. Therefore in **chapter 5**, IL-10 was evaluated *in vitro* and *ex vivo* for its potential protective effect on blood-induced joint damage.

Section III: Translation

For translation of *in vitro* findings to clinical practice in humans, results often first need to be confirmed in *in vivo* animals studies. Experimental joint bleeding in dogs is one of the animal *in vivo* models for blood-induced arthropathy[53-55;85-87]. However, while *in vitro* exposure of human and canine cartilage to blood results in severe long-lasting adverse changes in cartilage[44], an *in vivo* joint haemorrhage in the canine knee joint demonstrates similar adverse effects, but significantly less severe. This discrepancy needs to be elucidated, to be able to use the canine model for haemarthroses for translation to clinical practice. In **chapter 6** a high clearance rate was hypothesised as a possible explanation for this discrepancy and therefore this clearance rate was studied**.**

Besides the rate of clearance, another difference between *in vivo* and *in vitro* exposure of cartilage to blood was studied. Whereas in the *in vitro* explant culture system[88] the cartilage is exposed at all sides, in the *in vivo* situation cartilage is exposed to blood at the articular surface only. The objective of the study in **chapter 7** was to determine whether there is a difference in response of cartilage when submerged in blood compared to exposure at the articular surface only. Such a difference could add to the explanation of the observed discrepancy between the *in vitro* and *in vivo* results.

Section IV: Clinical practice

The knowledge of the previous studies added to translation of findings from *in vitro* studies to clinical practice. However, in clinical practice, in contrast to osteoarthritis and rheumatoid arthritis, haemophilic arthropathy has limited outcome parameters. Therefore, in **chapter 8** a first attempt was made to evaluate the use of serum and urine biomarkers of cartilage and bone turnover in evaluation of blood-induced arthropathy.

In **chapter 9** an improvement in evaluation of radiographic joint damage was subject of study. A digital image analysis program for knee radiographs (Knee Image Digital Analysis; KIDA) developed for knee osteoarthritis[89], was applied to varies degrees of haemophilic arthropathy of the knee and compared to the Pettersson scoring system[57], currently being the golden standard advised by the World Federation of Haemophilia (WFH).

Chapter 10 summarizes and integrates the results of all chapters and places them in a broader perspective. Additionally this chapter provides leads for the future in both research and clinical practice.

REFERENCES

1. Brown TJ, Laurent UB, Fraser JR. Turnover of hyaluronan in synovial joints: elimination of labelled hyaluronan from the knee joint of the rabbit. Exp.Physiol 1991;76:125-134.
2. Garnero P. Noninvasive biochemical markers in osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg VM, eds. Osteoarthritis.: Wolters Kluwer Lippincott Williams & Wilkins; 2008:215-232.
3. Tarner IH, Harle P, Muller-Ladner U, Gay RE, Gay S. The different stages of synovitis: acute vs chronic, early vs late and non-erosive vs erosive. Best.Pract.Res.Clin.Rheumatol. 2005;19:19-35.
4. Edwards JC. The nature and origins of synovium: experimental approaches to the study of synoviocyte differentiation. J.Anat. 1994;184 (Pt 3):493-501.
5. Muir H. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 1995;17:1039-1048.
6. Archer CW, Francis-West P. The chondrocyte. Int.J.Biochem.Cell Biol. 2003;35:401-404.
7. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 1998;47:477-486.
8. Cremer MA, Rosloniec EF, Kang AH. The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. J.Mol.Med. 1998;76:275-288.
9. Eyre DR, Weis MA, Wu JJ. Articular cartilage collagen: an irreplaceable framework? Eur.Cell Mater. 2006;12:57-63.
10. Eyre D. Collagen of articular cartilage. Arthritis Res. 2002;4:30-35.
11. Halasz K, Kassner A, Morgelin M, Heinegard D. COMP acts as a catalyst in collagen fibrillogenesis. J.Biol.Chem. 2007;282:31166-31173.
12. Roughley PJ, Poole AR, Mort JS. The heterogeneity of link proteins isolated from human articular cartilage proteoglycan aggregates. J.Biol.Chem. 1982;257:11908-11914.
13. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J. 1992;6:861-870.
14. Knudson CB, Knudson W. Cartilage proteoglycans. Semin.Cell Dev.Biol. 2001;12:69-78.
15. Roughley PJ, Lee ER. Cartilage proteoglycans: structure and potential functions. Microsc.Res.Tech. 1994;28:385-397.
16. Garnero P, Rousseau JC, Delmas PD. Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. Arthritis Rheum. 2000;43:953-968.
17. Sandell L, Heinegard D, Hering T. Cell biology, biochemistry, and molecular biology of articular cartilage in osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter J, Goldberg VM, eds. Osteoarthritis.: Wolters Kluwer Lippinschot Williams & Wilkins; 2008:73-106.
18. Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. Dev.Dyn. 2006;235:176-190.
19. Bonewald L. Osteocytes as multifunctional cells. J.Musculoskelet.Neuronal.Interact. 2006;6:331-333.
20. Miyamoto T, Suda T. Differentiation and function of osteoclasts. Keio J.Med. 2003;52:1-7.
21. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
22. Rodriguez-Merchan EC. Effects of hemophilia on articulations of children and adults. Clin.Orthop. 19967-13.
23. Gilbert MS. Musculoskeletal complications of haemophilia: the joint. Haemophilia. 2000;6 Suppl 1:34-37.
24. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
25. Hoots WK. Pathogenesis of hemophilic arthropathy. Semin.Hematol. 2006;43:S18-S22.
26. Arnold WD, Hilgartner MW. Hemophilic arthropathy. Current concepts of pathogenesis and management. J.Bone Joint Surg.Am. 1977;59:287-305.
27. 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;33:1406-1415.

28. Roy S, Ghadially FN. Synovial membrane in experimentally-produced chronic haemarthrosis. Ann.Rheum.Dis. 1969;28:402-414.
29. Madhok R, York J, Sturrock RD. Haemophilic arthritis. Ann.Rheum.Dis. 1991;50:588-591.
30. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. Clin.Orthop.Relat Res. 19976-11.
31. Valentino LA, Hakobyan N, Rodriguez N, Hoots WK. Pathogenesis of haemophilic synovitis: experimental studies on blood-induced joint damage. Haemophilia. 2007;13 Suppl 3:10-13.
32. Rodriguez-Merchan EC. Haemophilic synovitis: basic concepts. Haemophilia. 2007;13 Suppl 3:1-3.
33. Mainardi CL, Levine PH, Werb Z, Harris ED, Jr. Proliferative synovitis in hemophilia: biochemical and morphologic observations. Arthritis Rheum. 1978;21:137-144.
34. Dabbagh AJ, Trenam CW, Morris CJ, Blake DR. Iron in joint inflammation. Ann.Rheum.Dis. 1993;52:67-73.
35. Hough AJ, Banfield WG, Sokoloff L. Cartilage in hemophilic arthropathy. Ultrastructural and microanalytical studies. Arch.Pathol.Lab Med. 1976;100:91-96.
36. 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;80:540-545.
37. Roosendaal G, Mauser-Bunschoten EP, de Kleijn P et al. Synovium in haemophilic arthropathy. Haemophilia. 1998;4:502-505.
38. Morris CJ, Blake DR, Wainwright AC, Steven MM. Relationship between iron deposits and tissue damage in the synovium: an ultrastructural study. Ann.Rheum.Dis. 1986;45:21-26.
39. Morris CJ, Wainwright AC, Steven MM, Blake DR. The nature of iron deposits in haemophilic synovitis. An immunohistochemical, ultrastructural and X-ray microanalytical study. Virchows Arch.A Pathol.Anat.Histopathol. 1984;404:75-85.
40. Nishiya K. Stimulation of human synovial cell DNA synthesis by iron. J.Rheumatol. 1994;21:1802-1807.
41. Wen FQ, Jabbar AA, Chen YX et al. c-myc proto-oncogene expression in hemophilic synovitis: in vitro studies of the effects of iron and ceramide. Blood 2002;100:912-916.
42. Hakobyan N, Kazarian T, Jabbar AA, Jabbar KJ, Valentino LA. Pathobiology of hemophilic synovitis I: overexpression of mdm2 oncogene. Blood 2004;104:2060-2064.
43. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
44. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
45. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
46. 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;24:1350-1354.
47. 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;42:784-790.
48. Burkhardt H, Schwingel M, Menninger H, Macartney HW, Tschesche H. Oxygen radicals as effectors of cartilage destruction. Direct degradative effect on matrix components and indirect action via activation of latent collagenase from polymorphonuclear leukocytes. Arthritis Rheum. 1986;29:379-387.
49. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicol.Lett. 1995;82-83:969-974.
50. Morris CJ, Earl JR, Trenam CW, Blake DR. Reactive oxygen species and iron--a dangerous partnership in inflammation. Int.J.Biochem.Cell Biol. 1995;27:109-122.
51. Henle ES, Linn S. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J.Biol.Chem. 1997;272:19095-19098.
52. Tiku ML, Liesch JB, Robertson FM. Production of hydrogen peroxide by rabbit articular chondrocytes. Enhancement by cytokines. J.Immunol. 1990;145:690-696.
53. Roosendaal G, Tekoppele JM, Vianen ME et al. Articular cartilage is more susceptible to blood induced damage at young than at old age. J.Rheumatol. 2000;27:1740-1744.

54. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.
55. Hooiveld MJ, Roosendaal G, Vianen ME et al. 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.
56. Hooiveld MJ, Roosendaal G, Jacobs KM 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;50:2024-2031.
57. Pettersson H, Ahlberg A, Nilsson IM. A radiologic classification of hemophilic arthropathy. Clin.Orthop.Relat Res. 1980153-159.
58. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12 Suppl 3:117-121.
59. Green PM, Naylor JA, Giannelli F. The hemophilias. Adv.Genet. 1995;32:99-139.
60. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. Lancet 2003;361:1801-1809.
61. Ingram GI. The history of haemophilia. J.Clin.Pathol. 1976;29:469-479.
62. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. N.Engl.J.Med. 2001;344:1773-1779.
63. Biggs R, Douglas AS, Macfarlane RG et al. Christmas disease: a condition previously mistaken for haemophilia. Br.Med.J. 1952;2:1378-1382.
64. Rodriguez-Merchan EC. Common orthopaedic problems in haemophilia. Haemophilia. 1999;5 Suppl 1:53-60.
65. Pool JG, Gershgold EJ, Pappenhagen AR. High-potency antihaemophilic factor concentrate prepared from cryoglibulin precipitate. Nature 1964;203:312.
66. Plug I, van der Bom JG, Peters M et al. Mortality and causes of death in patients with hemophilia, 1992-2001: a prospective cohort study. J.Thromb.Haemost. 2006;4:510-516.
67. Roosendaal G, Lafeber F. Prophylactic treatment for prevention of joint disease in hemophilia--cost versus benefit. N.Engl.J.Med. 2007;357:603-605.
68. Manco-Johnson MJ, Abshire TC, Shapiro AD et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N.Engl.J.Med. 2007;357:535-544.
69. Raffini L, Manno C. Modern management of haemophilic arthropathy. Br.J.Haematol. 2007;136:777-787.
70. van Valburg AA, Wenting MJ, Beekman B et al. Degenerated human articular cartilage at autopsy represents preclinical osteoarthritic cartilage: comparison with clinically defined osteoarthritic cartilage. J.Rheumatol. 1997;24:358-364.
71. Poole AR, Kobayashi M, Yasuda T et al. Type II collagen degradation and its regulation in articular cartilage in osteoarthritis. Ann.Rheum.Dis. 2002;61 Suppl 2:ii78-ii81.
72. Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasheri A. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet.J. 2003;166:140-158.
73. Horton WE, Bennion P, Yang L. Cellular, molecular, and matrix changes in cartilage during aging and osteoarthritis. J.Musculoskelet.Neuronal.Interact. 2006;6:379-381.
74. Lorenz H, Richter W. Osteoarthritis: cellular and molecular changes in degenerating cartilage. Prog.Histochem.Cytochem. 2006;40:135-163.
75. Aigner T, Kim HA, Roach HI. Apoptosis in osteoarthritis. Rheum.Dis.Clin.North Am. 2004;30:639-53, xi.
76. Thomas CM, Fuller CJ, Whittles CE, Sharif M. Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis.Cartilage. 2007;15:27-34.
77. Martin JA, Buckwalter JA. The role of chondrocyte senescence in the pathogenesis of osteoarthritis and in limiting cartilage repair. J.Bone Joint Surg.Am. 2003;85-A Suppl 2:106-110.
78. Phillips DM, Haut RC. The use of a non-ionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants. J.Orthop.Res. 2004;22:1135-1142.
79. Rundell SA, Baars DC, Phillips DM, Haut RC. The limitation of acute necrosis in retro-patellar cartilage after a severe blunt impact to the in vivo rabbit patello-femoral joint. J.Orthop.Res. 2005;23:1363-1369.

80. Baars DC, Rundell SA, Haut RC. Treatment with the non-ionic surfactant poloxamer P188 reduces DNA fragmentation in cells from bovine chondral explants exposed to injurious unconfined compression. Biomech.Model.Mechanobiol. 2006;5:133-139.
81. Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu.Rev.Immunol. 2001;19:683-765.
82. Pestka S, Krause CD, Sarkar D et al. Interleukin-10 and related cytokines and receptors. Annu.Rev.Immunol. 2004;22:929-979.
83. 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;39:829-835.
84. Wang Y, Lou S. Direct protective effect of interleukin-10 on articular chondrocytes in vitro. Chin Med.J.(Engl.) 2001;114:723-725.
85. Convery FR, Woo SL, Akeson WH, Amiel D, Malcom LL. Experimental hemarthrosis in the knee of the mature canine. Arthritis Rheum. 1976;19:59-67.
86. Madhok R, Bennett D, Sturrock RD, Forbes CD. Mechanisms of joint damage in an experimental model of hemophilic arthritis. Arthritis Rheum. 1988;31:1148-1155.
87. Tessier JE, Salimi Z, Restrepo GL, Samuels LD, Joist JH. Experimental hemarthrosis in dogs: progressive synovitis monitored with technetium-99m pyrophosphate joint imaging. Haemostasis 1988;18:83-90.
88. 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;29:287-299.
89. Marijnissen AC, Vincken KL, Vos PA et al. Knee Images Digital Analysis (KIDA): a novel method to quantify individual radiographic features of knee osteoarthritis in detail. Osteoarthritis.Cartilage. 2008;16:234-243.

Chapter 2

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 & Rheumatism, 2007 Jan;56(1):199-207

N.W.D. Jansen[1,2]
G. Roosendaal[2]
J.W.J. Bijlsma[1]
J. DeGroot[3]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Business Unit BioSciences, Inflammatory & Degenerative Diseases, TNO Quality of Life, Leiden, The Netherlands

ABSTRACT

Background

Joint bleeding, or haemarthroses, leads in time to severe joint damage. This study was carried out to test the *in vitro* thresholds of exposure time and concentration that lead to irreversible joint damage, to add to the discussion on the usefulness of aspiration of the joints after a haemorrhage.

Methods

Explants of healthy human articular cartilage tissue were cultured in the presence or absence of 50% volume/volume (v/v) blood for 1, 2, 3 or 4 days or in the presence of 0, 5, 10, 20, 30 or 50% v/v blood for four days, followed by a 12-day period of recovery after withdrawal of blood. The effect of blood exposure on cartilage was determined by measuring the rate of proteoglycan synthesis as well as the release and content of cartilage matrix proteoglycans and the activity of matrix metalloproteinases.

Results

Exposure of cartilage to 50% v/v blood led to adverse changes that were largely independent of the exposure time. The adverse effects persisted after an initial exposure of up to or exceeding 2 days. Exposure of cartilage for 4 days led to concentration-dependent adverse changes. These effects persisted when the concentration equalled or exceeded 10% v/v blood. Moreover, after 2 days of exposure to a blood load of 10% v/v, the adverse effects on cartilage were not reversible.

Conclusion

A 2-day exposure of cartilage *in vitro* to 10% v/v blood leads to prolonged impairment of joint cartilage. This suggests that aspiration of blood from the joint within 2 days after haemarthrosis should be considered to prevent blood-induced joint damage in the long term.

INTRODUCTION

Joint bleeding, or haemarthrosis, can occur after a joint trauma, such as ligament rupture or intra-articular fracture. Intra-articular bleeding also frequently occurs in the larger joints of patients with haemophilia. Moreover, in such patients, it has clearly been demonstrated that this frequent joint bleeding results in severe destruction of the joint later in life[1;2]. However, limited joint bleeding or even a single incident has also been demonstrated to lead to irreversible joint damage[3-5]. Blood-induced arthropathy has characteristics of both degenerative joint disease (such as osteoarthritis) and inflammation-induced joint damage (as in rheumatoid arthritis)[6]. Similar to osteoarthritis and rheumatoid arthritis, blood-induced arthropathy is initiated many years before clinical manifestations become evident[7;8]. Although it is a long-lasting process, it is reasonable to expect, based on these previous observations[3-5], that a minimal amount of joint bleeding is sufficient to induce severe joint damage in later years.

With regard to the mechanisms of blood-induced joint damage, our group has demonstrated that the monocytes/macrophages within the mononuclear cell population together with the red blood cells as present in blood are responsible for the irreversible inhibition of matrix synthesis. Small amounts of interleukin-1 (IL-1), which is produced by activated monocytes/macrophages will increase the production of hydrogen peroxide by chondrocytes. The hydrogen peroxide reacts with haemoglobin-derived iron from damaged and phagocytosed red blood cells, which results in the formation of hydroxyl radicals in the vicinity of chondrocytes. This leads to chondrocyte apoptosis and, as a consequence, to irreversible inhibition of cartilage matrix synthesis[3;4;9;10].

We have previously demonstrated that a 4-day exposure of cartilage to 50% volume/volume (v/v) blood results in long-lasting inhibition of cartilage matrix proteoglycan synthesis and a prolonged decrease in proteoglycan content[3;11]. In addition to type II collagen, proteoglycans are one of the main cartilage matrix components, both qualitatively and quantitatively[12]. A 4-day period of exposure is assumed to be the natural time span of evacuation of blood from a joint[13;14], and at least 50% v/v blood in a synovial joint is the blood load expected to present shortly after bleeding takes place[4]. These *in vitro* findings were confirmed by *in vivo* animal studies[11;15;16]. Although the effects revealed in the *in vivo* experiments in animals were less dramatic than has been observed in human joints, they clearly resulted in compromised joint cartilage, which is predictive of cartilage degenerative changes in the long term[11;15;16].

Although intra-articular bleeding is clearly harmful to joint cartilage, there is currently very little consensus regarding whether aspiration of blood from the joint after haemarthrosis is indicated. Studies by Ingram *et al.*[17;18] and Holdsworth *et al.*[19] showed an immediate relief of pain and increase in range of motion upon aspiration

of the joint after bleeding. These effects were not long lasting, since after several weeks, there were no significant differences between the patients with and those without aspirated joints. It is likely that the initial differences were related to capsular pressure. Moreover, in the nonaspirated group, blood is finally evacuated from the joint via natural processes, although prior to that time, the cartilage will begin to show signs of damage; however, these destructive processes will only become clinically evident after several years.

In general clinical practice, it may be difficult to aspirate a joint immediately after the haemorrhage has occurred, because the patient may not immediately seek medical care. Moreover, physicians are reluctant to perform joint aspiration because of the potential for infection and, in case of haemophilia, because of additional sites of bleeding. Since there is circumstantial evidence to support the view that aspiration of a joint is indicated to prevent joint damage later in life[3-6;9-11;15;16], the present study was initiated to determine the thresholds of blood exposure time and blood load that lead to long-lasting cartilage damage. A possible difference between coagulating and non-coagulating blood was taken into account, because it is not known whether blood coagulates in a joint, and if so, to what extent. The results obtained by this *in vitro* study might be of use in the discussion of whether aspiration of blood from the joint after a haemorrhage is indicated and whether it would be feasible in clinical practice.

MATERIALS AND METHODS

Cartilage culture

Healthy full thickness human articular cartilage tissue was obtained *post mortem*, within 24 hours after death, from the humeral heads of donors who had no known history of joint disorders (mean ± SEM age at time of death 62.4 ± 2.7 years; n=25). In previous studies it has clearly been demonstrated that this macroscopically intact cartilage is a reliable source of histologically and biochemically normal, healthy cartilage[6;20]. Collection of the cartilage was carried out in accordance with the medical ethical regulations of the University Medical Center in Utrecht.

Slices of cartilage, excluding the underlying bone, were cut aseptically to the maximum possible thickness and kept in phosphate buffered saline (PBS; pH 7.4). Within 1 hour after dissection, the slices were cut into square pieces, weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottom microtiter plates in 200 µl per well. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 µg/ml), ascorbic acid (85 µM) and 10% heat-inactivated pooled human male AB^+ serum. Cultures were performed in a tissue incubator in an environment of 5% CO_2 in air, at 37°C.

For each experiment, fresh blood from healthy human donors was collected into vacutainer tubes (Becton Dickinson, UK) and immediately added to the cartilage tissue explants in culture medium. In experiments in which coagulating blood was used, the blood was added before coagulation started. Because the total volume was kept at 200 µl, part of the culture medium was replaced with whole blood. Previous experiments have shown that dilution of nutrients in the culture medium or the increase in serum components that results upon adding whole blood up to a maximum of 50% v/v have no influence proteoglycan turnover[3]. Cartilage chondrocytes are not in direct cell-cell contact with the blood cells, and the use of homologous blood instead of autologous blood is not of influence (data not shown).

After the cartilage tissue was exposed to blood, all of the adherent blood components were removed by two wash steps, each for 45 minutes under culture conditions. Subsequently, a group of the samples was analysed. The remaining samples were cultured for an additional period of 12 days in the absence of blood. In these cultures, medium was refreshed every four days. These prolonged cultures in the absence of blood gave the chondrocytes the ability to recover from the blood exposure, enabling the evaluation of the reversibility or irreversibility of the observed effects of blood exposure. After this recovery period, these cartilage samples were analysed as well.

Analyses of the cartilage samples directly after exposure and after 12 days of recovery included determination of proteoglycan synthesis rate and proteoglycan content as well as DNA content. In addition, culture medium was analysed for proteoglycan release and general matrix metalloproteinase (MMP) activity.

Experimental design

To study the effects of exposure time, cartilage samples were cultured in the presence or absence of 50% v/v blood (collected in vacutainers containing 170 IU Li-heparin/10 ml) for 1, 2, 3 or 4 days. To study the effects of blood concentration, cartilage samples were cultured for 4 days in the presence of 0, 5, 10, 20, 30 or 50% v/v blood (collected in vacutainers containing 170 IU Li-heparin/10 ml). To study the effects of a limited exposure time combined with low concentrations, cartilage was exposed for 2 days to 10% v/v blood. Both the direct effects as well as the effect after a recovery period were determined.

To study the effects of coagulating as compared with noncoagulating blood, cartilage samples were cultured in the presence or absence of 50% v/v blood for 2 and 4 days or for 4 days in the presence of 0, 10 or 50% v/v non-coagulating blood (collected in vacutainers containing 170 IU Li-heparin/10 ml) or the same concentrations of coagulating blood (collected in vacutainers without heparin). For these assays, only the effects after the recovery period were determined. The presence of heparin itself up to the concentrations in the final cultures does not influence the effects of blood on cartilage[3].

Analysis

The rate of proteoglycan synthesis in the joint cartilage was determined by measuring the rate of sulphate incorporation. $Na_2{}^{35}SO_4$ (DuPont, NEX-041-H, carrier free) was added in 10 µl aliquots of 74 kBq per well, and after 4 hours pulse labelling of the sulphated glycosaminoglycans (GAGs), the cartilage samples were washed twice with ice cold PBS and stored at -20°C. Thawed samples were digested with papain (Sigma (P-3125); 26.4 mg/ml in 50 mM phosphate buffer, pH 6.5, containing 2 mM N-acetylcysteine and 2 mM Na_2-EDTA) for 2 hours at 65°C. Papain digests were diluted to the appropriate concentrations for analysis of the proteoglycan synthesis rate and -content as well as the DNA content.

To determine the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (CPC, Sigma; C9002-100G). The precipitate was dissolved in 3M NaCl and the amount of radioactivity in the sample was measured, upon addition of Picofluor-40 (Packard), by liquid scintillation analysis. The rate of sulphate incorporation was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage, with results expressed as nmoles of sulphate per hour per gram wet weight of the cartilage tissue (nmol/h*g).

The proteoglycan content of the cartilage tissue digest was determined as the amount of GAGs in the cartilage tissue papain digests. GAGs were stained and precipitated with Alcian Blue[21-23] (Sigma A 5268). Staining for GAGs was measured as the change in absorbance at 620 nm, and chondroitin sulphate (Sigma (C4383),

The Netherlands) was used as a reference. Results are expressed as mg GAG per gram wet weight of the cartilage explants (mg/g).
The DNA content of the cartilage tissue digests, as a measure of cellularity, was determined in the cartilage samples using the fluorescent dye Hoechst 33258 (Calbiochem 382061). Calf thymus DNA (Sigma D-4764) was used as a reference. Results are expressed as mg DNA per gram wet weight of cartilage (mg/g). Regardless of the experimental conditions, there was no significant change in DNA content (cellularity) of the cartilage due to blood exposure.
Proteoglycan release was determined as the loss of GAGs in the culture medium. GAGs were stained and precipitated with Alcian Blue as described above. Results are expressed as mg GAGs released per gram wet weight of the cartilage (mg/g).
General MMP activity was determined in the culture supernatants using the internally quenched fluorogenic peptide substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) in the presence or absence of 12.5 mM BB94 (a general MMP inhibitor)[24-26]. The MMP activity in each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence over time, expressed as relative fluorescence units per second; RFU/s) between samples with and those without BB94, normalized against the wet weight of the cartilage (RFU/s per gram cartilage). The substrate was kindly provided by TNO Quality of Life (Leiden, The Netherlands).

Calculations and statistical analysis
Because of focal differences in the composition and bioactivity of the cartilage on the humeral heads, the results for 10 cartilage samples per parameter per donor, obtained randomly and each assessed seperately, were expressed as the mean ± SEM and taken as representative of the cartilage of that donor. The n values (ranging 4 - 6) indicate the number of experiments (namely the number of cartilage donors per experiment). The data were analysed using the nonparametric Wilcoxon signed rank test, two sided) for related samples, using SPSS software 11.5. P-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Prolonged blood-induced cartilage damage according to exposure time

Proteoglycan synthesis (figure 1) was completely inhibited when measured directly after exposure to 50% v/v blood. Even a 1-day exposure was able to induce these rigorous effects, with similar effects after 4 days of exposure. Cartilage under control conditions showed a slight increase in the rate of proteoglycan synthesis immediately in the first days of culture (not statistically significant), which could be attributed to the culture conditions[27].

The inhibition of proteoglycan synthesis induced in cartilage by blood exposure appeared to be reversible, depending on the initial duration of exposure. After an initial 1-day exposure of cartilage to blood followed by recovery from the exposure (i.e., after withdrawal of blood), almost complete reversibility of the inhibitory effects was observed (figure 1a), although the proteoglycan synthesis during recovery was still statistically significantly lower (-17%) than that in cartilage not exposed to blood ($p<0.05$). After an initial 2 day exposure followed by recovery, a 65% inhibition of proteoglycan synthesis ($p<0.05$) was found. The initial inhibition of proteoglycan synthesis upon 3 days or 4 days of blood exposure did not exhibit a significant recovery from exposure during the 12-day period in the absence of blood.

The release of proteoglycans was increased directly after exposure of cartilage to blood, independent of the duration of the exposure (figure 1b). Even a 1-day exposure resulted in a doubling of proteoglycan release. Surprisingly, after the 12-day recovery period, proteoglycan release was not different from that observed in nonexposed control cultures.

The activity of the cartilage matrix-degrading MMPs increased significantly upon exposure to blood (figure 1c). A clear dependency on exposure time was observed, since MMP activity increased with longer exposure time. After recovery, this effect was still present, although the differences were less striking. Even after 12 days of recovery, the initial 1-day blood exposure resulted in an almost doubling of MMP activity ($p<0.05$).

In addition, an exposure time-dependent decrease in proteoglycan content was observed (figure 1d). This decrease continued during the recovery period and ultimately resulted in a statistically significant impairment of matrix integrity (diminished proteoglycan content) after the cartilage had been initially exposed for 2 days or 3 days to 50% v/v blood.

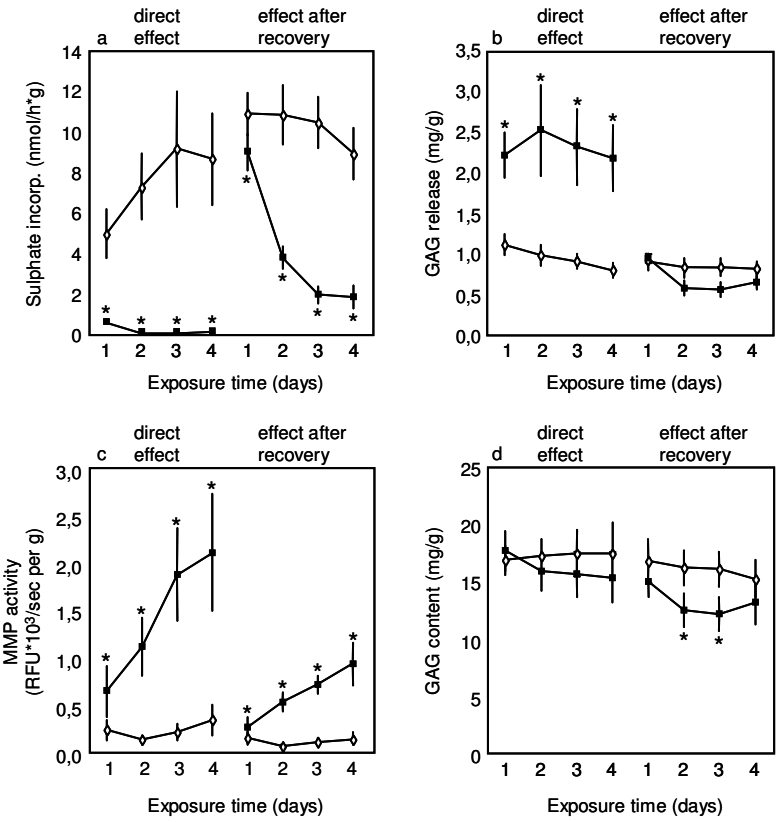

Figure 1: Exposure time-dependent effect of blood on cartilage
Proteoglycan synthesis rate (**a**), - release (**b**), and - content (**d**) and general MMP activity (**c**). Human healthy cartilage was exposed *in vitro* to 50% v/v blood for 1, 2, 3 or 4 days and either analyzed directly (left part of each panel) or after an additional 12 days recovery period in the absence of blood (right part of each panel). Open diamonds represent values of cartilage cultured in the absence of blood (controls); filled squares represent values of cartilage exposed to blood. Asterisks indicate differences of controls *versus* blood-exposed-samples (p-value of <0.05; n=6).

Prolonged blood-induced cartilage damage according to blood load

Exposure of cartilage for 4 days to 5% v/v blood led to a direct, almost complete inhibition of proteoglycan synthesis (figure 2a). Higher concentrations of blood also yielded inhibitory effects similar to those after exposure to the 5% concentration. This decrease in proteoglycan synthesis was sustained after a recovery period, although the effects of 5 and 10% v/v blood were less dramatic with 38% and 71% inhibition of proteoglycan synthesis, respectively (both $p<0.05$ compared with that in nonexposed cartilage). Exposure to higher concentrations of blood effectively limited the recovery of cartilage matrix synthesis; in fact, the proteoglycan synthesis was almost completely inhibited after 12 days of recovery (figure 2a; dashed line).

The release of proteoglycans (figure 2b) increased directly after the blood exposure. Surprisingly, this increase showed an inverse relationship with the blood load; GAG release after exposure to 5% v/v blood differed statistically significantly from the release after exposure to 50% v/v blood ($p<0.05$). The increase in the release of proteoglycans appeared to be completely reversible following recovery from exposure, even after an initial exposure to the highest (50% v/v) concentration of blood.

The MMP activity in the cartilage (figure 2c) showed a blood concentration-dependent increase directly after exposure. In addition, there was a concentration-dependent elevation of MMP activity after the recovery period, although the absolute values were lower compared with those measured directly after the exposure. Nevertheless, after exposure to cartilage to each incremental increase in concentration of blood, the MMP activity remained statistically significantly higher ($p<0.05$) than that in control cartilage cultures.

The proteoglycan content of cartilage after the initial exposure to 5% v/v blood was decreased 30%, compared with that in control cartilage, after recovery from the exposure (figure 2d). Higher blood concentrations only slightly diminished proteoglycan content further; at 50% v/v blood, there was a 34% decrease in proteoglycan content after recovery, compared with that in nonexposed cartilage.

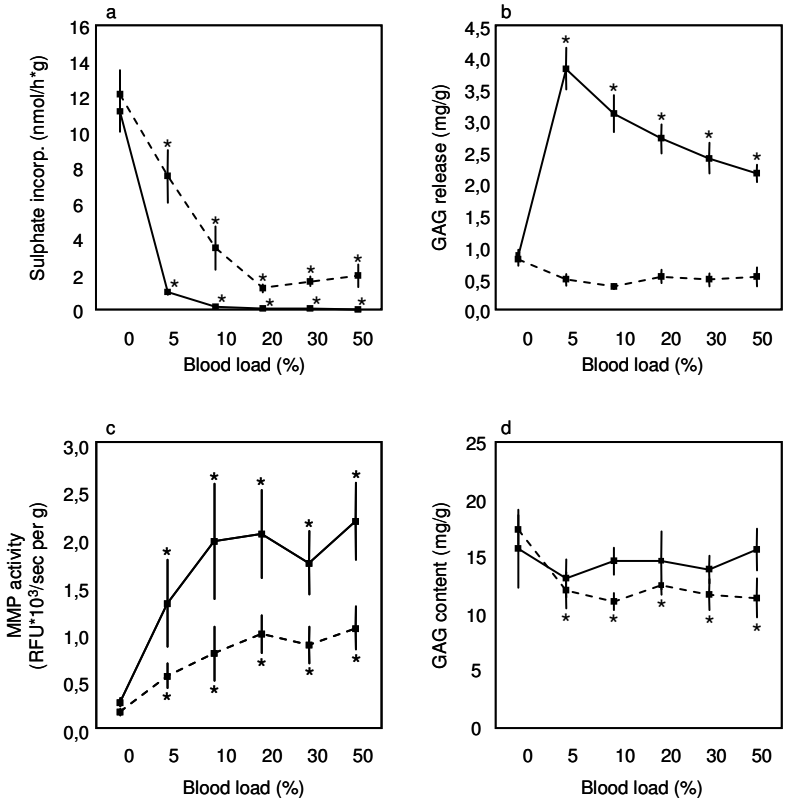

Figure 2: Concentration-dependent effect of blood on cartilage
Proteoglycan synthesis rate (**a**), - release (**b**), and - content (**d**) and general MMP activity (**c**). Human healthy cartilage was exposed *in vitro* for 4 days to 0, 5, 10, 20, 30 or 50% v/v blood, without (solid lines) or followed by a 12 days recovery period in the absence of blood (dashed lines). Asterisks indicate differences of blood-exposed samples *versus* controls (0% blood) (p-value of <0.05; n=5).

When cartilage was exposed for 2 days to 10% v/v blood (figure 3) and then allowed to recover for 12 days in the absence of blood, proteoglycan synthesis was still significantly diminished (figure 3a), MMP activity remained enhanced (figure 3c) and proteoglycan content decreased (figure 3d). Release of proteoglycan appeared to be only transiently increased after exposure to 10% v/v blood (figure 3b) and was completely restored after 12 days recovery.

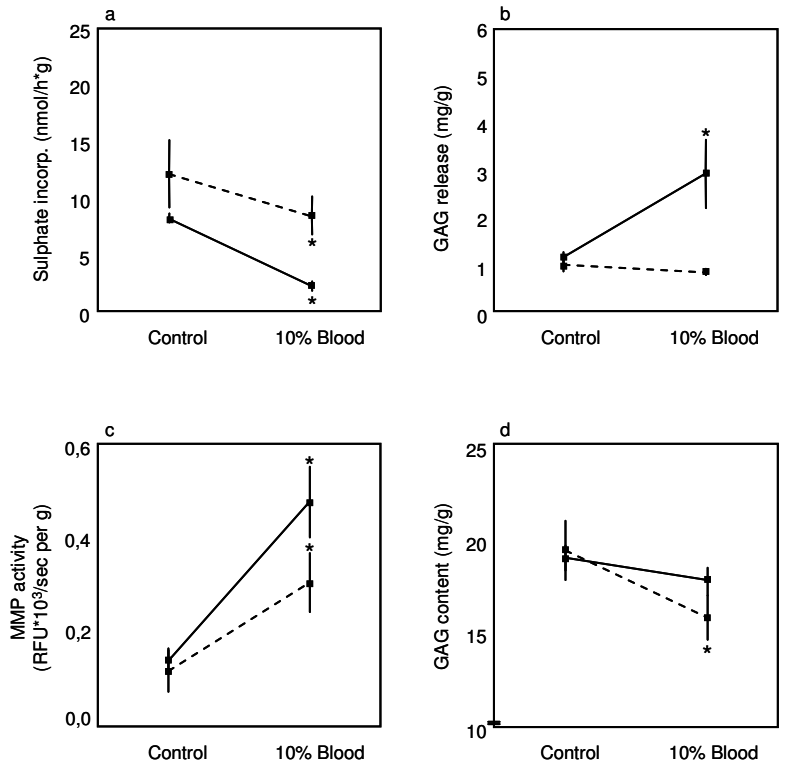

Figure 3: Effect of a 2-day exposure of cartilage to 10% v/v blood
Proteoglycan synthesis rate (**a**), - release (**b**), and - content (**d**) and general MMP activity (**c**). Human healthy cartilage was exposed *in vitro* for 2 days to 10 v/v blood, without (solid lines) or followed by a 12 days recovery period in the absence of blood (dashed lines). Asterisks indicate differences of blood-exposed samples *versus* controls (p-value of <0.05; n=6).

Similar adverse effects of coagulating and noncoagulating blood on cartilage

It is not known whether blood coagulates in the joint after a haemorrhage and if so to what extent and how quickly the coagulation occurs. The effects of coagulating blood were therefore compared with those of noncoagulating blood. For this study, only the effects after recovery from a 2- or 4-day exposure to 50% v/v blood and a 4-day exposure to 10 or 50% v/v blood exposure were analysed. In this set of experiments, the effects of noncoagulating blood were similar when compared with those of coagulating blood. As figure 4 clearly demonstrates, the adverse effects induced by blood on any of the parameters were not significantly different for cartilage tissue cultured with noncoagulating blood as compared with that cultured with coagulating blood.

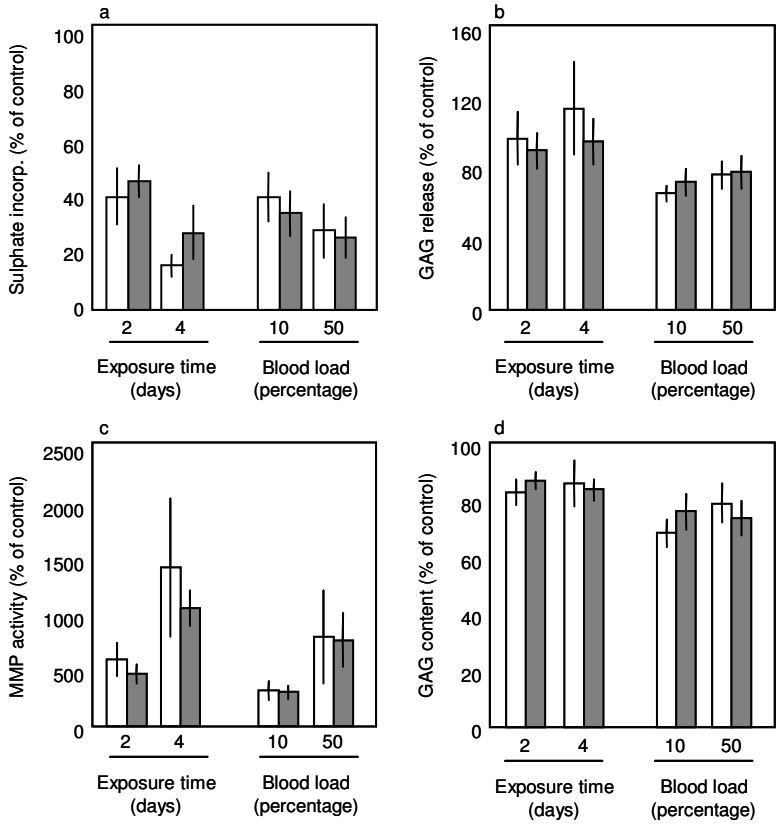

Figure 4: Effect of exposure of cartilage to either coagulating or non-coagulating blood
Proteoglycan synthesis rate (**a**), - release (**b**), and - content (**d**) and general MMP activity (**c**). Human healthy cartilage was exposed *in vitro* for 2 or 4 days to 10 or 50% v/v coagulating or non-coagulating blood, followed by a 12 days recovery period. The effects of blood exposure are depicted as a percentage of their control in the absence of blood. Open bars represent the values of cartilage exposed to non-coagulating blood; filled bars represent the values of cartilage exposed to coagulating blood. No statistical significant differences between both conditions were observed for any of the parameters (n=4).

DISCUSSION

There is no consensus as to whether aspiration of blood from the joint after haemarthrosis is indicated. This study was undertaken to provide information on the thresholds of blood concentration and exposure time that lead to prolonged cartilage damage. It was demonstrated that a 2-day exposure of cartilage to 10% v/v blood *in vitro* leads to prolonged biochemical impairment of joint cartilage, whereas a 1-day exposure to 50% v/v blood leads, for the most part, to only transient impairment of joint cartilage. In general, natural evacuation of blood from the joint after a haemorrhage takes more than one day. Based on the results from *in vivo* animal studies, it has been suggested that this process takes at least 4 days[13;14]. Similarly, the observations from treatment of patients with haemophilia indicate that 4 days is a reasonable time span for natural evacuation of blood.

Although the present results were determined *in vitro*, they suggest that aspiration of blood from a joint as soon as possible but at least within 48 hours after a joint haemorrhage should be considered in clinical practice, to prevent or diminish long-lasting impairment of the cartilage tissue. Nevertheless, the blood load (exposure time and/or dose) dependency of several of the adverse effects in the joint suggests that the sooner the aspiration takes place, the better. In addition to prevention of direct cartilage damage, aspiration of blood from a joint provides immediate relieve of pain and improves the range of motion[17-19]. It might also be expected that this aspiration decreases the risk of synovial triggering that leads to inflammatory activity[28-30]. We believe that to conclusively show the benefit of joint aspiration after a haemorrhage, a prospective, controlled, long-term clinical follow-up study is warranted to demonstrate protection from joint damage after joint bleeding, utilizing, for example, imaging techniques or analyses of serum markers for cartilage turnover.

Translation of these *in vitro* results has its restrictions, and issues to be considered are numerous. For instance, *in vitro,* the cartilage is exposed to blood at all cutting edges instead of solely at the articular surface as *in vivo;* this issue is currently under investigation. Furthermore synovial fluid can possibly interfere with the blood and neutralizing or potentiating factors might be present. Synovial tissue adds to cartilage damage by contributing to the inflammatory responses to blood exposure. Nevertheless, synovial tissue cells may phagocytose blood components and diminish the harmful effects. These issues have to be considered when translating the *in vitro* findings to clinical practice.

We have previously shown that exposure of cartilage to 50% v/v blood for 4 days leads to harmful effects on cartilage[3;4;9-11], namely the severe inhibition of proteoglycan synthesis and a direct increase in proteoglycan release and a decrease in the proteoglycan content. These effects were long-lasting, which could be attributed to the occurrence of chondrocyte apoptosis[9]. The present study extends these results by showing that the effects of exposing cartilage to blood can take

place within a shorter time period and after exposure to a lower blood load. Furthermore, our results indicating the reversibility of increased proteoglycan release, the exposure time- and blood load-dependent MMP activity, and the unresponsiveness of DNA content, are new findings that provide more insight regarding the mechanisms of blood-induced cartilage damage.

The observed effects of blood exposure on proteoglycan turnover cannot be attributed to changes in the cellularity of the cartilage, because no significant changes in DNA content were observed. In a previous study we have found that exposure of cartilage to blood results in chondrocyte apoptosis[9]. In that respect, one would anticipate a decrease in DNA content in cartilage upon exposure to blood. However, articular cartilage is not vascularized and does not contain mononuclear phagocytes. Therefore, apoptotic bodies, which still contain DNA that is detectable by our assay, are not cleared from the cartilage. This explains why the DNA content is not changed significantly as a consequence of blood exposure.

The effects observed after recovery from the exposure provide an indication of the irreversibility of the effects. It could be speculated that the effects would have largely been normalized when the recovery period was expanded. However, it has been shown previously that the harmful effects on synthesis persist for a at least 10 weeks after an initial 4-day 50% v/v blood load[4;11]. It was more feasible in the present study to conduct a follow-up of just two weeks because of the large numbers of experiments performed.

There was an inverse relation between the direct proteoglycan release and the concentration of blood added. A similar inverse relation was found for the exposure time although this was less striking. These findings can be explained by the fact that the release of proteoglycans was measured directly after the blood exposure, and not during the blood exposure itself. We assume that the amount of proteoglycans released during the blood exposure is higher when the blood load is higher, but that the amount that can be released directly after the exposure is lower with increasing concentrations of blood.

Furthermore, the release of proteoglycans completely normalized after the recovery period. As we have shown previously[4], the pro-inflammatory mediators IL-1β and tumor necrosis factor α (TNFα) play a role in the transient inhibition of proteoglycan synthesis measured directly after blood exposure. These cytokine-induced changes in proteoglycan synthesis were, however, not responsible for the prolonged inhibition of the rate of synthesis. It is reasonable to expect that the direct effects of exposure of cartilage to blood on proteoglycan release are induced by proinflammatory cytokines, and therefore that these effects are also transient.

In this study, we used a general assay for MMP activity in which the focus was largely based on the activity of collagenases, to provide data supplementary to the proteoglycan biochemistry data. Although some of the MMP activity measured may have originated from the original blood exposure, the actual MMP measurements

were performed in blood-free culture supernatants of the cartilage (shortly after the blood was withdrawn).

Our results also revealed that exposure of cartilage to blood leads to an exposure time - and blood load-dependent increase in MMP activity, both directly and, although at lower levels, after the recovery period. Active MMPs play a role by contributing to the breakdown of extracellular matrix, and release of proteoglycans is a consequence of this breakdown of cartilage matrix. It would therefore be expected that there would be a correlation between the activity of MMPs and the release of proteoglycans. However, this correlation was not evident in our experiments. The fact that the MMP assay used measures primarily collagenases and gelatinases[24-26] could explain the absence of a correlation between proteoglycan release and MMP activity. Collagen damage was not determined in this study.

Proteoglycans can also be degraded by other enzymes. The ADAMTS are a family of proteolytic enzymes that degrade extracellular matrix[31-35]. Among other subtypes, ADAMTS-1, -4 and -5 have been identified as aggrecanases that are of importance in the breakdown of aggrecan, the most prominent proteoglycan in cartilage[36-39]. It has also been shown that proteoglycans that were released from bovine or porcine articular cartilage after exposure to catabolic stimuli, e.g. retinoic acid, IL-1 or TNFα, were generated by the action of aggrecanases and not MMPs[31]. Therefore, the increase in the release of proteoglycans as a consequence of the exposure of cartilage to blood is probably mediated by aggrecanases, whereas the increase of MMP activity leads to damage of the collagen component of cartilage. Apparently, MMP activity remains for a prolonged period of time after blood exposure, combined with sustained inhibition of proteoglycan synthesis, and these changes result in persistent impairment of cartilage matrix integrity, whereas aggrecanase activity is transient, induced by transient (blood-induced) cytokines such as IL-1 and TNFα[4].

It is not known if and to what extent blood coagluates in the joint after a haemorrhage. However, we can rule out coagulation as a source of bias in the present study because we demonstrated that all the adverse effects induced by blood were similar after exposure to noncoagulating blood as compared with coagulating blood.

Thus, the results of the present study, although determined *in vitro,* demonstrate that aspiration of blood from a joint after haemarthrosis, even 24 hours after the incident, should be evaluated in clinical practice. This approach has the potential to prevent cartilage damage that will become clinically visible later in life.

REFERENCES

1. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
2. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
3. 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;24:1350-1354.
4. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
5. Tajima T, Yoshida E, Yamashita A et al. Hemoglobin stimulates the expression of matrix metalloproteinases, MMP-2 and MMP-9 by synovial cells: a possible cause of joint damage after intra-articular hemorrhage. J.Orthop.Res. 2005;23:891-898.
6. Roosendaal G, van Rinsum AC, Vianen ME et al. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-153.
7. Ghosh P, Smith M. Osteoarthritis, genetic and molecular mechanisms. Biogerontology. 2002;3:85-88.
8. van Valburg AA, Wenting MJ, Beekman B et al. Degenerated human articular cartilage at autopsy represents preclinical osteoarthritic cartilage: comparison with clinically defined osteoarthritic cartilage. J.Rheumatol. 1997;24:358-364.
9. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
10. 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;42:784-790.
11. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
12. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 1998;47:477-486.
13. Muirden KD, Peace G, Rogers K. Clearance of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. II. Autoradiographic and histological studies. Ann.Rheum.Dis. 1969;28:630-636.
14. Muirden KD. Clearnace of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. I. Relationship to the anaemia of rheumatoid arthritis. Ann.Rheum.Dis. 1969;28:548-551.
15. Hooiveld MJ, Roosendaal G, Jacobs KM 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;50:2024-2031.
16. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.
17. Ingram GI, Mathews JA, Bennett AE. A controlled trial of joint aspiration in acute haemophilic haemarthrosis. Br.J.Haematol. 1972;23:649-654.
18. Ingram GI, Mathews JA, Bennett AE. Controlled trial of joint aspiration in acute haemophilic haemarthrosis. Ann.Rheum.Dis. 1972;31:423.
19. Holdsworth BJ, Clement DA, Rothwell PN. Fractures of the radial head--the benefit of aspiration: a prospective controlled trial. Injury 1987;18:44-47.
20. van Valburg AA, Wenting MJ, Beekman B et al. Degenerated human articular cartilage at autopsy represents preclinical osteoarthritic cartilage: comparison with clinically defined osteoarthritic cartilage. J.Rheumatol. 1997;24:358-364.
21. Mastbergen SC, Bijlsma JW, Lafeber FP. Selective COX-2 inhibition is favorable to human early and late-stage osteoarthritic cartilage: a human in vitro study. Osteoarthritis.Cartilage. 2005;13:519-526.
22. Whiteman P. The quantitative determination of glycosaminoglycans in urine with Alcian Blue 8GX. Biochem.J. 1973;131:351-357.
23. Whiteman P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem.J. 1973;131:343-350.

24. Riley GP, Curry V, DeGroot J et al. Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. Matrix Biol. 2002;21:185-195.
25. Beekman B, Drijfhout JW, Bloemhoff W et al. Convenient fluorometric assay for matrix metalloproteinase activity and its application in biological media. FEBS Lett. 1996;390:221-225.
26. Beekman B, Drijfhout JW, Ronday HK, Tekoppele JM. Fluorogenic MMP activity assay for plasma including MMPs complexed to alpha 2-macroglobulin. Ann.N.Y.Acad.Sci. 1999;878:150-158.
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;19:123-129.
28. Rodriguez-Merchan EC. The destructive capabilities of the synovium in the haemophilic joint. Haemophilia. 1998;4:506-510.
29. 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;80:540-545.
30. Roosendaal G, Mauser-Bunschoten EP, de Kleijn P et al. Synovium in haemophilic arthropathy. Haemophilia. 1998;4:502-505.
31. Little CB, Flannery CR, Hughes CE et al. Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan in vitro. Biochem.J. 1999;344 Pt 1:61-68.
32. Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms involved in cartilage proteoglycan catabolism. Matrix Biol. 2000;19:333-344.
33. Arner EC. Aggrecanase-mediated cartilage degradation. Curr.Opin.Pharmacol. 2002;2:322-329.
34. Mort JS, Billington CJ. Articular cartilage and changes in arthritis: matrix degradation. Arthritis Res. 2001;3:337-341.
35. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res.Ther. 2003;5:94-103.
36. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J. 1992;6:861-870.
37. Knudson CB, Knudson W. Cartilage proteoglycans. Semin.Cell Dev.Biol. 2001;12:69-78.
38. Nietfeld JJ. Cytokines and proteoglycans. Experientia 1993;49:456-469.
39. Roughley PJ, Lee ER. Cartilage proteoglycans: structure and potential functions. Microsc.Res.Tech. 1994;28:385-397.

Chapter 3

Degenerated cartilage is as vulnerable to blood-induced damage as healthy cartilage is

Annals of the Rheumatic Diseases, Epub 4 jan 2008

N.W.D. Jansen[1,2]
G. Roosendaal[2]
J.W.J. Bijlsma[1]
J. DeGroot[3]
M. Theobald[2]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Business Unit BioSciences, Inflammatory & Degenerative Diseases, TNO Quality of Life, Leiden, The Netherlands

ABSTRACT

Background
Joint bleeds have a direct adverse effect on joint cartilage, leading to joint deterioration, and ultimately to disability. Because degenerated cartilage has a limited repair capacity, it was hypothesized that degenerated cartilage is more susceptible to blood-induced cartilage damage than healthy cartilage is.

Methods
Healthy, degenerated (pre-clinical osteoarthritic) and osteoarthritic (clinically defined) human cartilage was exposed to 10% v/v whole blood for a period of two days, followed by a recovery period of 12 days in the absence of blood. The effect of blood exposure on cartilage was determined by measuring the proteoglycan synthesis rate, - release, and - content, as well as protease (MMP) activity.

Results
In general, blood exposure led to a decrease in proteoglycan synthesis rate, an increase in the release of proteoglycans and in MMP activity, and therefore ultimately in a decrease of the proteoglycan content of the tissue. Impaired cartilage was as least as susceptible to this blood-induced damage as healthy cartilage was.

Conclusion
These results demonstrate that degenerated cartilage is not explicitly more susceptible to blood-induced damage than healthy cartilage is. Even when taking into account that these are just *in vitro* findings, it remains of great importance, also in already affected joints, to prevent joints bleeds and when joint bleeds do occur, to treat them adequately.

INTRODUCTION

Articular cartilage becomes damaged when it is exposed to blood as occurs during a joint bleed[1-3]. This damage is the consequence of the formation of hydroxyl radicals in the vicinity of the articular chondrocytes, leading to apoptosis of these chondrocytes[4]. These hydroxyl radicals form when hydrogen peroxide, synthesized by chondrocytes upon stimulation by IL-1, originating from activated monocytes/macrophages as present in the blood, reacts with haemoglobin-derived iron from damaged and phagocytosed red blood cells. The apoptosis of the chondrocytes leads to disturbance and impairment of the cartilage matrix turnover and hence to cartilage matrix damage[4-9].

Previously it was shown, that *in vitro* exposure of healthy cartilage to blood leads to a severe decrease in the synthesis rate of proteoglycans, one of the main matrix components of cartilage[4-7;9-11]. Furthermore, the release of these proteoglycans from the cartilage increases upon exposure to blood. Together, this results in a decrease in proteoglycan content. Also the activity of matrix metalloproteinases (MMPs), primarily collagenases[12;13], increases after the exposure of cartilage to blood[14] implicating that also collagen is affected upon blood exposure. These effects are long-lasting, even after a transient exposure of cartilage to a low concentration (10% volume/volume) of blood for a short period of time (2 days)[14]. During a joint haemorrhage the actual exposure time and blood concentration is estimated to be far higher, over 50% v/v for 4 days[9].

Despite the *in vitro* results described above, surprisingly the current general opinion amongst physicians remains that a few joint bleeds are acceptable. However, cartilage already affected by a previous joint bleed, trauma or degenerative processes as seen e.g. in osteoarthritis, might even be more susceptible to blood-induced damage than healthy cartilage is. The rational for this is that osteoarthritic cartilage is characterized by a disturbed proteoglycan turnover, damaged collagen, and has been associated with chondrocyte apoptosis[9;15-18] and thus is likely to have an impaired repair capacity. This may implicate that in the case of degenerated/osteoarthritic cartilage, joint bleeds are more deleterious and specifically in these cases should be prevented or, when they occur, treated appropriately to avoid extra stimulation of the progressive degeneration with consequently severe joint damage in later years. We therefore investigated whether degenerated (pre-clinical osteoarthritic)[19] and osteoarthritic (clinically defined) cartilage are more susceptible to blood-induced damage than healthy cartilage is.

MATERIALS AND METHODS

Cartilage culture

Three types of human cartilage were obtained: healthy (66.8 ± 5.5 years; m/f = 4/2); degenerated[19] (pre-clinical osteoarthritic; 78.4 ± 3.6 years; m/f = 3/4) and osteoarthritic (clinically defined; 66.5 ± 4.3 years; m/f = 2/4) articular cartilage. Healthy and degenerated cartilage were obtained *post mortem* from humeral heads within 24 hours after death of the donor. Healthy cartilage had a glossy, white, completely smooth surface and a healthy appearance. Degenerated cartilage had a macroscopic fibrillation of the surface and came from older donors without documented clinical history of joint disorders[19]. Osteoarthritic (OA) cartilage was obtained from the femoral knee condyles during joint replacement surgery. Collection of the cartilage was according to the medical ethical regulations of the University Medical Center Utrecht.

Slices of cartilage were cut aseptically as thick as possible, excluding the underlying bone and kept in phosphate buffered saline (PBS, pH 7.4). Within 1 hour after dissection, the slices were cut into square pieces, weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottomed microtiter plates in 200 µl culture medium per well according to standard procedures[14]. This culture system has been used previously and was demonstrated to be a reliable culture system to compare human healthy, degenerated (pre-clinical osteoarthritic), and osteoarthritic (end stage) cartilage tissue[19-21].

For each experiment fresh blood from healthy human donors was collected into heparinized vacutainer tubes (170 IU Li-heparin/10 ml) and added in 10% v/v to the cartilage tissue explants immediately after it was obtained.

After an exposure of two days, the cartilage was washed twice in fresh culture medium for 45 minutes under culture conditions to remove all the adherent blood components. Subsequently part of the samples was analysed. The remaining samples were cultured for an additional period of 12 days in the absence of blood. In these cultures, medium was refreshed every four days. These prolonged cultures in the absence of blood gave the chondrocytes the ability to recover from the blood exposure, enabling the evaluation of the (ir)reversibility of the observed effects of blood exposure. After this recovery period, these cartilage samples were analysed as well.

Analysis

Synthesis rate of sulphated proteoglycans was determined by a 4 hour pulse labelling with $^{35}SO_4$. After precipitation of the proteoglycans from a papain digest of the cartilage tissue, radioactivity was counted by liquid scintillation analysis and normalized to the specific activity of the medium. The rate of sulphate incorporation is

expressed as nmoles of sulphate incorporated per hour per gram wet weight and per mg DNA of cartilage tissue.
For the proteoglycan content glycosaminoglycans (GAGs) of the proteoglycans were stained and precipitated with Alcian Blue and determined by colorimetric assay using chondroitin sulphate as a reference[22-24]. Proteoglycan content is expressed in mg GAG per gram wet weight of cartilage tissue.
DNA content as a measure of the cellularity was determined fluorometrically using Hoechst 33258 staining with calf thymus DNA as a reference. DNA content is expressed as milligram DNA per gram wet weight of cartilage tissue.
Proteoglycan release was determined by the loss of GAGs in the culture medium from day 3-6 (direct effect) and from day 11-14 (effect after recovery), determined as described above and expressed as mg GAG per wet weight or mg DNA of the cartilage samples.
General MMP activity was determined in the culture supernatants using the conversion of an internally quenched fluorogenic substrate TNO211-F in the presence or absence of a general MMP inhibitor (BB94)[12;13;25]. MMP activity is expressed as relative fluorescence units per second (RFU/s) between samples with and without BB94 to correct for non-MMP protease activity, and normalized to the wet weight and DNA content of the cartilage tissue.
To determine the severity of cartilage degeneration, histological sections of the cartilage were graded for features of degeneration. Safranin-O-fast green-iron haematoxylin stained sections from three formalin fixed tissue samples from each donor were graded according to the modified[20] criteria of Mankin[26].

Calculations and statistical analysis

Because of focal differences in composition and bioactivity of cartilage specifically for degenerated and osteoarthritic tissue, the results for 10 cartilage samples per parameter per donor, obtained randomly and handled individually, were averaged and taken as a representative value for the cartilage of that donor. The n-value (6-7) indicates the number of experiments (viz. the number of cartilage donors). The effect of exposure of cartilage to blood per type of cartilage were analyzed using a non-parametric test for related samples (Wilcoxon signed rank test, two-sided), whereas the comparisons between the types of cartilage were analyzed using a non-parametric test for unrelated samples (Mann Whitney U, two-sided). Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Characteristics of the three types of cartilage

Cartilage integrity

Representative micrographs of the healthy, degenerated, and osteoarthritic cartilage are given in figure 1a. Note the presence of chondrocyte clusters, the decrease in proteoglycan staining and the surface irregularities in the degenerated and osteoarthritic cartilage. These characteristics were more prominent in the osteoarthritic cartilage than in the degenerated cartilage. The average histological grade (figure 1b) and proteoglycan content (figure 1c), all under control conditions (no addition of blood), supported the difference in degree of tissue integrity between the three types of cartilage, with degenerated cartilage giving intermediate values.

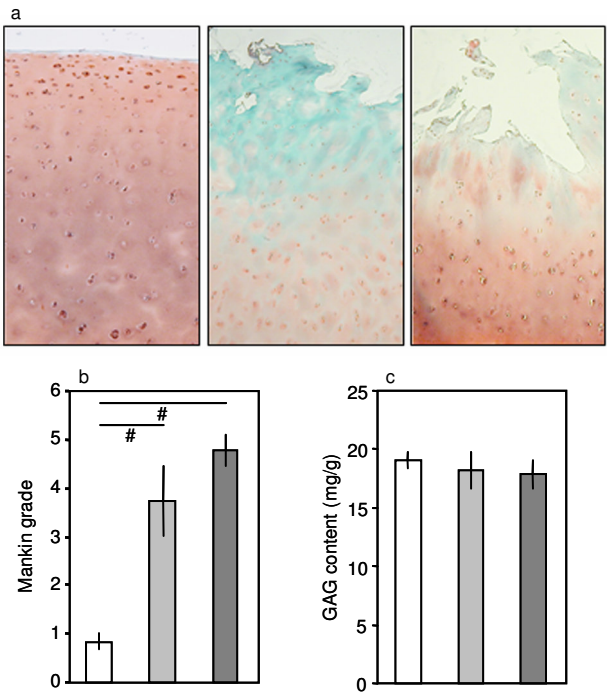

Figure 1: Characteristics of the three types of cartilage: cartilage integrity
Representative light micrographs of healthy (**a**; left panel), degenerated (**a**; middle panel) and osteoarthritic cartilage (**a**; right panel) and graphs depicting the average Mankin grade (**b**) and average proteoglycan content (**c**). The samples depicted in **a** were scored 0, 5, and 6 respectively according to the modified Mankin grade[20;26]. Original magnification 4x. White bars indicate the values of healthy cartilage (n=6), light grey bars those of degenerated cartilage (n=7) and dark grey bars those of osteoarthritic cartilage (n=6). The lines with a wicket indicate a statistically significant difference ($p<0.05$) between the different types of cartilage.

Cartilage chondrocyte activity

The parameters related to chondrocyte activity showed clear differences between the three types of cartilage. The DNA content and proteoglycan synthesis rate were lower for osteoarthritic cartilage than for degenerated cartilage, and degenerated cartilage had a lower DNA content and synthesis rate than healthy cartilage (figure 2a and 2b, respectively). Release of proteoglycans was higher for osteoarthritic than for degenerated, than for healthy cartilage (figure 2c). Overall MMP activity demonstrates a different pattern; the highest activity was observed for the degenerated cartilage and this enhanced activity was lost for the osteoarthritic cartilage. Note that these differences, not statistically significant, may be irrelevant because blood could increase MMP activity multi-fold (see figure 3d).

When the proteoglycan synthesis rate and release were normalized to cellularity (DNA content), the differentiation between the three types of cartilage remained similar (table 1). Interestingly, for MMP activity, when normalized to DNA content, there was a gradual increase with increasing severity of cartilage damage as to be expected.

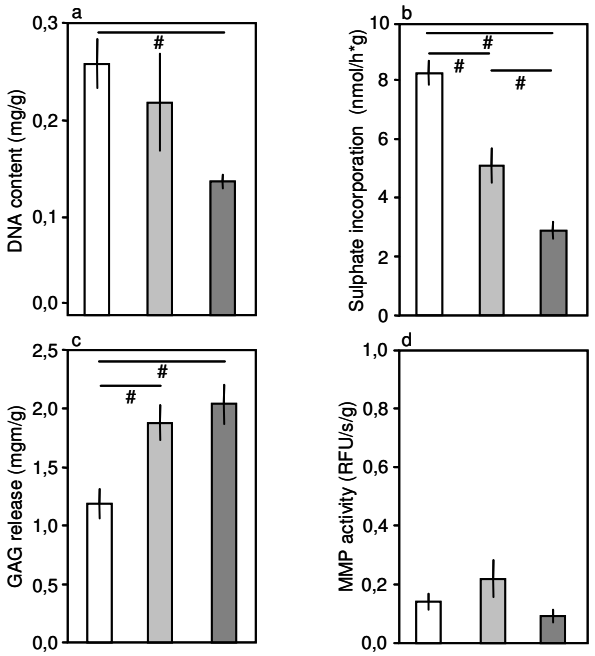

Figure 2: Characteristics of the three types of cartilage: cartilage chondrocyte activity

DNA content (**a**), proteoglycans synthesis rate (**b**), proteoglycan release (**c**) and MMP activity (**d**), normalized to the wet weight of the cartilage, are depicted. White bars indicate the values of healthy cartilage (n=6), light grey bars those of degenerated cartilage (n=7) and dark grey bars those of osteoarthritic cartilage (n=6). The lines with a wicket indicate a statistically significant difference ($p<0.05$) between the different types of cartilage.

Type of cartilage	PG synthesis (nmol/h*mg)	PG release (mg/mg)	MMP activity (RFU/sec/mg)
Healthy	34.2 ± 2.4	4.5 ± 0.5	0.6 ± 0.2
Degenerated	28.7 ± 4.2	10.4 ± 2.0*	1.1 ± 0.4
Osteoarthritic	21.9 ± 2.6*	14.0 ± 1.4*	1.6 ± 0.1

Table 1: Characteristics of the three types of cartilage: cartilage chondrocyte activity normalized to cellularity (DNA content) of the cartilage
Proteoglycans synthesis rate, proteoglycan release and MMP activity, normalized to the DNA content of the cartilage of healthy, degenerated and osteoarthritic cartilage. Asterisks indicate a statistically significant ($p<0.05$) difference compared to healthy cartilage.

Effect of exposure of cartilage to blood

Cartilage integrity

In figure 3 and table 2 the effects of blood on cartilage, both directly after a 2-day blood exposure of 10% v/v and after an additional 12-day recovery period in the absence of blood, are shown. Depicted is the change in outcome parameter compared to their controls i.e. cartilage treated identically but not exposed to blood. There was no statistically significant change in the DNA content of cartilage when the cartilage had been exposed to blood, neither directly after blood exposure nor after recovery (table 2).

Proteoglycan content was not changed statistically significant directly after a 2-day blood exposure (figure 3a), but the adverse changes in proteoglycan turnover upon blood exposure, as depicted in figure 3b and 3c, resulted in a statistically significant decrease in proteoglycan content between minus 10-20% of their controls after the recovery period. This decrease in proteoglycan content was not statistically significant different between the healthy, degenerated, and osteoarthritic cartilage.

Cartilage chondrocyte activity

The proteoglycan synthesis rate was decreased directly after blood exposure with ±75% for healthy, degenerated, and osteoarthritic cartilage when compared to their untreated controls (figure 3b). This decrease was slightly larger for osteoarthritic cartilage than for degenerated cartilage ($p<0.026$). After recovery, the proteoglycan synthesis rate was still statistically significantly decreased, although less outspoken than directly after the blood exposure. There was no statistically significant difference in the effect of blood exposure between the three types of cartilage after recovery. Similar results were obtained when proteoglycan synthesis rate was expressed per DNA content of the cartilage (table 2).

Effect	Type of cartilage	DNA (%)	PG synthesis (%)	PG release (%)	MMP activity (%)
Direct effect	Healthy	6.23 ± 2.4	-73.7 ± 5.3*	159 ± 38*	282 ± 72*
	Degenerated	9.22 ± 7.3	-71.2 ± 6.3*	125 ± 28*	489 ± 184*
	Osteoarthritic	2.77 ± 5.7	-82.3 ± 1.6*	121 ± 16*	1027 ± 353*#
Effect after recovery	Healthy	-7.22 ± 3.0	-21.7 ± 5.0*	-1.54 ± 11.2	223 ± 57*
	Degenerated	-7.01 ± 8.1	-8.7 ± 11.1	11.0 ± 10.6	221 ± 81*
	Osteoarthritic	-6.72 ± 3.1	-3.6 ± 11.2	37.8 ± 19.3*	514 ± 137*

Table 2: Effect of exposure of the three types of cartilage to blood
Human healthy, degenerated, and osteoarthritic cartilage tissue explants were exposed *in vitro* for 2 days to 10% v/v blood, followed by a 12-day recovery period. The DNA content, proteoglycan synthesis rate, and release, as well as MMP activity, the latter three normalized to the DNA content of the cartilage, are all depicted as a percentage of their control in the absence of blood, both directly after the blood exposure at day 2 as well as after the recovery period at day 14. An asterisk indicates a statistically significant ($p<0.05$) change upon blood exposure. The wicket indicates a statistically significant ($p<0.05$) difference in the effect of blood exposure of degenerated or osteoarthritic cartilage compared to healthy cartilage.

The release of proteoglycans was increased to approximately two times the control values directly after blood exposure, whereas after recovery the proteoglycan release did not differ from the untreated controls (figure 3c). The effect of blood on proteoglycan release was similar for the three types of cartilage. When proteoglycan release was expressed per DNA content of the cartilage samples, similar results were obtained (table 2) except for the fact that the release of proteoglycans from osteoarthritic cartilage was still statistically significantly enhanced after the recovery period.

Blood exposure also led to a statistically significant increase in MMP activity (figure 3d) for healthy, degenerated, and osteoarthritic cartilage, both directly after exposure to blood and after recovery, although after the recovery at lower levels. The increase in MMP activity compared to their untreated controls was statistically significantly higher for osteoarthritic cartilage than for healthy cartilage ($p<0.004$). This difference was still present after recovery, although not statistically significant anymore. When expressed per DNA content similar results were observed (table 2); the direct effect of blood on osteoarthritic cartilage being statistically significantly greater than for healthy cartilage (#).

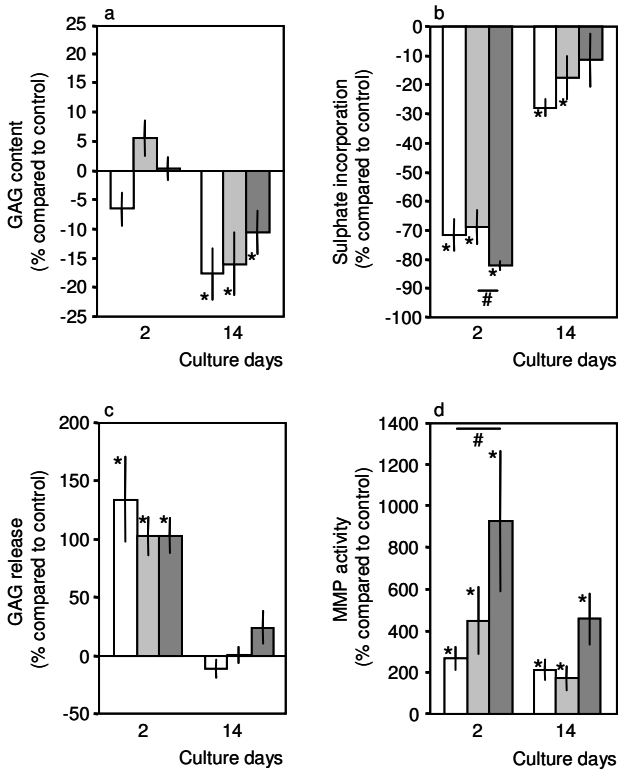

Figure 3: Effect of exposure of the three types of cartilage to blood

Human healthy (white bars; n=6), degenerated (light grey bars; n=7), and osteoarthritic (dark grey bars; n=6) cartilage tissue explants were exposed *in vitro* for 2 days to 10% v/v blood, followed by a 12-day recovery period. The proteoglycan content (**a**), - synthesis rate (**b**), - release (**c**), and MMP activity (**d**), all normalized to wet weight of the cartilage, are depicted as a percentage of their control in the absence of blood, both directly after the blood exposure at day 2 as well as after the recovery period at day 14. An asterisk indicates a statistically significant ($p<0.05$) change upon blood exposure. The lines with a wicket indicate a statistically significant ($p<0.05$) difference in the effect of blood exposure between the different types of cartilage.

DISCUSSION

Joint bleeds, even a limited number, ultimately lead to severe joint damage[1-3]. Currently the general opinion amongst physicians is that a few joint bleeds are acceptable. Although even for a first bleed under healthy conditions this might be disputed, it could well be that degenerated cartilage, e.g. by a previous joint bleed or as a result of biomechanical influences as in osteoarthritis, is even more susceptible to blood-induced damage, because of a decreased capacity to recover from the blood-induced damage. However, the present study could not demonstrate a clear difference in susceptibility of degenerated cartilage to blood exposure compared to healthy cartilage. Degenerated cartilage was at least as susceptible to blood-induced damage as healthy cartilage is. The only differences observed (increased inhibition of proteoglycan synthesis and larger increase in MMP activity; figure 3b and d) were, despite statistical significance, small. Since for none of the parameters the opposite effect was found, impaired cartilage is clearly not less vulnerable. Thus, also in case of impaired cartilage, as seen for instance in osteoarthritis, joint bleeds should be prevented if possible and when they do occur, should be treated properly.

Due to practical limitations, normal and degenerated cartilage were obtained from the humeral head, whereas osteoarthritic cartilage was obtained from the femoral condyles. With respect to differences in the vulnerability to blood of cartilage from different joints or from donors with different gender, to our knowledge no information is available in literature. However, in all our previous experiments over the past years we have never seen differences in susceptibility of cartilage to blood between cartilage from different joints or from donors with different gender. Irrespective, both items are a limitation in the experimental set-up.

Joint bleeds can occur because of a trauma, such as ligament rupture or intra-articular fracture, and occur spontaneously in patients suffering from the clotting disorder haemophilia. Prevention of joint bleeds is difficult. One can think of avoidance of circumstances with a high risk of such injury, like some kind of sports[27], but when joints bleeds do occur, proper treatment is needed. As we previously speculated[14], aspiration of blood from a joint after a joint bleed, as soon as possible ideally, but at least within 48 hours, can probably diminish the harmful effects of a joint bleed. Studies by Ingram *et al.*[28;29] and Holdsworth *et al.*[30] have shown an immediate relief of pain and increase in range of motion upon aspiration of a joint after a joint bleed. Furthermore there is circumstantial evidence that aspiration of a joint is indicated to prevent joint damage later in life[4-9;31-33]. The present study demonstrates that this is also indicated for already harmed joints as in osteoarthritis. Although there is ample evidence from human *in vitro* studies and animal *in vivo* studies, a prospective controlled long-term clinical follow-up study, using advanced imaging techniques and analyses of serum and/or urine markers of cartilage

turnover, might be indicated to demonstrate (protection of) joint damage after joint bleeds.

In case of haemophilia, prophylactic treatment as early as possible is preferable, but very expensive[34]. Therefore, sometimes it is thought that in patients with already damaged joints, there is less need for proper prophylactic treatment, because the damage has already occurred. With this study we demonstrate that blood has a similar devastating effect on already damaged cartilage when compared to healthy cartilage. Therefore, it may be concluded that optimal prophylactic treatment, also when joint damage is already present, remains of importance.

In the present study we have used pre-clinical osteoarthritic degenerated cartilage and osteoarthritic cartilage as was defined and described by van Valburg *et al*.[19]. The biochemical and histochemical characteristics of the three types of cartilage were very similar to those described in that study. In general the characteristics of degenerated cartilage were intermediate compared to those of healthy and osteoarthritic cartilage. In addition to that study, here we demonstrate the intermediate position of degenerated cartilage for proteoglycan release.

The control levels of the MMP activity of the three types of cartilage are not statistically different from each other. However, there was a tendency towards a higher activity of these matrix degrading enzymes in degenerated cartilage compared to healthy cartilage and osteoarthritic cartilage. Literature is inconclusive in this respect. Although in literature osteoarthritis is mostly linked to an increase in MMP activity[35;36], also a decrease in the expression or activity of several MMPs has been described[37;38]. The use of different substrates and/or assays to measure the MMP activity may account for these differences. Nevertheless, exposure of cartilage to blood consistently leads to a significant increase in MMP activity, and this increase is larger for degenerated than for healthy cartilage and larger for osteoarthritic cartilage than for degenerated cartilage.

The data suggest a certain degree of reversibility of the effects of blood upon recovery. However, previous experiments have demonstrated that the harmful effects of blood exposure are long-lasting up to at least 10 weeks, with only very minor recovery[5]. It cannot be excluded that the shorter exposure time with the lower concentrations of blood lead to reversibility of the observed effects. Additional studies are indicated in this respect.

The average age of the donors of the degenerative cartilage was higher than those of the donors of healthy and osteoarthritic cartilage, although the difference was not statistically significant. It can not be excluded that age interferes with susceptibility to blood exposure as has been demonstrated for dog cartilage[39;40]; the younger the animals the more susceptible to blood-induced damage. It could be that a higher vulnerability of younger cartilage has clouded a potential higher vulnerability of degenerated cartilage, although this cannot be concluded based on the presented results.

In the present study we used 10% v/v blood exposure for 2 days. This concentration and exposure time is based on a previous study in which we demonstrated that this combination is the minimum leading to prolonged (at least 12 days) adverse changes in cartilage[14]. Whether a higher concentration and longer exposure time would have resulted in differences in susceptibility between the three types of cartilage can only be speculated on. It could be that the decrease in proteoglycan content is greater in impaired cartilage than in healthy cartilage, because in this study a tendency towards more severe effects on the proteoglycans synthesis rate and the MMP activity in osteoarthritic cartilage were observed, which ultimately can result in a decrease of the proteoglycan content. However, such studies need to be performed to be conclusive in this respect.

Taken together, it is clear that joint bleeds have detrimental effects and impaired cartilage is at least as susceptible to blood-induced cartilage damage as healthy cartilage is. Therefore, joint bleeds should be prevented when possible and because complete prevention of joint bleeds is not realistic, research must be performed to develop proper treatment of joint bleeds.

REFERENCES

1. Hoots WK. Pathogenesis of hemophilic arthropathy. Semin.Hematol. 2006;43:S18-S22.
2. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
3. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
4. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
5. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
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;42:784-790.
7. 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;24:1350-1354.
8. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.
9. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
10. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 1998;47:477-486.
11. Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr.Course Lect. 1998;47:487-504.
12. Beekman B, Drijfhout JW, Bloemhoff W et al. Convenient fluorometric assay for matrix metalloproteinase activity and its application in biological media. FEBS Lett. 1996;390:221-225.
13. Beekman B, Drijfhout JW, Ronday HK, Tekoppele JM. Fluorogenic MMP activity assay for plasma including MMPs complexed to alpha 2-macroglobulin. Ann.N.Y.Acad.Sci. 1999;878:150-158.
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. 2006;56:199-207.
15. Aigner T, Kim HA, Roach HI. Apoptosis in osteoarthritis. Rheum.Dis.Clin.North Am. 2004;30:639-53, xi.
16. Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasheri A. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet.J. 2003;166:140-158.
17. Horton WE, Bennion P, Yang L. Cellular, molecular, and matrix changes in cartilage during aging and osteoarthritis. J.Musculoskelet.Neuronal.Interact. 2006;6:379-381.
18. Lorenz H, Richter W. Osteoarthritis: cellular and molecular changes in degenerating cartilage. Prog.Histochem.Cytochem. 2006;40:135-163.
19. van Valburg AA, Wenting MJ, Beekman B et al. Degenerated human articular cartilage at autopsy represents preclinical osteoarthritic cartilage: comparison with clinically defined osteoarthritic cartilage. J.Rheumatol. 1997;24:358-364.
20. Lafeber FP, van der Kraan PM, van Roy HL et al. Local changes in proteoglycan synthesis during culture are different for normal and osteoarthritic cartilage. Am.J.Pathol. 1992;140:1421-1429.
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;29:287-299.
22. Mastbergen SC, Bijlsma JW, Lafeber FP. Selective COX-2 inhibition is favorable to human early and late-stage osteoarthritic cartilage: a human in vitro study. Osteoarthritis.Cartilage. 2005;13:519-526.
23. Whiteman P. The quantitative determination of glycosaminoglycans in urine with Alcian Blue 8GX. Biochem.J. 1973;131:351-357.
24. Whiteman P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem.J. 1973;131:343-350.

25. Riley GP, Curry V, DeGroot J et al. Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. Matrix Biol. 2002;21:185-195.
26. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J.Bone Joint Surg.Am. 1971;53:523-537.
27. Buckwalter JA, Martin JA. Sports and osteoarthritis. Curr.Opin.Rheumatol. 2004;16:634-639.
28. Ingram GI, Mathews JA, Bennett AE. A controlled trial of joint aspiration in acute haemophilic haemarthrosis. Br.J.Haematol. 1972;23:649-654.
29. Ingram GI, Mathews JA, Bennett AE. Controlled trial of joint aspiration in acute haemophilic haemarthrosis. Ann.Rheum.Dis. 1972;31:423.
30. Holdsworth BJ, Clement DA, Rothwell PN. Fractures of the radial head--the benefit of aspiration: a prospective controlled trial. Injury 1987;18:44-47.
31. Hooiveld MJ, Roosendaal G, Jacobs KM 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;50:2024-2031.
32. Roosendaal G, van Rinsum AC, Vianen ME et al. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-153.
33. Tajima T, Yoshida E, Yamashita A et al. Hemoglobin stimulates the expression of matrix metalloproteinases, MMP-2 and MMP-9 by synovial cells: a possible cause of joint damage after intra-articular hemorrhage. J.Orthop.Res. 2005;23:891-898.
34. Roosendaal G, Lafeber F. Prophylactic treatment for prevention of joint disease in hemophilia--cost versus benefit. N.Engl.J.Med. 2007;357:603-605.
35. Martel-Pelletier J, Welsch DJ, Pelletier JP. Metalloproteases and inhibitors in arthritic diseases. Best.Pract.Res.Clin.Rheumatol. 2001;15:805-829.
36. Tchetverikov I, Lohmander LS, Verzijl N et al. MMP protein and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis, and osteoarthritis. Ann.Rheum.Dis. 2005;64:694-698.
37. Aigner T, Zien A, Gehrsitz A, Gebhard PM, McKenna L. Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum. 2001;44:2777-2789.
38. Bau B, Gebhard PM, Haag J et al. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum. 2002;46:2648-2657.
39. Hooiveld MJ, Roosendaal G, Vianen ME et al. 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.
40. Roosendaal G, Tekoppele JM, Vianen ME et al. Articular cartilage is more susceptible to blood induced damage at young than at old age. J.Rheumatol. 2000;27:1740-1744.

Chapter 4

The non-ionic surfactant poloxamer P188 does not prevent blood-induced cartilage damage

Submitted

N.W.D. Jansen[1,2]
G. Roosendaal[2]
J.W.J. Bijlsma[1]
M. Theobald[2]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands

ABSTRACT

Background
Exposure of cartilage to blood as happens during joint bleeding, leads to irreversible disturbance of proteoglycan turnover due to chondrocyte death. Because complete prevention of joint bleeding is not possible, there is a need for treatment modalities that prevent blood-induced joint damage. The non-ionic surfactant poloxamer P188 (pluronic) has been reported to prevent chondrocyte death due to trauma. Therefore, it was evaluated whether P188 can prevent blood-induced cartilage damage.

Methods
Healthy human articular cartilage explants were exposed to blood (10 or 50% v/v), P188 (8 mg/ml) or both. The proteoglycan synthesis rate, - release, and - content were assessed.

Results
The characteristic changes in proteoglycan turnover upon exposure of cartilage to blood, being an increase in release and decrease in synthesis rate and content, was not influenced by the presence of P188 under conditions that have been demonstrated to prevent chondrocyte death due to trauma.

Conclusion
Based on these studies, P188 is not expected to be effective in treatment of blood-induced cartilage damage and therefore was not studied further.

Ample evidence is gained over the past years, by both human *in vitro* and animal *in vivo* studies, that blood has a direct harmful effect on cartilage[1]. Although the observed effects after a first (or limited number of) joint bleed(s) will not be directly visible in clinical practice, they clearly will compromise the joint, limiting the regenerative capacity needed in case of additional stresses on the cartilage, such as loading or additional haemorrhages.

The most comprehensible example of blood-induced joint damage is seen in haemophilia. The repeated joint bleeds lead to joint damage within several years. This haemophilic arthropathy (HA) due to recurrent haemarthroses is the main cause of morbidity in patients suffering from haemophilia. And even despite the significant improvement of treatment with clotting factor, joint bleeds still occur. A recent study by Manco-Johnson in the New England Journal of Medicine clearly demonstrated this. As the editorial on this study stated[2], it will be impossible, due to high costs, to treat all patients with haemophilia in such a way (complete clotting factor substitution) that joint bleeding will be banned completely. Therefore a search of treatment modalities to prevent haemarthropathy is warranted. However, thus far there is no treatment at all for blood-induced joint damage. There is even hardly any research on possible treatment modalities. This might be due to the significant delay between the actual bleeding and the clinical outcome, the relative low incidence of severe haemophilia, and the relatively recent gained knowledge on the mechanisms of blood-induced joint damage[1].

As is demonstrated by several human *in vitro* studies, exposure of cartilage to blood results in adverse changes in chondrocyte activity, fully independent of synovial tissue and inflammatory mediators. Hydroxyl radicals are formed when hydrogen peroxide production by chondrocytes is increased upon stimulation by pro-inflammatory cytokines such as IL-1, originating from activated blood monocytes/ macrophages as present in the blood within the joint after a haemarthros. Hydrogen peroxide reacts with haemoglobin-derived iron from damaged and phagocytosed red blood cells in the vicinity of chondrocytes, leading to the formation of radicals that induce cell death, as was shown amongst others by TUNEL staining[3]. Inhibitors of radical formation and of apoptosis were able to partially inhibit the harmful effects of blood on cartilage. Chondrocyte death leads to significant long-lasting disturbance of matrix turnover, because chondrocytes maintain cartilage matrix integrity on their own. Moreover, due to chondrocyte death, cartilage will be impaired in its regenerative capacity needed to recover from stresses from biomechanical and biochemical origin. This is because chondrocytes in adult cartilage hardly proliferate; the chondrocytes present have to do the job for a lifetime. For this reason, chondrocyte death has to be prevented as good as possible. In this respect, recent studies on the potential chondro-protective capacity of poloxamer 188 (P188 or pluronic) drew the attention.

P188 is a poloxamer surfactant belonging to the class of water-soluable multi-block copolymers that have important surface-active properties. P188 is a tri-block copolymer having an average molecular weight of 8400 Dalton. It consists of two hydrophilic sites with in-between a hydrophobic site. This enables the surfactant to directly insert into membrane pores due to loss of membrane integrity as a result of trauma. In this way, it can protect against cell death. Therefore, P188 has been evaluated in survival of many different cell types, amongst which chondrocytes.
Effects of P188 on cartilage chondrocytes was initiated by the group of Haut in search of treatment for post-traumatic cartilage damage. In their first report[4], they showed that addition of P188 added directly after induction of chondrocyte trauma, by applying high unconfined static compression to bovine cartilage explants, saved chondrocytes from early death. The number of live cells was significantly higher in P188 treated cartilage (approximately 75%) than in the controls (approximately 50%) after trauma. In 2005 the group reported similar results on rabbit cartilage tissue *in vivo*. Half of the dead chondrocytes could be saved by intra-articular injection of P188 just before blunt trauma. In both studies they used histochemistry by use of a Live/Dead Cytotoxicity Kit as the only outcome parameter.
Because the induced chondrocyte necrosis by mechanical trauma was supposed to be different from the apoptosis upon blood exposure of cartilage, these studies were ignored in the search of treatment modalities for haemarthropathy. However, in 2006 Haut reported that P188 was also effective in reducing the percentage of cells with DNA fragmentation as shown by TUNEL staining in impacted explants[5]. These recent data suggest that early P188 intervention was effective in preventing DNA fragmentation of injured chondrocytes. As described above DNA fragmentation in chondrocytes as measured by TUNEL staining was also observed upon exposure of cartilage tissue to blood. Moreover, it appeared upon a more detailed literature survey that P188 could also reseal cell membranes damaged by oxidative agents and thereby provide neuroprotection. These latter two observations, the effect on apoptosis and on cell death induced by oxygen species, tempted us to study P188 as a potential treatment modality in case of blood-induced cartilage damage, because these mechanisms are thought to be involved in this damage.

Human cartilage tissue explants were exposed to 10% volume/volume (v/v) blood for 2 days, previously shown to lead to long-lasting cartilage damage. To half of the blood-exposed cartilage explants, 8 mg/ml P188 was added just prior to the addition of blood. The cartilage was loaded 10 cycles with a hand-held stainless steel platen at approximately 1 MPa, needed to get an optimal effect of P188, as described by Haut[4]. The experiment was performed with cartilage of 5 different donors (n=5) and average values are presented in figure 1. Although the characteristic inhibition of proteoglycan synthesis, increase in - release and decrease in - content after blood exposure was observed, no effect was evoked by the addition of P188.

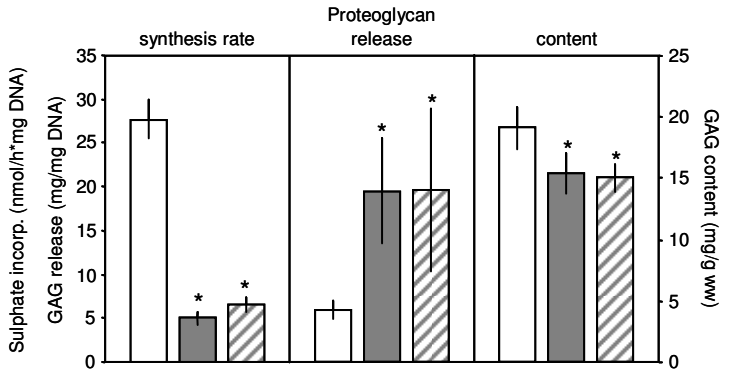

Figure 1: Effect of P188 on blood-induced cartilage damage
Proteoglycan synthesis rate, - release and - content of cartilage exposed to 10% v/v blood (filled grey/dashed bars) in the absence (filled grey bars) or presence (dashed bars) of 8 mg/ml P188. Asterisks indicate statistical significant change compared to control cartilage (white bars). There is no effect of P188 on blood-induced changes in proteoglycan turnover of healthy human articular cartilage.

It could have been that this minimal condition that leads to long-lasting disturbance of proteoglycan turnover did not lead to sufficient chondrocyte apoptosis as was demonstrated by exposure to 50% v/v blood. However, when these experiments were repeated with an exposure of the cartilage to 50% v/v blood, again no effect was found of P188 on the blood-induced changes in proteoglycan turnover (n=3; data not shown).
A check whether P188 had a direct effect on cartilage (potentially adverse, shading a potential beneficial effect) revealed no effect of P188 on the three cartilage parameters (figure 2).

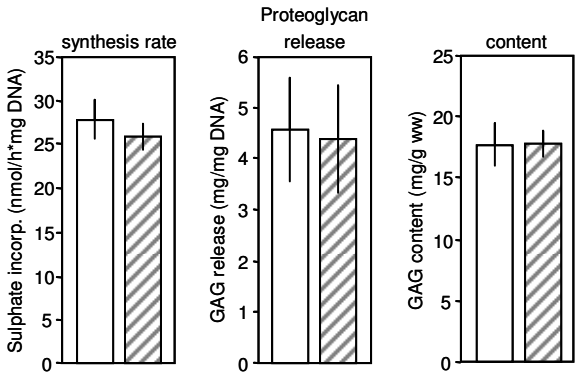

Figure 2: Effect of P188 on proteoglycan turnover
Proteoglycan synthesis rate, - release and - content of cartilage cultured in the absence (white bars) or presence of 8 mg/ml P188 (dashed bars). There is no effect of P188 on the proteoglycan turnover of healthy human articular cartilage.

From these studies it was concluded that blood-induced chondrocyte damage, despite a role for apoptosis induced by radicals, can not be diminished or prevented by P188. Why is P188 unable to save the chondrocytes from blood-induced damage? There may be numerous reasons that can be speculated on.
One of the options is that blood is present for 2 days, inducing chondrocyte damage, whereas in the studies of Haut a short trauma is used to induce the cell damage. Additionally P188 may have been inactivated by blood components: plasma and/or numerous red blood cells as present in the culture may have interfered with the activity of P188. In this respect higher concentrations P188 could have been tested, but in all cartilage studies of the group of Haut, P188 was added in a single dose only (the same dose as we used). A third explanation may be that the histochemical analyses used by Haut do not represent the actual chondrocyte activity. It might well be that despite the fact that cells were stained as live and did not show TUNEL staining, they were still unable to synthesize proteoglycans adequately. This can be verified by performing these histochemical assays with our cartilage samples. However, even when this indeed is true, chondrocytes appearing to be alive, but unable to synthesize matrix proteins, P188 would have been of no use, as live chondrocyte without proper metabolic activity can not prevent cartilage from degradation. Even if all these problems can be overcome, additional studies are needed to study whether P188 is able to restore damage in addition to prevention of damage. However, because not the slightest effect of P188 was observed, and our primary goal was a search for treatment modalities preventing blood-induced joint damage, no additional experiments have been performed to verify these speculations.
Although this study is far from complete, it was concluded that P188 is not a potential treatment modality in case of blood-induced joint damage that needs further exploration. Other options have to be explored to be able to treat joint bleeds to prevent subsequent joint damage.

REFERENCES

1. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12 Suppl 3:117-121.
2. Roosendaal G, Lafeber F. Prophylactic treatment for prevention of joint disease in hemophilia--cost versus benefit. N.Engl.J.Med. 2007;357:603-605.
3. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
4. Phillips DM, Haut RC. The use of a non-ionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants. J.Orthop.Res. 2004;22:1135-1142.
5. Baars DC, Rundell SA, Haut RC. Treatment with the non-ionic surfactant poloxamer P188 reduces DNA fragmentation in cells from bovine chondral explants exposed to injurious unconfined compression. Biomech.Model.Mechanobiol. 2006;5:133-139.

Chapter 5

Interleukin-10 protects against blood-induced joint damage

Accepted pending revision
British Journal of Haematology

N.W.D. Jansen[1,2]
G. Roosendaal[2]
M.J.J. Hooiveld[3]
J.W.J. Bijlsma[1]
J.A.G. van Roon[1]
M. Theobald[2]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Haematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Metabolic & Respiratory Diseases, GlaxoSmithKline, Zeist, The Netherlands

ABSTRACT

Background
Despite prophylactic treatment, haemophilia patients suffer from spontaneous joint bleeds, which lead to severe joint damage. Also after joint trauma, an intra-articular haemorrhage can add to joint damage in time. In search of possible interventions to prevent or limit the damaging effects of joint bleeds, we evaluated interleukin-10 (IL-10).

Methods
Human articular cartilage tissue explants were cultured in the presence or absence of 50% v/v blood (or its cellular components) for 4 days (an expected blood load *in vivo* after a joint haemorrhage), followed by a recovery period of 12 days. IL-10 was added in 1 or 10 ng/ml (pharmacological dosages reached during treatment with IL-10). Additionally, cartilage and synovial tissue obtained from joints with end stage haemophilic arthropathy were cultured in the presence of IL-10 (10 ng/ml).

Results
IL-10 protected cartilage from the damaging effects of blood exposure, measured by its effects on proteoglycan turnover. In addition, IL-10 beneficially influenced cartilage from patients with haemophilic arthropathy and reduced the production of the inflammatory cytokines IL-1β and TNFα by haemophilic synovial tissue.

Conclusion
Taken together, although effects were obtained *in vitro*, IL-10 protects against blood-induced joint damage and might be further evaluated as candidate in treatment of tissue damaging effects of joint haemorrhages.

INTRODUCTION

Recurrent joint bleeds, or haemarthroses, ultimately lead to joint destruction. Joint bleeds can occur after joint trauma or major joint surgery and can occur spontaneously in patients with severe haemophilia. Such patients often develop severe arthropathy in time[1-3]. Because of the delay between joint bleeding and the subsequent clinically apparent joint damage, it is difficult to establish the exact pathogenetic mechanism of blood-induced arthropathy. For a long time it was believed that the synovial tissue was first and foremost affected by joint bleeds and that the observed cartilage damage was due to, and thus secondary to, the changes of the synovial tissue[4;5]. More recently, it is shown that joint bleeds also directly, and probably first, affect the articular cartilage[6-9].

The synovial tissue plays a major role in the absorption of blood from the joint cavity after a joint bleed. This triggers the synovial tissue but initially leads only to transient effects[10]. After several joint bleeds a degradation product of red blood cells, haemosiderin, accumulates in the synovial tissue. This triggers inflammatory activity of the synovial tissue, leading to expansion and more permanent recruitment of inflammatory cells[4;5;11]. This chronic inflammatory response adds to progressive joint damage, among others by release of tissue destructive enzymes and cytokines[4;5].

Exposure of cartilage to blood (independent of synovial tissue and inflammatory mediators) results in adverse changes in chondrocyte activity[6-9]. *In vitro* studies have shown that this is caused by the induction of apoptosis of chondrocytes by hydroxyl radicals formed upon exposure to blood[12]. These hydroxyl radicals are formed when hydrogen peroxide production by chondrocytes is increased upon stimulation by pro-inflammatory cytokines such as IL-1, originating from activated blood monocytes/macrophages as present in the blood within the joint. H_2O_2 reacts with haemoglobin-derived iron from damaged and phagocytosed red blood cells in the vicinity of chondrocytes leading to the formation of radicals that induce apoptosis[12;13].

Taken together, the mechanism of blood-induced joint damage includes both degenerative (cartilage-mediated) and inflammatory (synovium-mediated) components. Both these processes finally occur in parallel and although influencing each other, they probably do not depend on each other.

Surprisingly, despite this knowledge, the current general opinion amongst physicians is that a few joint bleeds are acceptable, and consequently, blood-induced joint damage is still frequently seen. Treatments to prevent this damage are still lacking. Therefore, we searched for possible treatment modalities that are able to diminish or even prevent joint damage caused by a joint haemorrhage. In this light, we evaluated the potential benefit of the immunoregulatory cytokine interleukin-10 (IL-10).

IL-10 is a multifunctional cytokine and has diverse effects on several haemopoietic cells. Its main function is to control inflammatory responses, which is achieved by the regulation of the activity of T-cells, monocytes, and macrophages. Based on *in vitro*

and animal *in vivo* findings[14-22] IL-10 was suggested as a candidate in treatment of the chronic inflammatory joint disease rheumatoid arthritis[23], and was tested in clinical trials as such[24;25]. Moreover, IL-10 was reported to have direct beneficial effects on cartilage[20;26].
Because blood-induced joint damage includes both inflammation-mediated mechanisms as well as cartilage-mediated processes, IL-10 might have multiple targets to modulate joint damage induced by haemarthroses. Therefore, IL-10 was evaluated for its potential protective properties in blood-induced joint damage.

MATERIALS AND METHODS

Cartilage and synovial tissue culture

Both healthy and impaired full thickness human articular cartilage tissue were obtained *post mortem* from humeral heads within 24 hours after death of the donor. Healthy cartilage had a glossy, white, smooth appearance. Impaired cartilage had macroscopic visible fibrillation of the surface, previously defined as pre-clinical osteoarthritic cartilage[27]. Damaged cartilage and inflamed synovial tissue of patients suffering from haemophilia were obtained during total joint replacement surgery. Cartilage was available from sixteen donors with healthy articular cartilage (age 68.1 ± 3.2 years; 13 male/3 female), six donors with impaired articular cartilage (age 69.3 ± 6.4 years; 2 male/4 female), and from 3 donors (age 45.7 ± 5.3 years; 3 male) with haemophilic arthropathy (HA). Collection of the cartilage and synovial tissue was according to the medical ethical regulations of the University Medical Center Utrecht.
Slices of cartilage were cut aseptically, as thick as possible, excluding the underlying bone and kept in phosphate buffered saline (PBS, pH 7.4). Within 1 hour after dissection, the slices were cut into square pieces, weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottomed microtiter plates in 200 µl per well. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 µg/ml), ascorbic acid (85 µM) and 10% heat inactivated pooled human male AB^+ serum. The cartilage explants were cultured in a tissue incubator under 5% CO_2 in air, at 37°C, and 95% humidity.
For each experiment, fresh blood from healthy human donors was collected into heparinized vacutainer tubes (Becton Dickinson, UK; 170 IU Li-heparin/10 ml) and added to the cartilage tissue explants immediately after it was obtained. Because the total volume was kept at 200 µl, part of the culture medium was replaced by full blood. Previous experiments have shown that dilution of nutrients in the culture medium or the increase in serum components upon adding whole blood up to max 50% volume/volume (v/v) do not influence proteoglycan turnover on their own.[9] Cartilage chondrocytes are not in direct cell-cell contact with the blood cells and

therefore, the use of homologous blood rather than autologous blood has no differential influence on the cultures and has been demonstrated in canine experiments (data not shown). Furthermore, we have shown previously that the addition of heparin itself does not influence the effect of the exposure of cartilage to blood[8].

In the first set of experiments, healthy cartilage was exposed to the combination of red blood cells (RBC) and mononuclear cells (MNC) (50% equivalent to whole blood) for 4 days with or without IL-10. These two cell types have been shown to be primarily responsible for the blood- induced cartilage damage[8;9]. The concentrations of IL-10, 1 and 10 ng/ml (Sigma, I9276) were chosen based on the serum levels measured in healthy individuals treated with IL-10[24;25]. In the second set of experiments healthy and impaired cartilage samples were exposed to whole blood (50% v/v), with or without IL-10 or exposed to IL-10 alone for four days. In a third set of experiments cartilage obtained from end stage haemophilic arthropathy was exposed for 4 days to 10 ng/ml IL-10.

After these 4 days of culture (the blood exposure and/or IL-10 treatment period), in all these three sets of experiments the cartilage was washed twice in fresh culture medium for 45 minutes under culture conditions to remove all the adherent blood components and IL-10. Subsequently samples were cultured for an additional period of 12 days in the absence of blood and IL-10. In these cultures, medium was refreshed every four days. These prolonged cultures in the absence of blood enabled the evaluation of the (ir)reversibility of the observed effects of blood with or without IL-10 and with IL-10 alone. The loss of proteoglycans from the matrix was determined during the first 4 days in the absence of blood, because previous experiments have demonstrated this to be the optimal time period to evaluate proteoglycan release[7]. Proteoglycan synthesis rate and - content as well as DNA content were analysed after the 12 days of recovery; on day 16.

From each donor with haemophilic arthropathy of whom synovial tissue was available at time of joint replacement surgery (age 53.5 ± 2.5 years; n=4 male) 4-10 synovial tissue samples weighing between 10 and 70 mg were cultured in 1 ml culture medium as described above with or without the addition of 10 ng/ml IL-10. After two days of culture, the supernatants of the synovial tissue cultures were harvested and rendered cell-free by centrifugation (5' at 3000 rpm) and stored at -80°C. Interleukin-1 beta (IL-1β) and tumor necrosis factors alpha (TNFα) were determined in these supernatants.

Analysis

Proteoglycan synthesis rate was evaluated by sulphate incorporation rate: $Na_2{}^{35}SO_4$ (DuPont, NEX-041-H, carrier free) was added in 10 µl aliquots of 74 kBq per well. After 4 hours pulse labelling of the sulphated glycosaminoglycans (GAGs) the cartilage samples were washed twice in ice cold PBS and stored at -20°C. Thawed

samples were digested with papain (Sigma P3125) as described previously.[7] Papain digests were diluted to the appropriate concentrations for the analysis of the proteoglycan synthesis rate and -content as well as DNA content.
For the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (CPC, Sigma; C9002). The precipitate was dissolved in 3M NaCl and the amount of radioactivity in the sample measured by liquid scintillation analysis. The rate of sulphate incorporation was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage and expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage tissue (nmol/h*g).
The proteoglycan content of the cartilage tissue digest was determined by the GAG content of the digest. GAGs were stained and precipitated with Alcian Blue[28;29] (Sigma A5268). Alcian Blue staining was determined by the change in absorbance at 620nm. Chondroitin sulphate (Sigma C4383) was used as a reference. The proteoglycan content was expressed in mg GAG per gram wet weight of the cartilage explants (mg/g).
The DNA content of the cartilage tissue digests was determined as a measure of the cellularity of the cartilage samples using the fluorescent dye Hoechst 33258 (Calbiochem 382061). Calf thymus DNA (Sigma D4764) was used as a reference. DNA content is expressed as milligram DNA per gram wet weight of cartilage (mg/g). In all conditions (experiments) there was no significant change in DNA content, viz. the cellularity, of the cartilage due to blood exposure.
Proteoglycan release was determined by the loss of GAGs in the culture medium from the first 4 days of recovery. GAGs were stained and precipitated with Alcian Blue as described above. The proteoglycan release was calculated as mg GAG released per gram wet weight of the cartilage (mg/g).
The concentrations of IL-1β and TNFα in the culture supernatants of the synovial tissue were determined with commercially available cytosets (Biosource) and performed according to the manufacturer's protocol.

Calculations and statistical analysis

Because of focal differences in composition and bioactivity of the cartilage, the results for 10 cartilage samples per parameter per donor, obtained randomly and handled individually, were averaged and taken as a representative value for the cartilage of that donor. For synovial tissue, 3 samples were handled individually and results averaged as representative value of a donor. The n-values indicate the number of experiments (viz. the number of cartilage or synovial tissue donors).
The data were analysed using a non-parametric test for related samples (Wilcoxon signed rank test), using SPSS 12.0.1 software. Differences were considered to be statistically significantly different when $p \leq 0.05$, and is indicated in the figures by an asterisk.

RESULTS

Direct beneficial effects of IL-10 on the matrix turnover of healthy and impaired cartilage

Proteoglycans are one of the main components of the cartilage matrix. Addition of IL-10 to healthy cartilage in the first four days of culture ultimately resulted in an increase in proteoglycan synthesis rate of +12% and +5%, an increase in proteoglycan content of +7% and +2%, and a decrease in proteoglycan release of -3% and -11%, for 1 and 10 ng/ml IL-10, respectively. Although these effects after the recovery period were not dose dependently and only statistically significant for 1 ng/ml IL-10 for proteoglycan synthesis rate ($p<0.01$) and for proteoglycan content ($p<0.02$), they were in all cases beneficial.

For impaired cartilage, similar effects were observed; +5%, +25%, and +3%, +9%, for proteoglycan synthesis rate and – content for 1 and 10 ng/ml, respectively, and -8% and -4% for proteoglycan release for both concentrations of IL-10, respectively. There was again a tendency towards beneficial effects on cartilage with a change in the measured parameters of a same magnitude as seen for healthy cartilage, but again dose independent, and for this type of cartilage only statistically significant for the increase in proteoglycan content at a concentration of 10 ng/ml ($p<0.02$).

IL-10 prevents cartilage damage induced by exposure to RBC + MNC

Exposure of cartilage to the combination of red blood cells (RBC) and mononuclear cells (MNC) leads to a disturbance in proteoglycan turnover. The proteoglycan synthesis rate decreases (figure 1; upper left panel), the proteoglycan release increases (figure 1; upper right panel), and the proteoglycan content decreases (figure 1; lower left panel), measured after twelve days of recovery in the absence of RBC+MNC after an initial 4-day exposure to 50% v/v; all statistically significant.

The presence of IL-10 during the exposure had a protective effect to this disturbance in proteoglycan turnover, statistically significant for 10 ng/ml for all three parameters. This effect was dose-dependent; the effects of 10 ng/ml IL-10 were more outspoken than the effects of 1 ng/ml.

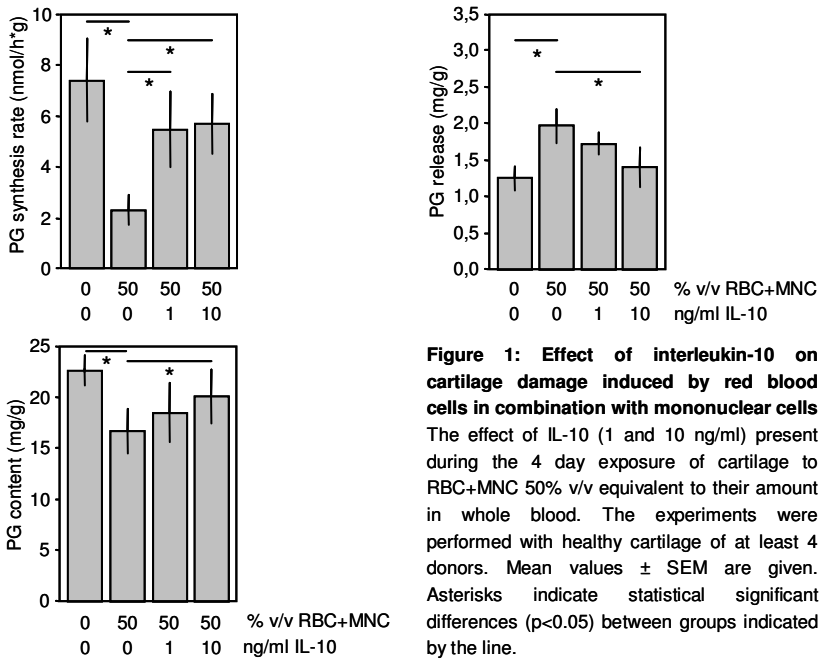

Figure 1: Effect of interleukin-10 on cartilage damage induced by red blood cells in combination with mononuclear cells
The effect of IL-10 (1 and 10 ng/ml) present during the 4 day exposure of cartilage to RBC+MNC 50% v/v equivalent to their amount in whole blood. The experiments were performed with healthy cartilage of at least 4 donors. Mean values ± SEM are given. Asterisks indicate statistical significant differences ($p<0.05$) between groups indicated by the line.

IL-10 inhibits blood-induced cartilage damage

When healthy cartilage was exposed to whole blood, similar effects as found for the exposure to RBC+MNC were seen (table 1). This was also observed for impaired cartilage i.e. cartilage with fibrillated surface and disturbed proteoglycan turnover (table 1).

	Healthy cartilage			Impaired cartilage		
	Control	Blood	p	Control	Blood	p
Synthesis rate (nmol/h*g)	6.99 ± 0.81	1.23 ± 0.19	0.00	5.99 ± 1.45	0.90 ± 0.24	0.02
Release (mg/g)	0.83 ± 0.14	2.03 ± 0.29	0.00	1.17 ± 0.19	2.52 ± 0.54	0.02
Content (mg/g)	20.11 ± 1.41	16.69 ± 1.81	0.00	15.73 ± 2.14	13.69 ± 2.31	ns

Table 1: Effect of blood on healthy and impaired cartilage
The effect of exposure of cartilage to 50% v/v blood for 4 days on the proteoglycan synthesis rate, - release and - content, determined after 12 additional days of recovery in the absence of blood. The experiments were performed with healthy cartilage of at least 8 donors and with impaired cartilage of at least 4 donors. Mean values ± SEM are given. p-values for differences between controls and the blood-exposed cartilage are given.

When IL-10 was present during the 4-day exposure of healthy cartilage to 50% v/v blood, the proteoglycan synthesis rate after the additional 12-day recovery period was on average +34% (1 ng/ml) and +132% (10 ng/ml) higher than the synthesis rate of the blood-exposed cartilage without IL-10 (figure 2a; left panel).
The increase in proteoglycan release as a consequence of the blood exposure was diminished with approximately -40% in the presence of 1 and 10 ng/ml of IL-10 during the exposure (figure 2a; middle panel). After recovery, proteoglycan content was higher (~+20%) in the cartilage samples co-cultured with 10 ng/ml IL-10 during the 4 day blood exposure (figure 2a; right panel) when compared to cartilage cultured with blood without IL-10. With 1 ng/ml IL-10 the effect was slightly less outspoken and not statistically significant for proteoglycan synthesis rate and – content.
These protective effects of IL-10 against blood-induced damage of healthy cartilage were also observed when already impaired cartilage was exposed to blood. The effects were less pronounced (figure 2b), but clearly dose dependent and also statistically significant for the 10 ng/ml IL-10.

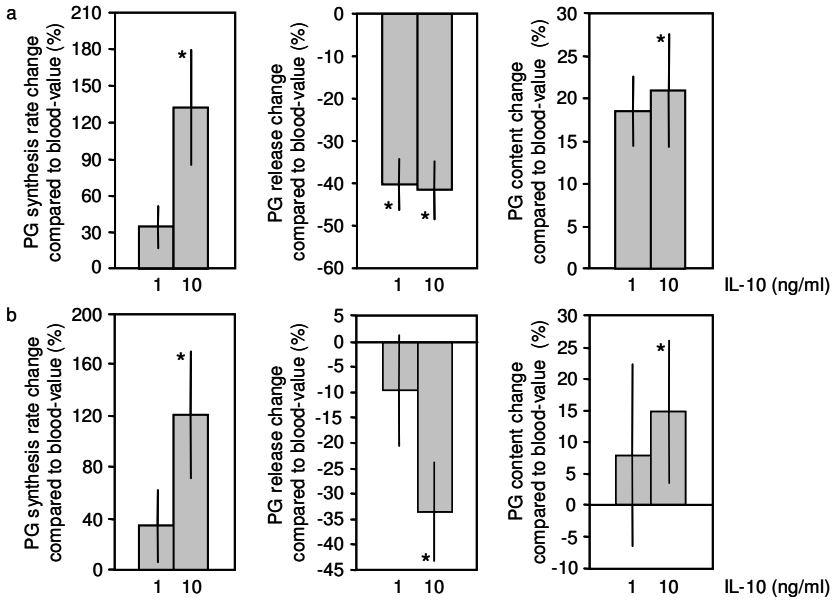

Figure 2: Effect of interleukin-10 on blood-induced cartilage damage
The effect of IL-10 (1 and 10 ng/ml) present during the 4-day exposure of cartilage to blood (50% v/v). The values are given as percentage compared to the control values viz. cartilage with blood, but without IL-10. The experiments were performed with healthy cartilage of at least 8 donors (upper panels; **a**) and impaired cartilage of at least 4 donors (lower panels; **b**). Mean values ± SEM are given. Asterisks indicate statistical significant difference compared to the controls without IL-10 ($p<0.05$).

IL-10 beneficially affects cartilage and synovial tissue from patients with haemophilic arthropathy

In figure 3a the effect of the addition of 10 ng/ml IL-10 in cultures of cartilage from patients who underwent total joint replacement surgery due to haemophilic arthropathy are presented. In the presence of 10 ng/ml IL-10 during the first four days of culture, the proteoglycan synthesis rate was increased after the recovery period. The proteoglycan release was diminished. The proteoglycan content was not statistically significantly affected, although a slight tendency towards an increase in content was seen. Taken together the addition of 10 ng/ml IL-10 in the first four days of culture of cartilage from patients with severe haemophilic arthropathy was beneficial for the proteoglycan turnover of this severely impaired cartilage.

Synovial tissue from patients with haemophilia that suffered from severe arthropathy produced abundant amounts of TNFα and IL-1β. IL-10 reduced this TNFα and IL-1β production significantly on average with -60% and -83%, respectively, both statistically significant (figure 3b).

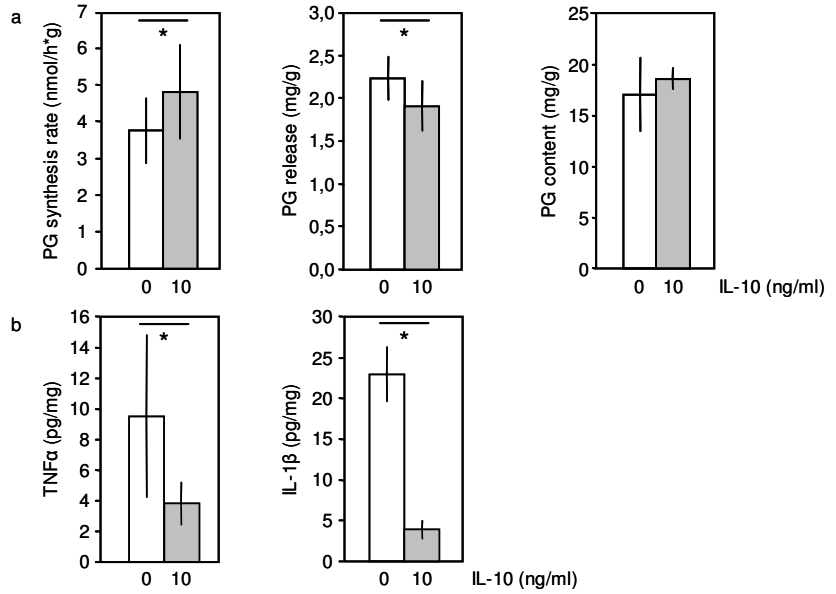

Figure 3: Effect of interleukin-10 on cartilage and synovial tissue from patients suffering from haemophilic arthropathy

a: The effect of IL-10 (10 ng/ml) on cartilage obtained from 3 patients with end-stage haemophilic arthropathy. Cartilage was cultured in the absence (white bars) or presence (grey bars) of 10 ng/ml IL-10 for four days, followed by a subsequent culture of 12 days in the absence of IL-10. Mean values ± SEM are given. Asterisks indicate statistical significant difference ($p<0.05$).

b: Synovial tissue from 4 patients with haemophilic arthropathy was cultured in the absence (white bar) or presence (grey bar) of 10 ng/ml IL-10. After two days, the culture supernatants were assessed for the amount of IL-1β and TNFα. Mean values ± SEM are given. Asterisks indicate statistical significant difference ($p<0.05$).

DISCUSSION

The present *in vitro* results demonstrate that IL-10 protects both healthy and already impaired joint cartilage, at least to a certain extent, from damage as result of a short exposure to blood. Moreover, joint cartilage already severely affected by joint bleeds *in vivo* in haemophilia patients was influenced beneficially by *ex vivo* treatment with IL-10. These protective and reparative effects of IL-10 on the matrix turnover of cartilage were accompanied by anti-inflammatory properties as demonstrated by a reduction of the release of IL-1β and TNFα, both important cartilage destructive and pro-inflammatory cytokines, from the synovial tissue from joints with haemophilic arthropathy.

The protective effect of IL-10 was dose-dependent and led to an almost normalization of proteoglycan synthesis rate, -release, and -content by addition of 10 ng/ml IL-10 to co-cultures of cartilage with RBC+MNC. Upon addition of IL-10, these parameters were not significantly different from those of the control cultures without RBC+MNC. When cartilage was exposed to whole blood with equivalent amounts of RBC and MNC, effects were also dose dependently, but did not lead to complete normalization with 10 ng/ml IL-10. This difference in efficacy might originate from the observation that the harmful effects of whole blood were slightly more severe than those of isolated RBC+MNC (see figure 1 and table 1), which fits previous observations[8;9]. Another explanation can be a potential neutralizing effect of whole blood on IL-10 activity; it is generally known that serum proteins will bind cytokines such as IL-10 decreasing its activity. No higher concentrations of IL-10 were tested, because the concentrations were based on the serum levels reached during *in vivo* human treatment. In this respect this approach allows a more realistic translation from our *in vitro* results to potential clinical application.

Importantly, the effects of IL-10, present during the first 4 days of culture (the actual exposure), were measured after a prolonged recovery period in the absence of IL-10. This direct short-term treatment is relevant to clinical practice because the first steps to clinical practice will most reasonably be intra-articular injections with IL-10. However, a shortcoming of the study set-up is that treatment in our *in vitro* approach started concomitant with the blood exposure. A lag time of at least several hours to a day would have mimicked clinical practice better. The possible decrease in the effect of IL-10 can possibly be overcome by the presence of IL-10 for a longer period of time, i.e. even when the bleeding has subsided completely. This, however, needs further *in vitro* and animal *in vivo* studies.

Severe joint destruction as a consequence of frequent joint bleeding is a major problem for patients suffering from severe forms of haemophilia[1-3]. Also joint bleeds as a result of joint trauma may add to joint damage in time[30-33]. In the process of blood-induced joint destruction, both the synovial tissue and the cartilage are involved. The synovial tissue accomplishes clearance of blood from the joint. This will

lead directly to the production of pro-inflammatory and cartilage destructive mediators[34]. Repeated exposure leads ultimately to chronically inflamed synovial tissue, amongst other by haemosiderin deposition, also leading to the formation of tissue destructive mediators, such as IL-1β and TNFα[4]. It was clearly demonstrated that IL-10 is able to significantly reduce the production of these inflammatory and tissue destructive cytokines produced by chronically inflamed synovial tissue, in addition to the beneficial effects of IL-10 on the proteoglycan turnover of the cartilage.

Previously, we have proposed a process leading to blood-induced arthropathy, which is schematically depicted and described in figure 4. On the basis of literature, the following is speculated: First, IL-10, being an anti-inflammatory cytokine, will have an anti-inflammatory effect on the synovial tissue. IL-10 has been reported to inhibit the release of pro-inflammatory and tissue destructive cytokines and proteases such as MMPs, the latter accompanied by enhancing tissue inhibitor of metalloproteinases-1 (TIMP-1)[35;36]. All these factors have been shown to be involved in blood-induced arthropathy[4;7;9].

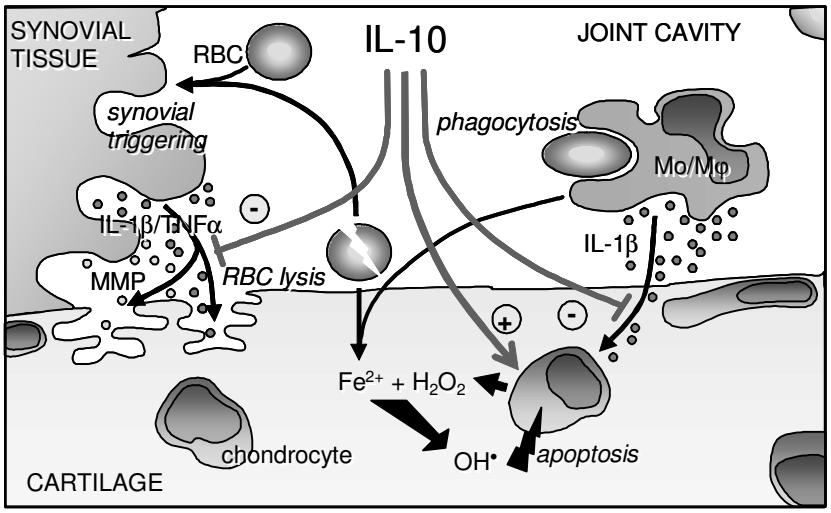

Figure 4: The mechanism of blood-induced arthropathy and IL-10
When blood is present in de joint cavity it will be taken up by the synovial tissue leading to synovial inflammation and thereby production of inflammatory cytokines and MMPs, which have a damaging effect on the cartilage. Erythrocytes are taken up by the macrophages, which upon this stimulation produce IL-1β activating the chondrocyte. By this, the chondrocyte produces increasing amounts of hydrogen peroxide and this hydrogen peroxide reacts with the iron, released from the erythrocytes, and hydroxyl radicals are formed which induce apoptosis of the chondrocyte. IL-10 can have an effect on at least three pathways in this process of blood-induced joint damage as indicated.

Second, IL-10 is able to inhibit macrophage activation. As a consequence of this, less IL-1 will be released by macrophages, diminishing the formation of hydrogen peroxide. This reduction of one of the components of the Fenton reaction results in a decrease in the formation of cytotoxic hydroxyl radicals. Of importance in this perspective is, that although IL-10 in general has an inhibiting effect on the macrophage, it does not lead to inhibition of phagocytosis by the macrophage[37]. Therefore, administration of IL-10 after a joint bleed will probably not lead to a lower rate of clearance of blood from the joint. On the contrary, there is even evidence that IL-10 can up-regulate phagocytosis under certain conditions[38], which would perhaps even enhance the clearance rate, which might be considered beneficial.

Third, in addition to neutralising the adverse effects of blood, IL-10 will have direct chondroprotective effects on cartilage. We demonstrated that IL-10 has a direct beneficial effect on proteoglycan turnover of healthy cartilage, supporting previous results[20;26], already affected cartilage, and even on end-stage haemophilic arthropathy cartilage. Inhibition of production of hydrogen peroxide by chondrocytes therefore is possibly not only due to a diminished availability of IL-1, but probably also to the direct influence of IL-10 on the chondrocyte.

Taken together, the protective and reparative effects of IL-10 on cartilage, accompanied by its anti-inflammatory properties, measured *in vitro* and *ex vivo,* warrants further studies to evaluate IL-10 as a candidate in treatment of tissue damaging effects of joint haemorrhages.

REFERENCES

1. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
2. Roosendaal G, Lafeber FP. Blood-induced joint damage in hemophilia. Semin.Thromb.Hemost. 2003;29:37-42.
3. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
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;80:540-545.
5. Roosendaal G, Mauser-Bunschoten EP, de Kleijn P et al. Synovium in haemophilic arthropathy. Haemophilia. 1998;4:502-505.
6. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
7. 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. 2006;56:199-207.
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;24:1350-1354.
9. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
10. Goddard NJ, Mann H. Diagnosis of haemophilic synovitis. Haemophilia. 2007;13 Suppl 3:14-19.
11. Rodriguez-Merchan EC. Pathogenesis, early diagnosis, and prophylaxis for chronic hemophilic synovitis. Clin.Orthop.Relat Res. 19976-11.
12. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
13. 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;42:784-790.
14. Apparailly F, Verwaerde C, Jacquet C et al. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. J.Immunol. 1998;160:5213-5220.
15. Isomaki P, Luukkainen R, Saario R, Toivanen P, Punnonen J. Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium. Arthritis Rheum. 1996;39:386-395.
16. Joosten LA, Lubberts E, Durez P 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;40:249-260.
17. Jorgensen C, Apparailly F, Couret I et al. Interleukin-4 and interleukin-10 are chondroprotective and decrease mononuclear cell recruitment in human rheumatoid synovium in vivo. Immunology 1998;93:518-523.
18. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J.Exp.Med. 1994;179:1517-1527.
19. Morita Y, Yamamura M, Kawashima M 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.
20. 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;39:829-835.
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. Arthritis Rheum. 2001;44:3-12.
22. Walmsley M, Katsikis PD, Abney E et al. Interleukin-10 inhibition of the progression of established collagen-induced arthritis. Arthritis Rheum. 1996;39:495-503.
23. Keystone E, Wherry J, Grint P. IL-10 as a therapeutic strategy in the treatment of rheumatoid arthritis. Rheum.Dis.Clin.North Am. 1998;24:629-639.

24. Chernoff AE, Granowitz EV, Shapiro L et al. A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. J.Immunol. 1995;154:5492-5499.
25. Fuchs AC, Granowitz EV, Shapiro L et al. Clinical, hematologic, and immunologic effects of interleukin-10 in humans. J.Clin.Immunol. 1996;16:291-303.
26. Wang Y, Lou S. Direct protective effect of interleukin-10 on articular chondrocytes in vitro. Chin Med.J.(Engl.) 2001;114:723-725.
27. van Valburg AA, Wenting MJ, Beekman B et al. Degenerated human articular cartilage at autopsy represents preclinical osteoarthritic cartilage: comparison with clinically defined osteoarthritic cartilage. J.Rheumatol. 1997;24:358-364.
28. Whiteman P. The quantitative determination of glycosaminoglycans in urine with Alcian Blue 8GX. Biochem.J. 1973;131:351-357.
29. Whiteman P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem.J. 1973;131:343-350.
30. Casteleyn PP, Handelberg F, Opdecam P. Traumatic haemarthrosis of the knee. J.Bone Joint Surg.Br. 1988;70:404-406.
31. Lohmander LS, Ionescu M, Jugessur H, Poole AR. Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. Arthritis Rheum. 1999;42:534-544.
32. Maffulli N, Binfield PM, King JB, Good CJ. Acute haemarthrosis of the knee in athletes. A prospective study of 106 cases. J.Bone Joint Surg.Br. 1993;75:945-949.
33. 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;33:1406-1415.
34. Jansen N.W.D., Roosendaal G, Wenting MJ et al. Very rapid clearance after a joint bleed in the canine knee can not prevent adverse effects on cartilage and synovial tissue. Manuscript in preparation 2008
35. 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;96:2304-2310.
36. Wang M, Hu Y, Shima I, Stearns ME. IL-10/IL-10 receptor signaling regulates TIMP-1 expression in primary human prostate tumor lines. Cancer Biol.Ther. 2002;1:556-563.
37. Grutz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. J.Leukoc.Biol. 2005;77:3-15.
38. Capsoni F, Minonzio F, Ongari AM et al. IL-10 up-regulates human monocyte phagocytosis in the presence of IL-4 and IFN-gamma. J.Leukoc.Biol. 1995;58:351-358.

Chapter 6

Very rapid clearance after a joint bleed in the canine knee can not prevent adverse effects on cartilage and synovial tissue

Submitted

N.W.D. Jansen[1,2]
G. Roosendaal[2]
M.J.G. Wenting[1]
J.W.J. Bijlsma[1]
M. Theobald[2]
H.A.W. Hazewinkel[3]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

ABSTRACT

Background

Joint bleeding leads to joint destruction. *In vitro* exposure of human and canine cartilage to blood results in long-lasting severe adverse changes in cartilage. An *in vivo* joint haemorrhage in the canine knee joint demonstrates similar adverse effects although significantly less outspoken. As a possible explanation for this discrepancy, we studied the clearance rate of blood from the canine knee joints.

Methods

Blood was injected into the knee joint of Beagle dogs, either 48 hours, 24 hours or 15 minutes before termination. The amount of red and white blood cells present in the joint cavity was determined. Chondrocyte activity and cartilage matrix integrity as well as cartilage destructive activity of synovial tissue were determined biochemically. Additionally, synovial tissue was analyzed by use of histochemistry.

Results

The amount of blood was decreased to <5% within 48 hours. Within this time period the cartilage was negatively affected and the synovial tissue showed cartilage destructive activity. Evaluation of the synovial tissue 15 minutes post-injection revealed countless numbers of intact RBC that were almost completely disappeared after 48 hours without significant recruitment of macrophages.

Conclusion

Blood is cleared very rapidly from the canine knee joint, but already has adverse effects on both cartilage and synovial tissue within that short time span. This rapid clearance can play a role in the discrepancy between long-term *in vitro* and *in vivo* effects of blood-induced joint damage since more than 10% v/v blood present for at least 48 hours is needed to induce long-term adverse effects *in vitro*.

INTRODUCTION

Recurrent joint bleeding leads to damage of articular cartilage and eventually to destruction of the joint[1-4]. In case of repeated joint bleeds as in the clotting disorder haemophilia, this is a generally observed phenomenon, but it can also occur after joint trauma. Insight into the pathogenetic mechanism of blood-induced arthropathy has evolved from various *in vitro* and animal *in vivo* studies. It is shown that blood has a direct effect on cartilage, which is caused by the combination of mononuclear cells (MNC) and red blood cells (RBC) as present in whole blood[5]. Via several steps apoptosis of the chondrocytes is induced[6], leading to serious disturbance of the cartilage matrix turnover. Furthermore, joint bleeding has an effect on the synovial tissue, which initially is expressed by hypertrophy of the synoviocytes, hypervascularization[3], even increasing the risk of new bleeds, and by inflammatory changes[7;8]. The inflammatory changes also contribute to the cartilage damage, by production of tissue-destructive enzymes and cytokines[9]. These processes ultimately lead to joint destruction.

In vitro studies revealed that an exposure of 50% volume/volume (v/v) blood for 4 days leads to long-lasting (up to 10 weeks) severe inhibition (on average >70%) of cartilage matrix proteoglycan synthesis for both human and canine full thickness cartilage explants[10]. This blood load was considered to be an underestimation of the blood load during a joint haemorrhage as seen in humans. Traumatic joint bleeds or joint bleeds due to haemophilia are believed, based on clinical practice, to reach a blood concentration far above 50% v/v and if the blood is not aspirated, it usually takes several days before the effusion subsides. Later *in vitro* studies revealed a threshold of 10% v/v blood for 2 days to be a minimum blood load to induce long-lasting disturbance of cartilage matrix turnover[11]. Lower concentrations or shorter exposure times led to less severe acute changes that were reversible.

In *in vivo* canine experiments two injections of blood in the knee, with two days in between, were assumed to mimic a blood load of 4 days of at least 50% v/v. This resulted in direct harmful effects as demonstrated by a decrease in proteoglycan synthesis, an increase in proteoglycan release and even a decrease in proteoglycan content and enhanced collagen damage[12]. These effects however, were significantly less severe than those *in vitro*. Even repeated (up to 12) intra-articular blood injections with intervals of 3-4 days did not result in the extreme effects as observed *in vitro*. Even more surprisingly, in later studies the effects appeared to be largely reversible within a follow-up of 10 weeks[10]. However, when the dogs were forced to load their affected joint, more permanent adverse changes occurred[13], from which it was concluded that one should take care with loading of a joint after an intra-articular haemorrhage.

Overall evaluation led to the conclusion that there is a significant discrepancy between the severity of the harmful effect of blood on cartilage *in vivo* when

compared to the effect *in vitro*. An explanation could be a much more rapid clearance of blood from the canine knee joint as originally anticipated on, resulting in a blood load below threshold values that induce long-lasting effects. Therefore, the present study was undertaken to study the clearance rate of blood from the canine knee joint in relation to its harmful effect.

MATERIALS AND METHODS

Experimental design

Six skeletally mature female Beagle dogs (mean age 8.2 ± 1.2 years) were obtained from the University Utrecht Central Animal Facilities. They were housed in groups of two and were fed a standard commercial diet with water *ad libitum*. The University Animal Experiments Ethical Committee gave approval for this study. Approximately four milliliter freshly collected autologous blood was injected into the knee while the dog was under short-term anesthesia (Domitor™/AntiSedan™). This was done either 48 hours (dogs 1-3; left), 24 hours (dogs 4-6; left) or 15 minutes (dogs 4-6; right) before the scheduled sacrifice of the dogs. The control joint (dogs 1-3; right) was not injected at all in order to prevent possible iatrogenic bleeding. Dogs were euthanized by intravenous injection of 10 ml Euthesate™ (200 mg/ml). The hind limbs were amputated and, upon minor arthrotomy, samples of the synovial fluid were taken. Thereafter, synovial tissue from the suprapatellar pouch and cartilage of the femoral condyles and tibial plateau of the knee joints were collected. All these latter procedures were carried out under laminar flow conditions immediately after the animals were euthanized. The amounts of red (RBC) and white blood cells (WBC) present in the joint fluid were determined immediately, by automated cell counting (Coulter Counter, Beckman).

Cartilage was cut as thick as possible, taking care to exclude the underlying bone, and was subsequently cut into square pieces, weighed aseptically (5.8 ± 0.3 mg) and incubated individually in 96-well round-bottomed microtiter plates in 200 µl culture medium per well. For the determination of the *ex vivo* proteoglycan synthesis rate as a measure of the chondrocyte matrix synthesis, the rate of sulphate incorporation was determined. To this end, $Na_2{}^{35}SO_4$ (DuPont, NEX-041-H, carrier free) was added in 10 µl aliquots of 148 kBq per well. After 4 hours pulse labelling of the sulphated glycosaminoglycans (GAGs), the cartilage samples were washed twice in ice cold PBS and stored at -20°C. Thawed samples were digested with papain (Sigma P3125) as described previously[11]. Papain digests were diluted to the appropriate concentrations for the analysis of the proteoglycan synthesis rate and DNA content.

For the determination of the proteoglycan release, cartilage explants were pre-cultured for one day to remove detached proteoglycans, resulting from cutting the cartilage samples. After this pre-culture, cartilage explants were put in fresh culture

medium and cultured for three days after which the amount of proteoglycans released in this 3-day culture period were determined as described below.
The synovial tissue was cut into 5 pieces, of which 3 pieces were fixed in 4% phosphate-buffered formalin, for histochemistry as described below and 2 pieces were cultured for four days in 5 ml culture medium. After culture, the supernatants were harvested and rendered cell free by centrifugation (1000xg, 10 min). The two supernatants of the synovial tissue samples of the same joint were pooled. These pooled supernatants were added to cultures of homologous (Beagle) cartilage from the hip joint, after one day of pre-culture of the cartilage, in 5% and 50% v/v concentration (diluted in culture medium). The change in proteoglycan synthesis rate (5% cultures), - release (50% cultures) and - content (50% cultures) of the cartilage exposed for four days to the synovial tissue culture supernatants compared to control cartilage (without the addition of supernatants) was considered as a measure for the catabolic (tissue destructive) properties of that synovial tissue sample.
Culture medium of both cartilage and synovial tissue cultures consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 µg/ml), ascorbic acid (85 µM) and 10% heat-inactivated Beagle serum. Cultures were performed in a tissue incubator under 5% CO_2 in air, at 37°C, and 95% humidity.

Cartilage analysis
For the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (CPC, Sigma; C9002). The precipitate was dissolved in 3M NaCl and the amount of radioactivity in the sample measured by liquid scintillation analysis. The rate of sulphate incorporation was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage and expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage tissue (nmol/h*g).
The proteoglycan content of the cartilage tissue digest was determined by the GAG content of the digest. GAGs were stained and precipitated with Alcian Blue[14;15] (Sigma A5268). Alcian Blue staining was determined by the change in absorbance at 620nm. Chondroitin sulphate (Sigma C4383) was used as a reference. The proteoglycan content was expressed in mg GAG per gram wet weight of the cartilage explants (mg/g).
The DNA content of the cartilage tissue digests was determined as a measure of the cellularity of the cartilage samples using the fluorescent dye Hoechst 33258 (Calbiochem 382061). Calf thymus DNA (Sigma D4764) was used as a reference. DNA content is expressed as milligram DNA per gram wet weight of cartilage (mg/g). In all conditions (experiments), there was no change in DNA content, viz. the cellularity, of the cartilage due to blood exposure.

Proteoglycan release was determined by the loss of GAGs in the culture medium. GAGs were stained and precipitated with Alcian Blue as described above. The proteoglycan release was calculated as mg GAG released per gram wet weight of the cartilage (mg/g).

Synovial tissue histology

For histochemistry, samples of synovial tissue, fixed in 4% phosphate-buffered formalin, were embedded in paraffin wax after standard processing. Deparaffined sections of synovial tissue were stained with either hematoxylin-eosine (HE) to quantify inflammation and the amount of RBC, with MAC387 (AbD Serotec MCA874G; human with canine cross-reactivity) for macrophages/monocytes or with Perls' Prussian Blue for iron deposits. Each specimen was analyzed by two independent observers who were blinded to the source of the samples. The severity of the inflammation was scored using the slightly modified[16] criteria described by Goldenberg and Cohen[17] for HE-sections. The presence of RBC were scored in the HE sections as follows: 0: no RBC; 1: single RBC throughout the tissue; 2: small clusters of RBC; 3: large clusters of RBC. In the sections stained with MAC387 or with Perls' Prussian Blue the amount of positive cells per mm^2 were determined.

Verification experiment

Two observations from this study were rather astonishing: the rapid clearance of RBC from the joint and the presence of huge amounts of intact RBC in the synovial tissue after 15 minutes. Therefore, the study was repeated for these two parameters. An identical experiment was performed (6 dogs aged 9.8 ± 0.2 months) and both RBC and WBC count in the synovial fluid was performed as well as histochemistry of the synovial tissue.

Calculations and statistical analysis

For the direct effects on cartilage, parameters were determined in six explants taken from fixed locations from the femoral condyles as well as in six from the tibial plateau. Because for all parameters, no differences in effect were found for the condyles and the plateau, the mean of the 12 samples was taken as representative for that particular knee.

For the cartilage destructive properties of the synovial tissue, each pooled supernatant was added to 8-10 cartilage explants. The mean of these 8-10 explants was taken as a representative for that synovial tissue culture supernatant.

The data were analysed using a parametric test for unrelated samples (independent samples T-test), using SPSS 12.0.1 software. Data are presented as means ± SEM of three joints for each time point and parameter. Asterisks indicate a p value ≤ 0.05.

RESULTS

Clearance

Directly after injection of autologous blood, the concentration of red blood cells (RBC) in the synovial fluid was $5.7*10^{12}$ per L (figure 1a). This number is within the normal range of RBC in whole blood of Beagles[18], suggesting that the joint cavity was almost completely filled with blood and that the volume of synovial fluid originally present was negligible. Twenty four hours after the injection of blood, the amount of RBC was decreased to $1.6*10^{12}$ per L, and 48 hours post-injection this amount was further decreased to $0.2*10^{12}$ per L, i.e. a decrease of 71% and 96%, respectively. Because of this rapid evacuation of RBC from the joint, this part of the study was repeated with six additional animals, providing almost identical results (bars in the background of figure 1a).

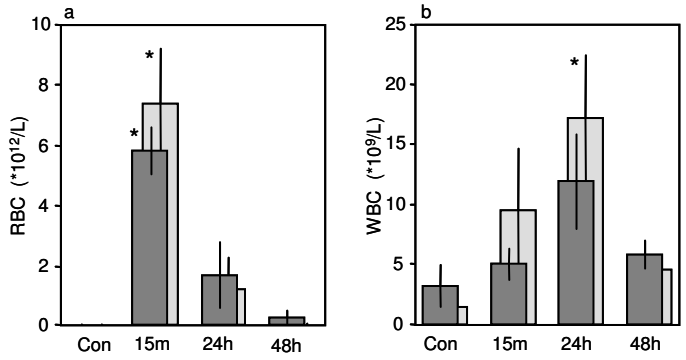

Figure 1: Red and white blood cells in the synovial fluid
Concentration of red blood cells (**a**) and white blood cells (**b**) as present in the synovial fluid, in joints without injection of blood (Con) and in the joints 15 minutes (15m), 24 hours (24h) or 48 hours (48h) after blood injection. Bars in the background are from the second (verification) experiment. Mean values ± SEM are given; n=3 for both sets of experiments. Asterisks indicate statistical significant differences ($p \leq 0.05$) compared to the control values.

Control joints, not containing any RBC, contained about $3.2*10^{9}$ white blood cells (WBC) per L (figure 1b), which is within the normal range of WBC in canine synovial fluid[19]. The amount of WBC measured 15 minutes after the injections, being $5.0*10^{9}$ per L, was within the normal range as present in whole blood of Beagles[18]. After 24 hours, it was increased up to $11.9*10^{9}$ per L, but after 48 hours decreased to $5.8*10^{9}$ per L, suggesting transient recruitment of WBC. These numbers were also obtained with the second set of animals (bars in the background of figure 1b).
There was no clear sign of clotting of the blood in the joint cavity at any time point.

Direct harmful effects on cartilage

Despite this very quick clearance, the short presence of blood led to adverse changes in cartilage proteoglycan turnover as depicted in figure 2. There was no significant effect of the presence of blood for 15 minutes on neither proteoglycan synthesis rate nor proteoglycan release. Twenty four hours post-injection, the proteoglycan synthesis rate (figure 2a) of cartilage was decreased with 22% compared to that in control cartilage (from the uninjected knee), although not statistically significant. This inhibition in proteoglycan synthesis rate was increased to 34% ($p \leq 0.05$) 48 hours post-injection, suggesting a time-dependent process. Similarly, a time-dependent increase in release of proteoglycans (figure 2b) was observed, +24%; +53% for 24 and 48 hours respectively, although not statistically significant.

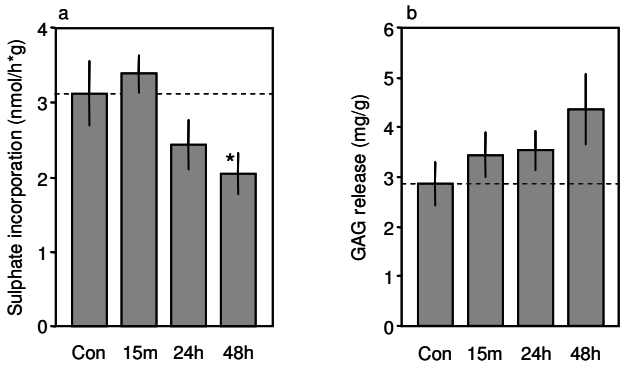

Figure 2: Effects of intra-articular blood injections on cartilage matrix turnover
Proteoglycan synthesis rate (**a**) and release of proteoglycans (**b**) were measured 15 minutes (15m), 24 hours (24h) and 48 hours (48h) after intra-articular injections of autologous blood. The control (Con) values are of cartilage of joints without injections. Mean values ± SEM are given; n=3. Asterisks indicate statistical significant differences ($p \leq 0.05$) compared to the control values.

Synovial tissue

Tissue destructive properties of synovial tissue

Culture supernatants of synovial tissue explants of the blood exposed joints were added to healthy canine hip cartilage to assess the effect of haemarthrosis on cartilage destructive properties of the synovial tissue. Addition of control synovial tissue culture supernatants led to the usually observed decrease in proteoglycan synthesis rate (figure 3a), but this decrease almost doubled when the synovial tissue had been exposed *in vivo* to blood for 24 hours or 48 hours.

Addition of control supernatants or supernatants from synovial tissue obtained 15 minutes post-injection had no effect on the proteoglycan release of healthy cartilage (figure 3b), but the release was tripled (+200%) when supernatants of the 24 hours or 48 hours ($p \leq 0.05$) *in vivo* blood-exposed synovial tissues were added.

The inhibition of synthesis rate and enhancement of release resulted in a decrease of the proteoglycan content of the cartilage (figure 3c), which appeared to be time-dependent as well. A decrease of more than 25% ($p < 0.05$) was observed when culture supernatants of synovial tissue from the 48 hours group were added.

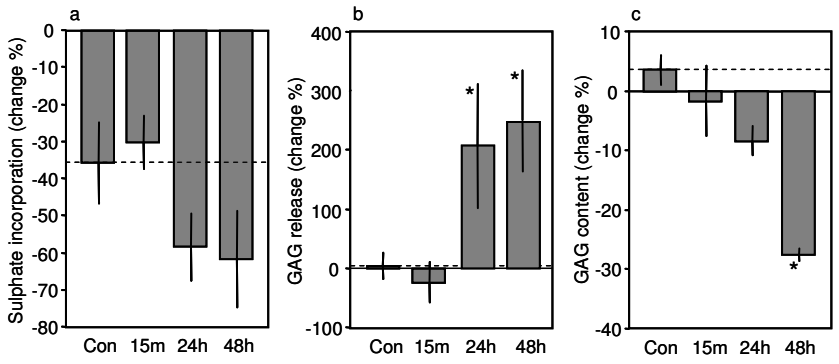

Figure 3: Effects of synovial tissue culture supernatants on the matrix turnover of healthy cartilage

The effect of intra-articular blood on the catabolic activity of synovial tissue was measured by the effects of supernatants of these synovial tissue cultures on the matrix turnover of healthy canine hip cartilage. The proteoglycan synthesis rate (**a**), - release (**b**), and - content (**c**) were measured. Synovial tissue originated from joints without blood injections (Con), or from synovial tissue 15 minutes (15m), 24 hours (24h), or 48 hours (48h) after the intra-articular injections with blood. Mean values ± SEM are given (n=3) as a percentage compared to cartilage cultures without the addition of culture supernatants. Asterisks indicate statistical significant differences ($p \leq 0.05$) compared to the control values of uninjected joints.

Histology

The synovial tissue was stained with either hematoxylin and eosin for evaluation of general inflammatory characteristics and RBC presence, with MAC387 for monocytes/macrophages, or with Perls' Prussian Blue for iron depositions. Photographs of histological sections at the four time points after blood exposure are shown in figure 4a-d.

Representative HE stained sections are depicted in figure 4a and b for the control joint and for tissue obtained 15 minutes after injection, respectively. Despite the synovial triggering, there was no clear sign of inflammation. The slightly modified[16] Goldenberg and Cohen[17] score (figure 5a) was not significantly influenced and was low compared to the maximum score of 10 that can be reached.

Striking was the enormous influx of RBC in the synovial tissue within 15 minutes (figure 4b and 5b). The inset clearly shows numerous intact RBC in between the synovial tissue cells. These numbers of RBC decreased quickly, in parallel with the RBC count in the joint cavity (figure 1a).

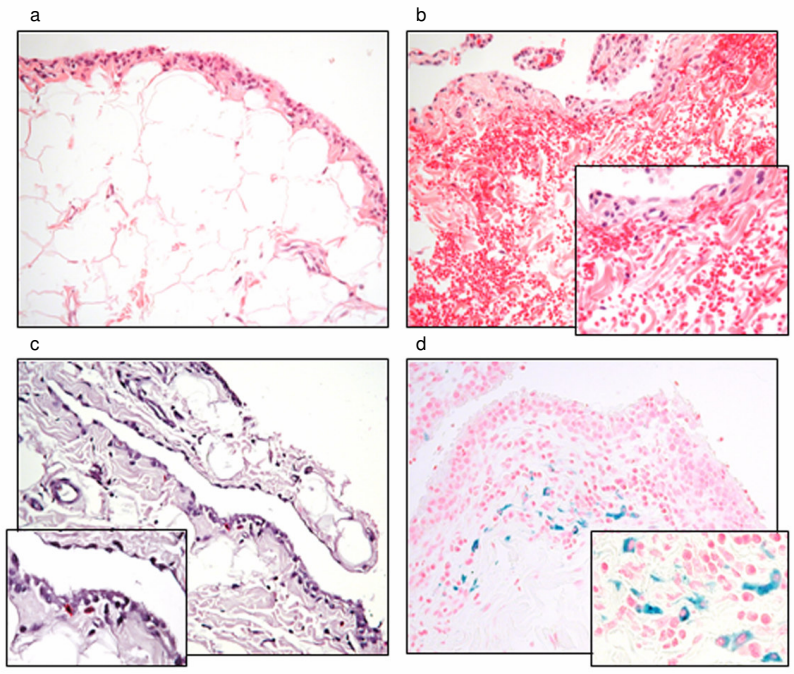

Figure 4: Histological sections of synovial tissue

Light micrographs of synovial tissue stained with hematoxylin and eosin (**a**, **b**), MAC387 (**c**) or Perls' Prussian Blue (**d**). Synovial tissue originated from joints without injections with blood (control; a), or from synovial tissue 15 minutes (b), 24 hours (c), or 48 hours (d) after blood injection. Original magnification: 20x.

The amount of MAC387 positive cells (figure 4c and 5c), representing monocytes/macrophages, was increased ($p \leq 0.05$) after 15 minutes and appeared to increase slightly in time. However, overall the amount of MAC387 positive cells was low.

The amount of Perl's Prussian Blue positive cells was the highest after 48 hours (figure 4d and 5d). Nevertheless, the amount of Perl's Prussian Blue-positive cells remained very low when compared to synovial tissue of joints exposed to blood frequently as in haemophilic arthropathy[9]. A representative photograph of a high amount of Perls Prussian Blue-positive cells is shown in figure 4d.

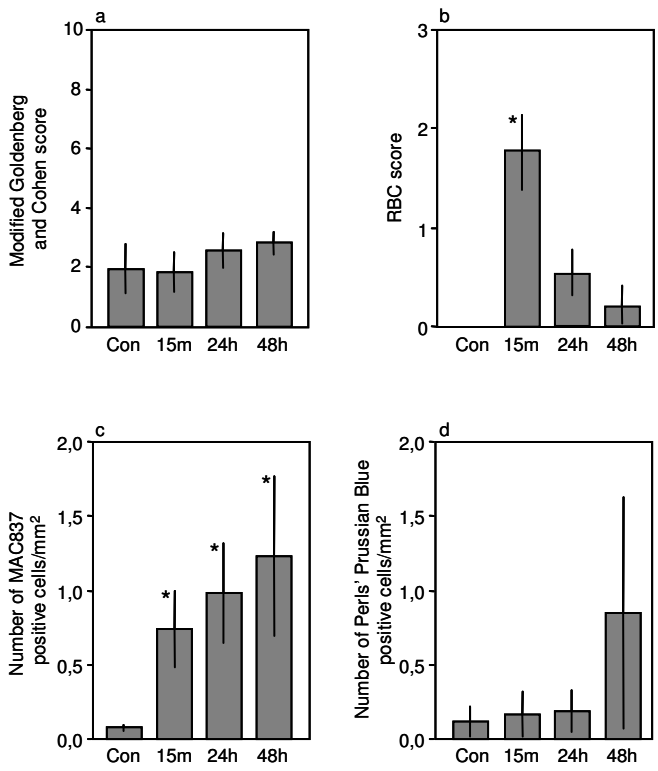

Figure 5: Histological scores

a: Modified Goldenberg and Cohen score for inflammation in HE-sections; **b**: score for the presence of RBC in HE sections; **c**: number of MAC387-positive monocytes/macrophages; **d**: number of Perls' Prussian Blue-positive cells for iron deposits. Synovial tissue originated from joints without blood injection (Control), or from synovial tissue 15 minutes (15m), 24 hours (24h), or 48 hours (48h) after intra-articular injection with blood. Mean values ± SEM are given of n=6, including the verification experiment.

DISCUSSION

This study shows that blood is cleared very rapidly from the canine knee joint: within 48 hours, the concentration of RBC in the joint cavity is decreased to less than 5%. The exact mechanism of this rapid clearance remains unsolved. Despite this quick clearance, adverse effects on both cartilage and synovial tissue are initiated.

The principal reason for this study was to elucidate the discrepancy between the severe harmful effects of blood on cartilage explants observed *in vitro* and the significantly less severe adverse effects observed in canine *in vivo* experiments. We hypothesized that an underestimation of the clearance rate could be one of the possible explanations, because rapid clearance leads to a shorter and less intense blood exposure. Recently we showed that *in vitro* the threshold for long-lasting effects is 10% v/v blood for 48 hours[11]. The present results demonstrate that *in vivo* in the canine joint the blood load is lowered below this level within this period (less than 5% in 48 hours). Moreover, in the *in vitro* situation, the blood is present in a fixed amount during the whole exposure period. In the *in vivo* situation, the blood decreases gradually in time. In the *in vitro* studies a clear time- and dose-dependency of the direct harmful effects of blood on cartilage was found[11]. At the threshold blood load (10% v/v for 48 hours), the effects are still long lasting but significantly reduced to less than 50% of the harmful effects observed with 50% v/v blood for 4 days. Thus, it may be concluded that indeed rapid clearance can be (at least in part) the explanation for the observed discrepancy between the *in vitro* and *in vivo* results of blood exposure.

Note that most of the *in vitro* experiments were conducted with homologous blood, whereas in the *in vivo* experiments autologous blood was used. However, the susceptibility of human and canine cartilage to homologous or autologous blood is similar (unpublished observations).

No reliable total volume measurements of joint fluid could be performed, relating the concentration of WBC to the exact volume of joint fluid. Thus, although not to be expected, it can not be excluded that the temporary increase in WBC count may in part be related to a decrease in fluid volume in the joint due to selective absorption of plasma/synovial fluid.

A potential flaw in the set up of the *in vitro* experiments may be that in the *in vivo* situation, only the articular surface is exposed to blood, whereas in the *in vitro* explant system the cartilage is exposed to all sides including the cutting edges. This can also contribute to the difference in the magnitude of the effect, and thereby also to the reversibility of this effect. This issue is currently subject of study and will be published separately.

The present observation may argue the validity of the previously published *in vitro* studies. Indeed the results of these studies have to be interpreted with caution when a translation to clinical practice is discussed. Irrespectively, it is clear that a short-

term exposure of cartilage to blood is harmful, not only *in vitro*, but also *in vivo*. The present study underscores this again by demonstrating the immediate significant adverse changes in both cartilage and synovial tissue. Although, based on previous canine *in vivo* research, most of these effects are not likely to be long-lasting, they can make the joint more vulnerable to a subsequent joint bleed or trauma, especially when this occurs within a period in which the joint has not fully recuperated. Furthermore, this (transiently) affected cartilage is less capable to withstand loading as we demonstrated *in vivo* before[13]. Moreover, some of the synovial changes such as iron deposition (haemosiderosis) may not be reversible and a permanent trigger for inflammatory activity.

A question that raised from the present study is how the quick clearance of blood from the joint is accomplished. Striking was the fact that the synovial tissue of the joints were loaded with RBC already 15 minutes after the haemarthrosis was induced, but that this amount of RBC was far less already after 24 hours and that almost no RBC were found in the synovial tissue 48 hours after injection. It has been suggested that RBC can be opsonised when entering the joint cavity and can be recognised as foreign by macrophages and synoviocytes[20]. However, although there was an increase in monocytes/macrophages, this increase was not of a magnitude expected to be able to remove this load of RBC.

Interestingly, also the degree of Perls' Prussian Blue staining was limited, suggesting only a limited deposition of iron in the form of haemosiderin in the synovial tissue. Although it is difficult to estimate how many RBC are needed to deposit sufficient iron-containing material to get the intensity and number of Perls'-positive cells as observed, it can hardly be that the increase in Perls'-staining in this study includes the iron from all the RBC cleared from the joint cavity and synovial tissue. In that case, it would have been more likely that a broader distribution of Perls'-staining was found. This suggests that rapid degradation is not taking place on a large scale. Apparently, other mechanisms of clearance must take place.

A possibility is that intact RBC re-enter the peripheral blood directly or via the lymphatic system. However, we can only speculate on this, for there is no clear evidence of the exact process. In future studies it would be interesting to label the blood cells before intra-articular injection to trace the route by which the cells are cleared from the joint cavity after a joint haemorrhage.

Previous clearance studies in rabbits[21;22] have shown a less quick clearance, varying from one- to two-third of the injected amount within one week. The contradiction with our quick clearance could originate from the limited discriminating ability of the technique used; the decrease in radioactivity signal upon injection of ^{59}Fe-labelled RBC was measured outside the joint using a probe, giving no information of the exact location of the radiolabelled RBC (synovial fluid, synovial tissue, or even peri-articular). Some tissue analysis was performed, however on most time points only from one animal, making it neither able for extensive analysis and conclusions, nor

for comparison with the present study. On the other hand, the extreme clearance rate in our study could be specific for the canine species.
Irrespectively, the rabbit study and clinical practice with haemarthroses in haemophiliacs were the premises for the estimation in our previous studies that 4 days 50% v/v blood mimics the blood load during a joint haemorrhage. It may indeed be argued (and needs to be studied) whether this extreme rapid clearance also occurs in the human joint, as clinical practice suggests differently. Based on clinical practice in the treatment of haemophilia patients (personal observation; GR), it is not likely that in humans the blood load is decreased below 10-20% within two days, meaning that in humans a single joint haemorrhage probably has more serious direct adverse effects, with a more long-lasting character, and predisposes for damage from subsequent influences such as load and repeated bleeding.
Taken together, rapid clearance of blood from the canine knee joint contributes to the discrepancy between the long-term *in vitro* and *in vivo* effects of blood-induced joint damage. More research is needed to fully elucidate the mechanisms of clearance. Irrespectively, blood has devastating effects on articular cartilage very rapidly, both directly and via the synovial tissue, and in this respect it remains important to prevent (traumatic) joint haemorrhages and if they occur, to treat them properly.

REFERENCES

1. Arnold WD, Hilgartner MW. Hemophilic arthropathy. Current concepts of pathogenesis and management. J.Bone Joint Surg.Am. 1977;59:287-305.
2. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
3. Madhok R, York J, Sturrock RD. Haemophilic arthritis. Ann.Rheum.Dis. 1991;50:588-591.
4. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
5. 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;24:1350-1354.
6. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
7. Rodriguez-Merchan EC. The destructive capabilities of the synovium in the haemophilic joint. Haemophilia. 1998;4:506-510.
8. Roosendaal G, Mauser-Bunschoten EP, de Kleijn P et al. Synovium in haemophilic arthropathy. Haemophilia. 1998;4:502-505.
9. 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;80:540-545.
10. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
11. 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. 2006;56:199-207.
12. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.
13. Hooiveld MJ, Roosendaal G, Jacobs KM 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;50:2024-2031.
14. Whiteman P. The quantitative determination of glycosaminoglycans in urine with Alcian Blue 8GX. Biochem.J. 1973;131:351-357.
15. Whiteman P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem.J. 1973;131:343-350.
16. Pelletier JP, Martel-Pelletier J, Ghandur-Mnaymneh L, Howell DS, Woessner JF, Jr. Role of synovial membrane inflammation in cartilage matrix breakdown in the Pond-Nuki dog model of osteoarthritis. Arthritis Rheum. 1985;28:554-561.
17. Goldenberg DL, Cohen AS. Synovial membrane histopathology in the differential diagnosis of rheumatoid arthritis, gout, pseudogout, systemic lupus erythematosus, infectious arthritis and degenerative joint disease. Medicine (Baltimore) 1978;57:239-252.
18. Vajdovich P, Gaal T, Szilagyi A, Harnos A. Changes in some red blood cell and clinical laboratory parameters in young and old Beagle dogs. Vet.Res.Commun. 1997;21:463-470.
19. Perman. Synovial Fluid. In: Kaneko, ed. Clinical Biochemistry of Domestic Animals.: Academic Press; 2008:749-783.
20. Vasilev VA, Vidinov NK. Comparative assessment of articular cartilage and synovial membrane in experimental haemarthrosis. Acta Biol.Hung. 1984;35:305-314.
21. Muirden KD, Peace G, Rogers K. Clearance of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. II. Autoradiographic and histological studies. Ann.Rheum.Dis. 1969;28:630-636.
22. Muirden KD. Clearance of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. I. Relationship to the anaemia of rheumatoid arthritis. Ann.Rheum.Dis. 1969;28:548-551.

Chapter 7

Blood-induced cartilage damage *in vitro*: articular exposure *versus* total exposure
an interim report

N.W.D. Jansen[1,2]
G. Roosendaal[2]
J.W.J. Bijlsma[1]
M. Theobald[2]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands

The results described in this chapter will be extended prior to submission for publication.

ABSTRACT

Background
Joint bleeds lead to joint destruction. Insight in the involved mechanisms was gained from *in vitro* and *in vivo* studies, but whereas in the *in vitro* explant culture system the cartilage is exposed to all sides, in the *in vivo* situation cartilage is exposed to blood at the articular surface only. The objective of this study was to determine whether there is a difference in response of cartilage when submerged in blood compared to exposure to just the articular surface.

Methods
Human full thickness articular cartilage tissue was exposed to 50% v/v blood for 4 days either to all sides in an explant culture system, or in a culture system enabling isolated articular exposure. Subsequently the cartilage was cultured for an additional 12 days without blood to exclude the direct reversible effect. After these 16 days, cartilage proteoglycan synthesis rate and - content were determined.

Results
Exposure of full thickness cartilage to the articular side alone led to less severe adverse effects on cartilage proteoglycan synthesis rate and - content than exposure to all surfaces including the five cutting edges**,** although the adverse effects of blood exposure were still obvious.

Conclusion
Exposure of cartilage to blood at the articular surface leads to less severe adverse effects than when cartilage explants are fully submerged in blood. This contributes to the explanation why blood-induced cartilage damage after an experimentally induced haemarthros *in vivo* is less severe compared to the previously studied *in vitro* effects of blood on cartilage. But also after articular exposure alone, blood has harmful effects on articular cartilage, and in this respect it is important to prevent (traumatic) haemarthroses and if they occur, to treat them properly.

INTRODUCTION

From clinical practice it is obvious that joint bleeds, and certainly repeated joint bleeds, eventually lead to severe joint damage; haemarthropathy[1-3]. However, there is mostly a large time span between the actual joint bleeds and the arthropathy as a resultant. This renders it difficult to gain insight in the exact pathogenetic mechanism, besides the ethical considerations and practical limitation of studies with humans. Nevertheless, insight in the pathogenesis of haemarthropathy is of great importance to be able to develop proper treatment modalities. Therefore, *in vitro* and animal *in vivo* studies have been performed in research on haemarthropathy[4-16].

In the *in vitro* studies, it has been shown that blood has a direct effect on cartilage. Exposure of full thickness articular cartilage explants to blood leads to an increase in the release of proteoglycans and a decrease in the synthesis rate of these molecules. The increased activity of proteases which degrade the cartilage matrix network of collagens and proteoglycans underlies the increase in proteoglycan loss. This leads to structural damage of the cartilage as demonstrated by a decrease of the total content of proteoglycans[9]. Apoptosis of chondrocytes, which are fully responsible for maintenance of the cartilage matrix, is involved in this process[5] and demonstrates the irreversibility of the process. The long term inhibition of cartilage matrix synthesis, even up to 70 days after an initial 4 day exposure, is not unique for human cartilage as it was also observed for canine cartilage[4].

Canine *in vivo* experiments have confirmed that cartilage is directly negatively affected by joint bleeds[7;13;17]. A decrease in proteoglycan synthesis rate, enhanced loss, diminished content, and collagen damage have been shown. Also the synovial tissue is adversely affected and contributes to the overall process leading to haemarthropathy; additions of culture supernatants of synovial tissue from joints after an experimentally induced joint bleed, to healthy cartilage *in vitro* leads to disturbance of the matrix turnover.

Although the results obtained *in vitro* are confirmed *in vivo*, these effects were significantly less outspoken than obtained *in vitro*. Most striking was the effect on proteoglycan synthesis: in general, this inhibition is close to -100% directly after an exposure to 50% v/v for 4 days *in vitro*. The inhibition of the proteoglycan synthesis rate after an experimentally induced haemorrhage *in vivo* (2 intra-articular blood injections with a 2-day interval) reached 31%[13]. In addition, the proteoglycan content decreased more outspoken *in vitro* than *in vivo*: after a 4-day exposure followed by a 12-day recovery period in the absence of blood, the proteoglycan content was diminished by ~20% *in vitro* and, although still statistically significant, by 8% *in vivo,* with a blood load assumed to be comparable. Although forced loading of the affected joint increased the adverse effects of joint haemorrhage[8], the effects remained far less outspoken than obtained *in vitro*.

Recently, we investigated the clearance rate of blood from a canine knee joint, to determine whether the experimentally induced haemarthros *in vivo* (2 injections of > 4 ml with a 2-day interval) was indeed of the same magnitude as was assumed, i.e. the same blood load and duration as in the *in vitro* condition (4 days; 50% v/v blood). It was found that the amount of blood decreased to < 5% within 2 days. Notwithstanding, within this time period the cartilage was negatively affected and the synovial tissue showed cartilage destructive properties. This rapid clearance can play a role in the discrepancy between long-term *in vitro* and *in vivo* effects of blood exposure since more than 10% v/v blood for 48 hours is needed to induce long-term adverse effects *in vitro*[18]. It is still unclear whether this extreme rapid clearance also occurs in the human joints, although clinical practice suggests differently.
Another difference between the *in vitro* and the *in vivo* situation is that whereas *in vivo* the cartilage is exposed to blood at the articular surface only, in the *in vitro* explant culture system[19], the articular full thickness cartilage tissue explants are exposed to the 5 additional cutting edges as well. The explants in culture are fully submerged in blood. This difference in exposure could add to the discrepancy between the relatively mild *in vivo* effects compared to the severe *in vitro* effects of blood on cartilage. A culture system was developed to address this issue, allowing comparison of articular exposure with full exposure.

MATERIALS AND METHODS

Cartilage culture

Healthy full thickness human articular cartilage tissue was obtained *post mortem* from humeral heads within 24 hours after death of the donor. The donors had no known history of joint disorders and showed no signs of degeneration. Cartilage was available from 24 donors (age 65 ± 2 years; 17 male/7 female). Collection of the cartilage was according to the medical ethical regulations of the University Medical Center Utrecht.

Slices of cartilage were cut aseptically, as thick as possible, excluding the underlying bone and kept in phosphate buffered saline (PBS, pH 7.4). Within 1 hour after dissection, the slices were either cut into square pieces (~2*3 mm surface, ~2 mm high) or punched with a biopsy punch (8 mm ∅; ~2 mm high). The explant cubes were weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottomed microtiter plates in 200 µl culture medium per well. The biopsy discs were put in 48-well tissue culture plate, the articular surface upwards, in 700 µl culture medium per well (figure 1a).

The discs were kept in the middle of the wells by use of Teflon rings with short spokes that allowed exposure to the side of the cartilage biopsy disc (figure 1b). On top of the cartilage discs Teflon cylinders (5 mm inner ∅) were placed, that could be adjusted in height by screwing them up or down in a stainless steel lid that was fixed to the 48-well tissue culture plate (figure 1b). By screwing the Teflon cylinder on top of the cartilage disc, isolated articular exposure could be realised within the Teflon cylinder (figure 1c).

Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 µg/ml), ascorbic acid (85 µM) and 10% heat inactivated pooled human male AB^+ serum. The cartilage was cultured in a tissue incubator under 5% CO_2 in air, at 37°C, and 95% humidity.

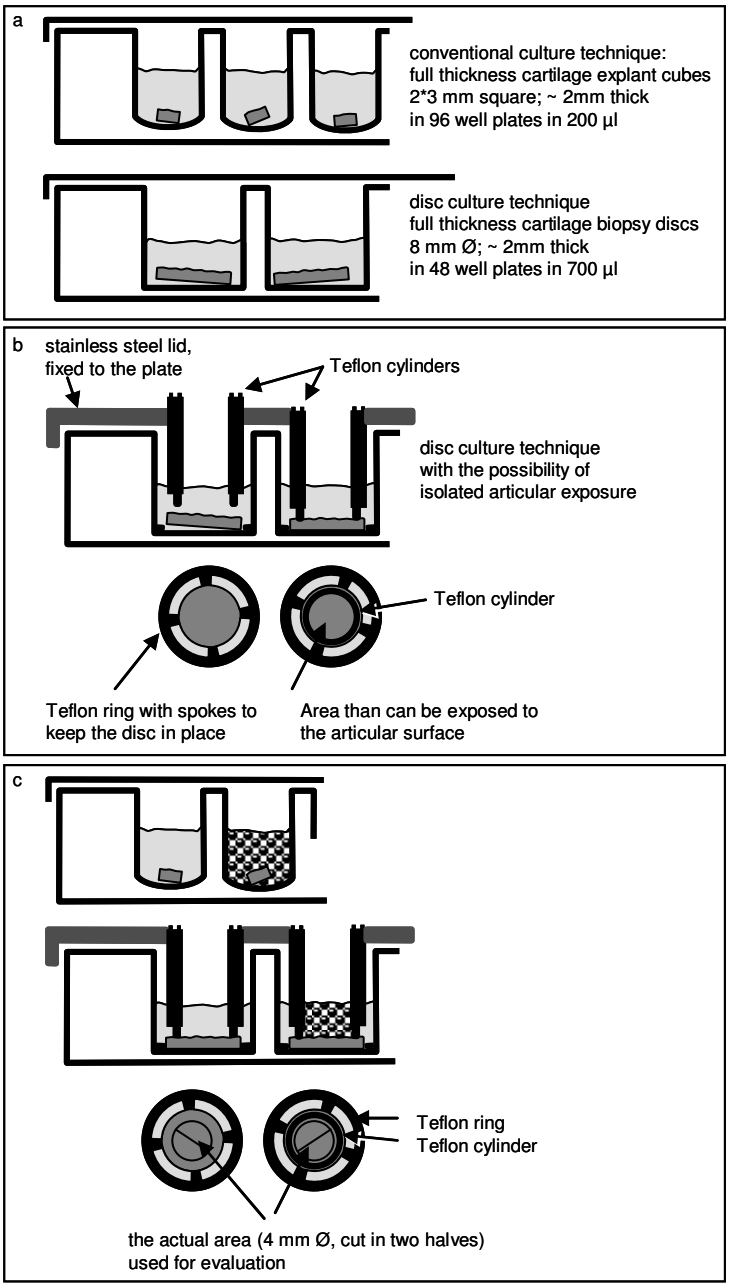

Figure 1: Culture systems and blood exposure

Schematic representation of **a:** explant cubes *versus* biopsy discs; **b:** biopsy discs with or without Teflon cylinder; **c:** total exposure of cartilage explant cubes *versus* articular surface exposure of biopsy discs to 50% v/v blood (◒).

Experimental set-up

Evaluation of the culture system
To determine whether the newly developed culture system had an influence on the proteoglycan synthesis rate and - content, first the culture of the discs was compared to that of the previously used full thickness cartilage explants (figure 1a).
To determine whether the Teflon cylinders have an effect on the cartilage, culture conditions with cylinders in tight contact with the cartilage was compared with identical culture conditions but without direct contact of the cylinder with the cartilage (figure 1b).

Exposure to blood
For each experiment, fresh blood from healthy human donors was collected into heparinized vacutainer tubes (Becton Dickinson, UK; 170 IU Li-heparin/10 ml) and added to the cartilage tissue explants in a 50% volume/volume (v/v) concentration, immediately after it was obtained. In case of blood exposure to the articular surface only, all culture medium in the cylinder, being approximately 100 µl, was replaced by 100 µl of medium with 50% v/v blood.
Previous experiments have shown that dilution of nutrients in the culture medium or the increase in serum components upon adding whole blood up to max 50% v/v do not influence proteoglycan turnover on their own[14]. Cartilage chondrocytes are not in direct cell-cell contact with the blood cells and therefore, the use of homologous blood instead of autologous blood is not of influence as has been demonstrated in canine experiments (data not shown). Furthermore, we have shown previously that the addition of heparin itself does not influence the effect of the exposure of cartilage to blood[12].

In all set-ups, after 4 days of culture, with or without blood, the cartilage was washed twice in fresh culture medium for 45 minutes under culture conditions to remove all the adherent blood components. In case of the biopsy discs the middle of the disc, i.e. the exposed part (figure 1c), was punched out with a 4 mm ∅ biopsy punch. This disc was cut in half, weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottomed microtiter plates in 200 µl culture medium. All cartilage samples, both cubes and half discs, were then cultured for an additional period of 12 days in the absence of blood. In these cultures, medium was refreshed every four days. These prolonged cultures in the absence of blood gave the chondrocytes the ability to recover from the blood exposure, excluding the direct reversible effects of blood exposure.
After these 16 days of culture 74 kBq $Na_2{}^{35}SO_4$ (DuPont, NEX-041-H, carrier free) was added in a volume 10 µl to each well for the evaluation of the proteoglycan synthesis rate. After 4 hours pulse labelling of the sulphated glycosaminoglycans

(GAGs), the cartilage samples were washed twice in ice cold PBS and stored at -20°C. Thawed samples were digested with papain (Sigma P3125) as described previously[9]. Papain digests were diluted to the appropriate concentrations for the analysis of the proteoglycan synthesis rate and - content as well as DNA content.

For the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (CPC, Sigma; C9002). The precipitate was dissolved in 3M NaCl and the amount of radioactivity in the sample measured by liquid scintillation analysis. The rate of sulphate incorporation was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage and expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage tissue (nmol/h*g).
The proteoglycan content of the cartilage tissue digest was determined by the GAG content of the digest. GAGs were stained and precipitated with Alcian Blue[20;21] (Sigma A5268). Alcian Blue staining was determined by the change in absorbance at 620nm. Chondroitin sulphate (Sigma C4383) was used as a reference. The proteoglycan content was expressed in mg GAG per gram wet weight of the cartilage explants (mg/g).
The DNA content of the cartilage tissue digests was determined as a measure of the cellularity of the cartilage samples using the fluorescent dye Hoechst 33258 (Calbiochem 382061). Calf thymus DNA (Sigma D4764) was used as a reference. DNA content is expressed as milligram DNA per gram wet weight of cartilage (mg/g). In all conditions (experiments) there was no significant change in DNA content, viz. the cellularity, of the cartilage due to blood exposure.

Calculations and statistical analysis
The n-values indicate the number of experiments (viz. the number of cartilage or synovial tissue donors). A non-parametric test for related samples (Wilcoxon signed rank test) was used to evaluate the effect of blood. The difference between total and articular surface exposure were analysed by a non-parametric test for unrelated samples (Mann Whitney U Test). Statistical analysis was performed using SPSS 12.0.1 software. Differences were considered to be statistically significant when $p \leq 0.05$, and is indicated in the figures by an asterisk.

RESULTS

Evaluation of the culture system

To study whether *in vitro* exposure of cartilage to blood at just the articular surface differs from the exposure at all sides, a culture system was developed which enables isolated articular surface exposure. For this system the use of biopsy discs of cartilage instead of the previously used explant cubes is required (figure 1a). The differences between these two types of culture had no effect on proteoglycan synthesis rate and – content (figure 2).

The articular surface exposure can be realised by placing a Teflon cylinder on top of the cartilage (figure 1b). The presence of the cylinder on top of the cartilage surface had no effect on proteoglycan synthesis rate or on proteoglycan content (figure 3).

Based on these two control studies, it was concluded that the culture system by which the articular surface exposure can be realised, is suitable for this study, since no significant differences in chondrocyte activity and matrix integrity characteristics with respect to proteoglycans were observed when compared to the frequently used original cartilage tissue explant culture system.

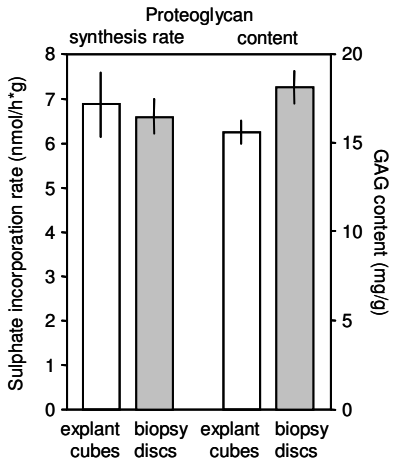

Figure 2: Explant cubes *versus* biopsy discs
Proteoglycan synthesis rate (left part of the graph) and - content (right part of the graph) were determined of cartilage cultured as explants cubes or as biopsy discs (see figure 1a). White bars indicate explant cubes; grey bars indicate biopsy discs. No statistically significant differences were found.

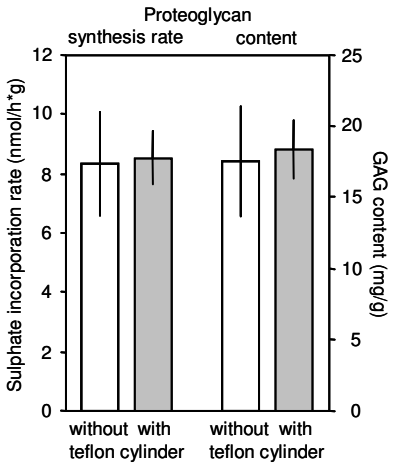

Figure 3: Biopsy discs with or without Teflon cylinder
Cartilage biopsy discs were cultured in the presence of the Teflon cylinder, either above ('without'; white bars) or on top of the cartilage ('with': grey bars) (see figure 1b). Effects on proteoglycan synthesis rate (left part of the graph) and proteoglycan content (right part of the graph) were determined. No statistically significant differences were found.

Exposure to blood

The effect of blood exposure on the articular surface alone in comparison to complete exposure (figure 1c) is depicted in figure 4a and b for proteoglycan synthesis rate and proteoglycan content, respectively. Total exposure led to an inhibition of -82% ($p<0.001$) of the proteoglycan synthesis rate. Also when only the articular surface was exposed to blood, an inhibition of proteoglycan synthesis rate was observed, although less pronounced; -52% ($p=0.034$). The proteoglycan synthesis rate of the cartilage exposed to blood at the articular surface was statistically significantly higher than that of the totally exposed cartilage ($p=0.048$). Figure 4b shows the proteoglycan content, which is decreased upon total exposure to blood with -19% ($p<0.001$). Exposure to the articular surface only led to a decrease of the proteoglycan content, with -10%, although not statistically significant from that of the fully exposed discs, less significant ($p=0.034$).

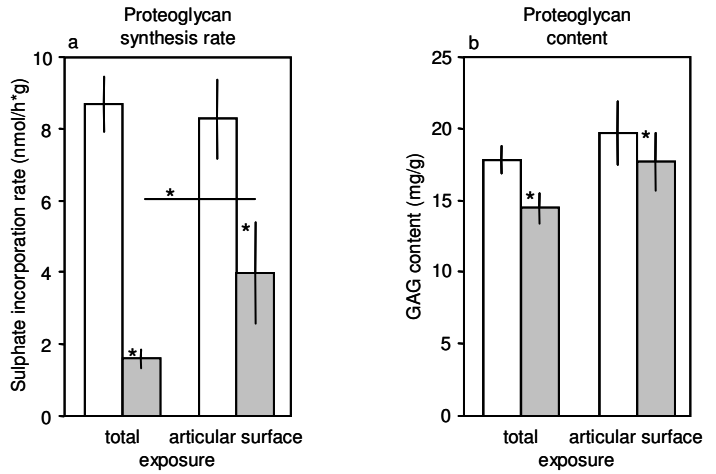

Figure 4: Total exposure *versus* articular surface exposure of cartilage to blood
a: proteoglycan synthesis rate; **b**: proteoglycan content. Cartilage was exposed to blood either totally (left part of the graph) or at the articular surface only (right part of the graph) (see figure 1c). White bars indicate the value of unexposed (control) cartilage; grey bars indicate cartilage exposed to blood. Asterisks indicate $p<0.05$.

DISCUSSION

For this study an adapted culture system was developed enabling isolated articular surface exposure of human articular cartilage. This culture system showed to be suited for cartilage culture and did not lead to changes in proteoglycan synthesis and content under control conditions. Exposure of cartilage to blood at the articular surface led to less severe effects on the proteoglycan synthesis rate and - content than when cartilage explants were exposed at all sides. This is probably part of the

explanation why blood-induced cartilage damage after an experimentally induced haemarthros *in vivo* is less severe compared to the *in vitro* effects of blood on cartilage. Irrespectively, blood still has devastating effects on articular cartilage.
These are the first results obtained with this newly developed culture system. The mechanisms behind the differences between full exposure and articular exposure can therefore only be speculated on and suggestions need additional studies that are ongoing.
The difference in the magnitude of the effect of blood exposure could be due to the smaller blood exposed surface/volume ratio of the cartilage. However, the mechanism of blood-induced cartilage damage is suggested to depend on IL-1 enhanced hydrogen peroxide production by chondrocytes leading to radical formation in the presence of catalytic iron retrieved from red blood cells in the vicinity of chondrocytes, leading to apoptosis of the chondrocytes[5;6]. Although a complex issue, it is not expected that the diffusion rate of IL-1 and the penetration rate of catalytic iron (the cartilage matrix is negatively charged) are rate limiting factors. It has been described that by diffusion small molecules take about 30 minutes to cover a distance of 1 mm[22]. Localisation of blood-induced cell apoptosis (at the surfaces and/or deeper layers) may provide answers in this respect.
More plausible would be the difference in integrity of the articular surface compared to the deeper layers[23]. It could well be that the articular surface is less penetrable than the artificial cutting edges of a cartilage explant. This specific characteristic of the articular surface has been suggested to be essential in protection of the matrix against all sorts of mediators[24]. Not in line with this explanation is that already damaged cartilage (osteoarthritic cartilage) with a completely disrupted articular surface is similarly vulnerable to blood than healthy cartilage[25]. In these studies, however, full exposure could have shaded possible differences between intact and damaged articular surfaces because the articular surface only forms 1/6 of the entire surface exposed. A comparison of articular surface exposure only of osteoarthritic and healthy cartilage may provide answers in this respect.
Other aspects that need additional study are the effects on release of proteoglycans, protease activity, and collagen damage, all previously been shown to be affected by exposure to blood, but are not studied using the present culture system yet.
Nevertheless, from this pilot study it can be concluded that exposure of cartilage to blood at the articular surface leads to adverse effects on cartilage although less severe than when cartilage explants are fully submerged in blood. This adds to the understanding why blood-induced cartilage damage after an experimentally induced haemarthros *in vivo* is less severe compared to the *in vitro* effects of blood on cartilage. Irrespectively, it is inevitable that exposure of just the articular cartilage surface to blood leads to cartilage damage that can lead to severe joint damage in time. Therefore, (traumatic) haemarthroses need to be prevented and if they occur, they need to be treated properly.

REFERENCES

1. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
2. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12 Suppl 3:117-121.
3. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
4. Hooiveld M, Roosendaal G, Vianen M et al. Blood-induced joint damage: longterm effects in vitro and in vivo. J.Rheumatol. 2003;30:339-344.
5. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
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;42:784-790.
7. Hooiveld MJ, Roosendaal G, Vianen ME et al. 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.
8. Hooiveld MJ, Roosendaal G, Jacobs KM 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;50:2024-2031.
9. 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. 2006;56:199-207.
10. Muirden KD, Peace G, Rogers K. Clearance of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. II. Autoradiographic and histological studies. Ann.Rheum.Dis. 1969;28:630-636.
11. Muirden KD. Clearnace of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. I. Relationship to the anaemia of rheumatoid arthritis. Ann.Rheum.Dis. 1969;28:548-551.
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;24:1350-1354.
13. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.
14. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
15. SWANTON MC. Hemophilic arthropathy in dogs. Lab Invest 1959;8:1269-1277.
16. Zeman DH, Roberts ED, Henk WG, Watson E. Macroscopic, microscopic and ultrastructural findings in experimental haemarthrosis of rhesus monkeys. J.Comp Pathol. 1989;101:117-129.
17. Roosendaal G, Tekoppele JM, Vianen ME et al. Articular cartilage is more susceptible to blood induced damage at young than at old age. J.Rheumatol. 2000;27:1740-1744.
18. Jansen N.W.D., Roosendaal G, Wenting MJ et al. Very rapid clearance after a joint bleed in the canine knee can not prevent adverse effects on cartilage and synovial tissue. Manuscript in preparation 2008
19. 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;29:287-299.
20. Whiteman P. The quantitative determination of glycosaminoglycans in urine with Alcian Blue 8GX. Biochem.J. 1973;131:351-357.
21. Whiteman P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem.J. 1973;131:343-350.
22. Maroudas A. Distribution and diffusion of solutes in articular cartilage. Biophys.J. 1970;10:365-379.
23. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 1998;47:477-486.
24. Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr.Course Lect. 1998;47:487-504.

25. Jansen NW, Roosendaal G, Bijlsma JW et al. Degenerated cartilage is as vulnerable to blood-induced damage as healthy cartilage is. Ann.Rheum.Dis. 2008.

Chapter 8

The combination of uCTX-II, sCOMP, and sCS846 reflects cartilage damage in haemophilic arthropathy

Accepted for publication provided adequately revised
Arthritis & Rheumatism

N.W.D. Jansen[1,2]
G. Roosendaal[2]
B. Lundin[3]
L. Heijnen[2,4]
E. Mauser-Bunschoten[2]
J. W.J. Bijlsma[1]
M. Theobald[2]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Department of Radiology, Lund University Hospital, Sweden
4. Rehabilitation Center De Trappenberg, Huizen, The Netherlands

ABSTRACT

Background

Haemophilic arthropathy, with characteristics of inflammatory (rheumatoid arthritis) and degenerative joint damage (osteoarthritis), occurs at an early age with minor co-morbidity, and is restricted to 3 pairs of large joints. It might be that haemophilic arthropathy can be used to identify and evaluate biomarkers of cartilage and bone turnover.

Methods

Blood and urine were collected from 36 patients suffering from haemophilic arthropathy in various degrees. Commercially available serum and urine biomarkers most frequently reported on (uCTX-I, uCTX-II, sCTX-I, sCTX-II, sCOMP, sC1,2C, sC2C, and sCS846), were evaluated. From all patients, radiographs of ankles, knees, and elbows were evaluated for the degree of joint damage, using the Pettersson score, specific for haemophilic arthropathy, and based on cartilage and peri-articular bone changes.

Results

uCTX-II ($R=0.387$; $p=0.01$), sC1,2C ($R=0.314$; $p=0.04$), and sCS846 ($R=0.312$; $p=0.03$) showed correlations with the overall Pettersson score and with narrowing of joint space width ($R=0.348$, $R=0.284$, $R=0.424$, respectively; all $p<0.05$). Specific bone changes, such as osteoporosis and erosions, did not correlate with either of the bone markers. Combined indexes of different markers, based on regression analysis, increased the correlation up to $R=0.672$ ($p\leq0.001$) for the combination of uCTX-II, sCOMP, and sCS846.

Conclusion

These results support that a combination of biomarkers relate significantly better to severity of joint damage than individual biomarkers. In this study, the combination of uCTX-II, sCOMP, and sCS846 correlates best with the degree of arthropathy. Haemophilic arthropathy may be of use in future studies to identify and evaluate biomarkers of cartilage and bone turnover.

INTRODUCTION

In the field of osteoarthritis (OA) and rheumatoid arthritis (RA) there is ample research on biomarkers for their applicability to determine the severity and/or progression of joint damage and to evaluate the effect of (new) treatment modalities[1]. These biomarkers are molecules or fragments released into biological fluids during the process of tissue turnover. For OA and RA biomarkers are studied that are considered indicative of degradation or synthesis of cartilage, bone, and synovial tissue, all involved in the joint deterioration in these joint disorders. Blood and urinary biomarkers are of specific interest, because of their easy clinical applicability, but also biomarkers present in synovial fluid are subject of research.

Thus far, results of studies on possible use of these biomarkers in OA and RA have not been very conclusive, although some biomarkers have been identified that show correlation with the severity (cross-sectional) or even progression (longitudinal) of joint degeneration. In this respect, most evidence is gained for u(rinary)CTX-II and s(erum)COMP. CTX-II is a biomarker for collagen type II degradation, considered to be a key event in cartilage degeneration[2]. For both RA and OA it has been shown that CTX-II is elevated compared to normal individuals and that CTX-II levels correlate with the extent of joint destruction and predict progression[3-7]. Additionally, a change in uCTX-II levels in response to treatment can be predictive of long-term radiographic progression in RA[8]. COMP[9], Cartilage Oligomeric Matrix Protein, is a member of the thrombospondin family. It binds to type II collagen fibres and thereby stabilises the collagen fibre network. COMP is not exclusively found in cartilage, but is also found in tendons, menisci, and synovial tissue[10;11]. COMP has been shown to be increased in the serum of patients with early OA or RA[12-14] and to correlate with progression of disease[14-16]. On the other hand, COMP has been described to be decreased in a later stage of RA[17].

Whereas CTX-II is a marker of collagen type II and hence for cartilage, CTX-I is a marker of collagen type I[18] and therefore is considered as a marker for bone. There has been less focus on CTX-I compared to CTX-II. High levels of CTX-I at baseline predict an increased risk of radiological progression over 4 years in patients with early RA[5].

Other biomarkers that are currently more frequent subject of investigation in the field of OA and RA are C1,2C, C2C, and CS846. C1,2C[19;20] can originate from cleavage of both collagen type I and type II, whereas C2C[21] originates solely from collagen type II. CS846 is a marker of (effective or ineffective) synthesis of chondroitin sulphate and is shown to be elevated in OA cartilage[22] and synovial fluid[23] and is correlated with progression of joint damage in RA[24].

However, in general, the value of these biomarkers seems still limited, due to conflicting results on their predictive value for joint degeneration in OA and RA and even on their ability to differentiate between health and disease. For individuals they

are as yet of no use and at a group level the best results are obtained when very well defined specific populations are compared and evaluated, but even in such studies a large variation in outcome exists.

A type of joint degeneration that has characteristics of both OA and RA is haemophilic arthropathy (HA). HA is the consequence of recurrent joint bleeds in haemophilia and is the main cause of morbidity in haemophilia[25]. This blood-induced arthropathy has features of both degenerative joint disease such as OA, as well as of inflammatory joint disease such as RA[26]. The inflammatory response of the synovial tissue leads to cartilage destruction[27-29] and, independent of the synovial tissue, blood has direct adverse effects on cartilage[30;31]. Exposure of cartilage to blood leads to disturbance of the turnover of proteoglycans and to degradation of collagen, the main cartilage matrix components[32]. Ultimately, the whole joint deteriorates, including changes in the underlying bone.

Since haemophilia is an inherited disorder, initiation of joint damage caused by joint bleeds often starts at very early age. Patients with haemophilia can have severely destructed joints, which require joint replacement surgery, already at the age of 40, while patients with OA or RA in need for joint replacement generally are older. Moreover, HA is not characterized by systemic chronic inflammation as seen in RA. Due to the age difference and absence of a systemic inflammatory component, patients with HA are not familiar with all kinds of co-morbidities such as liver or kidney dysfunction. Liver and kidney are involved in the secondary processing of turnover products released from cartilage and bone and with that will have an effect on biomarker formation and excretion[33]. HA is also not subject to aggressive treatment as seen in RA. Another issue in favor of HA in light of research on biomarkers is the fact that HA is largely restricted to three major joints; the ankle, elbow, and knee joint. The Pettersson score[34;35], which is a sum of scores for knees, ankles and elbows, provides a good view of the total joint damage in the patient.

The presence of co-morbidities, due to age and disease characteristics influencing biomarker metabolism, aggressive medication, and the involvement of multiple small joints in OA and RA expectedly hampers research on biomarkers in these diseases. In this respect HA restricted mainly to 3 pairs of large joints, occurring at a relatively young age, without a systemic inflammatory component, and not treated with aggressive medication, may be helpful in research on identification and evaluation of biomarkers of cartilage and bone turnover. Additionally, these biomarkers may be useful to predict and monitor HA. To our knowledge, biomarkers have never been studied in this disease thus far.

The present study was undertaken to evaluate whether commonly used and commercially available biomarkers of cartilage and bone in serum and/or urine are associated with the severity of joint damage in patients with various degrees of haemophilic arthropathy.

MATERIALS AND METHODS

Blood and urine

From 36 random patients suffering from haemophilia (31 A; F-VIII deficiency and 5 B; F-IX deficiency); mean age 35 ± 2 years) blood and urine was collected. Exclusion criteria were HIV infection, presence of antibodies against supplemented clotting factor (inhibitor patients) or any joint bleed in the last 3 months. Ten millilitre of blood was collected in vacutainer tubes (Becton Dickinson, UK). Eight IU of recombinant clotting factor concentrate (VIII or IX) was added to ensure proper coagulation. Blood was kept at room temperature for at least one hour. After coagulation, the blood was centrifugated at 1000 g for 10 minutes and serum aliquots were stored at -80°C until use. Second morning urine was kept at 4°C for max 8 hours, before aliquots were stored at -80°C. Sampling of blood and urine was performed twice, with one month in between.

This study was conducted according to the declaration of Helsinki and received approval from the Medical Ethical Review Board. All patients gave written informed consent.

Radiographs

Radiographs of ankles, knees and elbows were taken according to standard procedures of the University Medical Center Utrecht. In case radiographs had been taken more than 12 months before blood and urine sampling, new radiographs were taken. All radiographs were scored according to the Pettersson method[34;35]. This scoring is advised by the World Federation of Haemophilia (WFH) to quantify radiographic joint damage in case of HA. The scoring is based on 8 criteria: osteoporosis, enlarged epiphysis, irregular subchondral surface, narrowing of joint space, subchondral cyst formation, erosions of joint margins, gross incongruence of articulating bone ends, and deformity (angulation and/or displacement between articulating bones), by use of an ordinal scale from 0 to max 2 per criterion. The maximum score per joint is 13 with a maximum total patient score of 78 being the sum of the six index joints. In this study, joints with arthrodeses (n=12 of 216) or arthroplasty (n=5 of 216) were given 0 points instead of a maximum score, because of the absence of cartilage and as such not contributing to the generation of most biomarkers.

Biomarkers assays

All biomarkers were determined by using commercially available ELISA kits, according to the manufacturer's protocol. Urine samples were assessed for the presence of CTX-I (CrossLaps; Nordic Bioscience) and CTX-II (CartiLaps; Nordic Bioscience) and were corrected for the amount of creatinine (Cayman Chemicals; urine dillution). Serum samples were assessed for the presence of CTX-I

(CrossLaps; Nordic Bioscience), CTX-II (CartiLaps; Nordic Bioscience), COMP (Anamar), C1,2C (IBEX), C2C (IBEX), and CS846 (IBEX). The various assays all detected specific epitopes. The CTX-I (C-terminal telopeptide of type I collagen) assay is based on an antiserum reactive with a peptide sequence specific for a part of the C-terminal telopeptide α1-chain of human type 1 collagen[18;36]. The CTX-II (C-terminal telopeptide of type II collagen) assay measures C-telopeptide fragments created by the cleavage of type II collagen by collagenases[3]. The COMP assay measures both intact and fragmented COMP[12]. The C1,2C (Col 2 ¾C_{short})[19;20] assay measures the neoepitope created by the cleavage of mainly type II, but also type collagen I, by collagenases). The C1,2C is the secondary cleavage product from the long C2C cleavage product. The C2C (Col 2 ¾C_{long}) assay measures the neoepitope created by the cleavage of type II collagen by collagenases[21]. The CS846 (chondroitin sulphate 846 epitope) assay measures an epitope on the chondroitin chains of the proteoglycan aggrecan[22].

Calculations and statistical analysis

Correlation between joint damage as measured with the Pettersson scores and biomarker-levels were done using a non-parametric Spearman correlation analysis. Stepwise linear regression analysis was performed in search of the combination of biomarkers best correlating with the Pettersson score. Based on the outcome of this regression analyses, combined scores were calculated. This was done by calculation of the average level of a biomarker. The ratio of the value of a biomarker of each individual patient compared to this mean was determined for each relevant biomarker. The mean of these ratios for different biomarkers of each patient was plotted against the Pettersson score. Using this approach each biomarker in the combined score was given the same weight. Statistical analysis was done with SPSS 12.0.1 software. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Correlation of joint damage with biomarkers

Pettersson scores ranged from 0 to 61 points, with a mean ± SD of 22 ± 17 points. In general the duplicate samples, taken with a months interval, provided similar levels of each biomarker for each patient. There were no reported haemarthroses between the two sampling moments. Therefore, the average of both time points have been taken as the value for each patient.

From all biomarkers, uCTX-II, C1,2C and CS846 showed a statistically significant correlation with the total Pettersson score (table 1). Joint space narrowing (JSN) is the most important indicator of cartilage loss. Correlations of all biomarkers with only the score for JSN were statistically significant for the same three biomarkers, and additionally for COMP (table 1). The scatter plots of these four biomarkers against the total Pettersson score and against the score for JSN are depicted in figure 1 and 2, respectively. Although the correlations were statistically significant, the correlation coefficients were low, ranging from 0.284 to 0.424.

There were no correlations between (partial) bone biomarkers (CTX-I and C1,2C) and Pettersson score, not even for the bone specific parameters ‘osteoporosis’ and ‘erosions of joint margins’ (table 1).

Pettersson		uCTX-I	uCTX-II	COMP	sCTX-I	sCTX-II	C1,2C	C2C	CS846
Total									
	R	0.227	0.387	0.264	0.161	0.102	0.314	0.040	0.312
	p	0.092	0.010*	0.060	0.175	0.284	0.033*	0.409	0.032*
JSN									
	R	0.203	0.348	0.284	0.094	0.113	0.291	-0.015	0.424
	p	0.117	0.019*	0.047*	0.292	0.262	0.045*	0.465	0.005*
Osteoporosis									
	R	0.039	0.232	0.213	0.144	0.060	0.191	0.051	0.268
	p	0.410	0.087	0.106	0.200	0.368	0.136	0.383	0.057
Erosions of joint margins									
	R	0.035	0.084	-0.106	0.048	0.018	0.081	0.157	-0.206
	p	0.419	0.312	0.268	0.391	0.459	0.322	0.181	0.115

Table 1: Correlation of Pettersson score and biomarker levels

The mean of two samples collected with one month in between of each biomarker for each patient was correlated with radiographic joint damage by use of the total Pettersson score, and with narrowing of the joint space width (JSN), osteoporosis, and erosions of joint margins, being three of the eight parameters of the Pettersson scoring system, representing more specifically cartilage and bone changes. Spearman correlation coefficients and p-values are given.

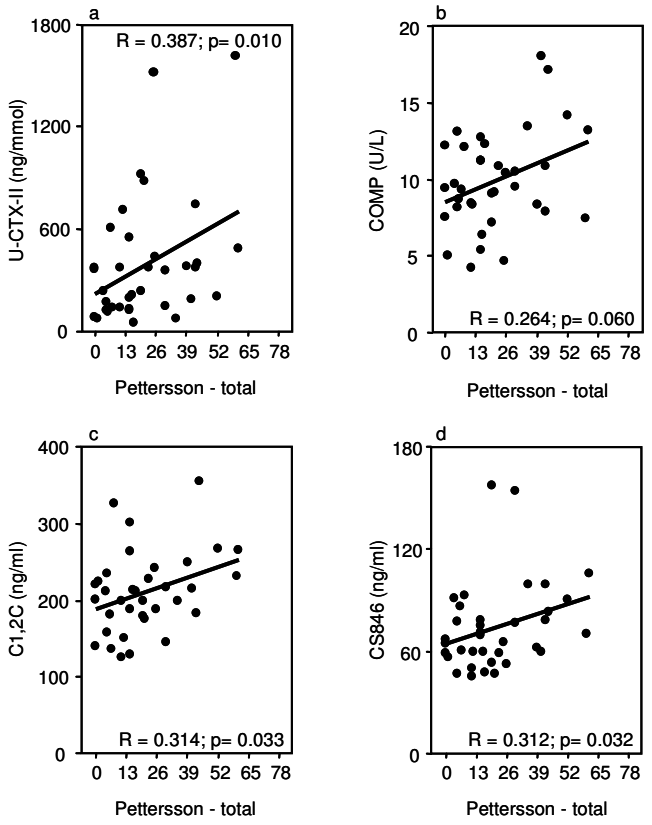

Figure 1: Radiographic joint damage correlated with biomarker levels
Pettersson score for radiographic joint damage (of ankle, knee, and elbow) *versus*, **a:** uCTX-II; **b:** COMP; **c:** C1,2C; **d:** CS846 is shown. Spearman correlation coefficients and p-values are given.

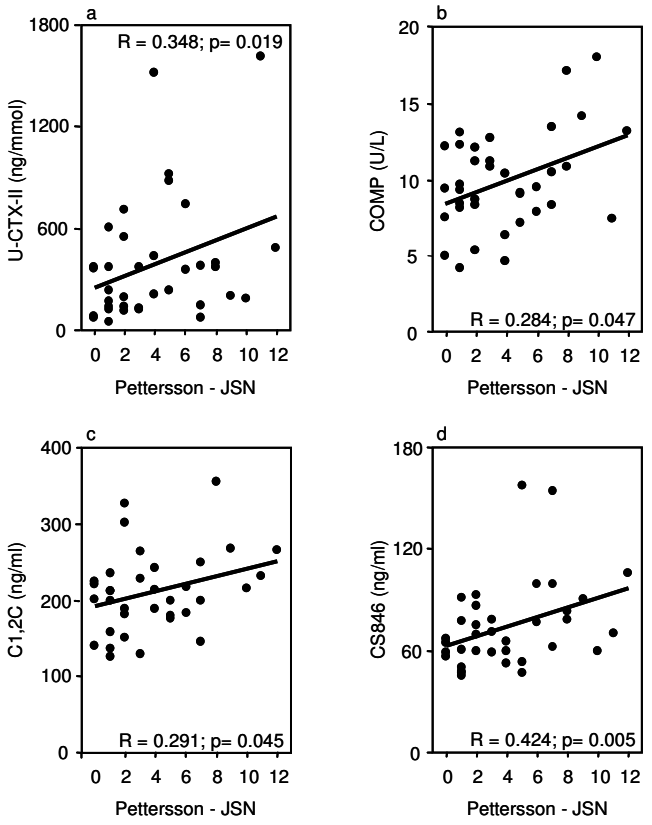

Figure 2: Joint space narrowing correlated with biomarker levels
Radiographic joint space narrowing (JSN) (of ankle, knee, and elbow) as determined by the Pettersson score *versus* **a**: uCTX-II; **b**: COMP; **c**: C1,2C; **d**: CS846 is shown. Spearman correlation coefficients and p-values are given.

Regression analysis

To determine which combination of biomarkers best correlate with the amount of joint damage, stepwise linear regression analysis was performed. With the total Pettersson score as dependent variable, the combination of uCTX-II, COMP, and CS846 showed the highest correlation. The addition of COMP to uCTX-II in the model increased multiple correlation coefficient to 0.660 and this was even increased to 0.728 after addition of CS846.

With JSN as dependent variable, the combination of the same biomarkers showed the highest correlation, with an ultimate multiple correlation coefficient of 0.754 including all three biomarkers.

Because of the difference in absolute value of these biomarkers, the ratio of the value of a biomarker from each individual patient compared to the mean of that specific biomarker was calculated. The mean of these ratios for the three biomarkers for each patient was plotted against the total Pettersson score and the score for JSN (figure 3). The Spearman correlation coefficient of the combination of the three biomarkers with total Pettersson score was clearly higher than that of each biomarker individually (R = 0.672; p≤ 0.001). This was also true for the correlation with the score for JSN (R = 0.698; p≤ 0.001).

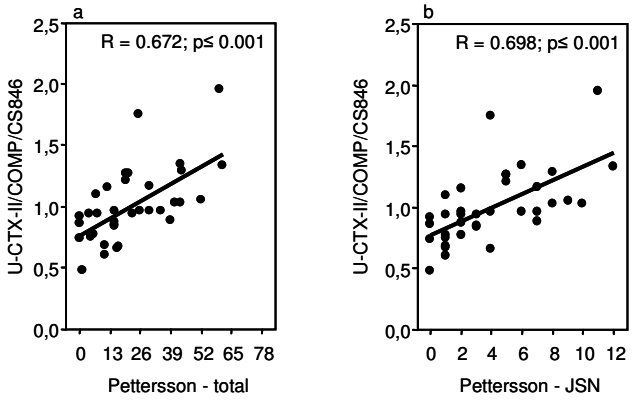

Figure 3: Radiographic joint damage correlated with the combined index of uCTX-II, COMP, and CS846 levels

From each of the biomarkers the value of each patient was normalized to the mean value of that biomarker for all patients, giving each biomarker the same weight in a combined index. The mean of these ratios for the three biomarkers is plotted against radiographic joint damage by use of the total Pettersson score (**a**), and narrowing of the joint space width (JSN; **b**), being one of the eight parameters of the Pettersson scoring system. Spearman correlation coefficients and p-values are given.

As C1,2C also showed a statistically significant correlation with both total Pettersson score as well as with the score for JSN, also a ratio including this biomarker in addition to uCTX-II, COMP, and CS846 was calculated and plotted against total Pettersson score and the score for JSN (figure 4). However, addition of C1,2C did not contribute significantly to the correlation of the combination of biomarkers derived from regression analyses with total Pettersson score or with the score for JSN.

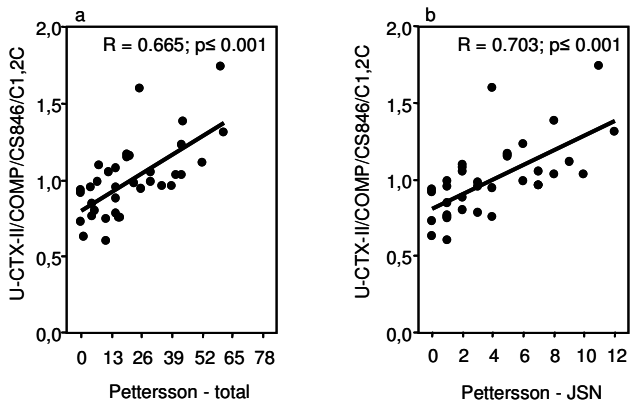

Figure 4: Radiographic joint damage correlated with the combined index of uCTX-II, COMP, C1,2C, and CS846 levels

From each of the biomarkers the value of each patient was normalized to the mean value of that biomarker for all patients, giving each biomarker the same weight in a combined index. The mean of these ratios for the four biomarkers is plotted against radiographic joint damage by use of the total Pettersson score (**a**), and narrowing of the joint space width (JSN; **b**), being one of the eight parameters of the Pettersson scoring system. Spearman correlation coefficients and p-values are given.

DISCUSSION

In this study it is shown that cartilage markers uCTX-II, sC1,2C, sCS846, and sCOMP correlate with radiographic joint damage in patients with haemophilia. Most importantly, a combined index of selected biomarkers, based on stepwise regression analyses, revealed that the combination of uCTX-II, sCS846, and sCOMP had the best correlation with the overall radiographic joint damage in general and for the specific feature JSN. C1,2C appeared of no additional value to this combination of biomarkers. Interestingly, the bone markers CTX-I and C1,2C did not correlate with Pettersson parameters describing bone changes such as osteoporosis and erosions. These results demonstrate that HA might be of use in the identification and evaluation of biomarkers of cartilage turnover, but apparently not for biomarkers of bone turnover.

Joint damage is the consequence of a combination of processes, involving both synthesis and degradation of matrix components, in various joint tissues, like cartilage and bone. In this study, the combination of biomarkers that correlates best with the radiographic joint damage and of cartilage loss, indicated by JSN, in particular, contains biomarkers of both degradation (uCTX-II and COMP) and synthesis (CS846). Furthermore, the biomarkers originate from different cartilage matrix components: collagens (uCTX-II), proteoglycans (CS846) and a non-collagenous, non-proteoglycan component of cartilage, COMP.

Some biomarkers have been shown to be dependent on gender, age or circadian rhythm[4;37;38]. In the present study, gender could not interfere, because all patients were male, since haemophilia is an X-chromosome linked disease. Most patients suffered from haemophilia A, while only a minority suffered from haemophilia B. There is no known difference in the (development of) arthropathy between haemophilia A and B and therefore no difference regarding biomarkers is expected.

Besides the fact that some biomarkers correlate with age, age is also known to be positively correlated with joint damage in patients with haemophilia[39]. In this study, a minor, though significant correlation of the total Pettersson score with age was found ($R=0.341$; $p=0.021$). Furthermore, uCTX-I, uCTX-II, COMP, and sCTX-I correlated with age in this study ($R=-0.459$; $p=0.01$, $R=-0.387$; $p=0.004$, $R=0.400$; $p=0.003$, $R=0.482$; $p<0.001$, respectively). Addition of the variable age to the multiple regression analysis however, yields the same combination of biomarkers for Pettersson outcome with age on a third place. A large influence of the circadian rhythm is not expected in this study, since sampling was performed in the morning. It might be that small variations were reduced by using the average of two samples each taken in the morning but with a month in-between.

In the Pettersson scoring system, there is no definition how to score arthrodesis and arthroplasty. In this study, joints after arthrodeses or arthroplasty were given 0 points, because of the very low amount or even total absence of cartilage being able to

contribute to the amount of cartilage biomarkers. However, in these conditions bone may still be active or may even be more active. This could explain the absence of a correlation for the bone biomarkers. However, when the patients with arthrodeses, ankylosis or arthroplasty (n=9) were left out of the statistical analysis, still no correlations with bone biomarkers were found, neither for the total radiographic joint damage, nor for bone specific items such as osteoporosis or erosions. Exclusion of these patients from the analyses did not change the (combination of) biomarkers correlating with total joint damage.

The good correlation of the combined index of biomarkers with the radiograpic joint damage, and JSN in specific, might be due to the fact that joint damage in patients with heamophilia occurs mostly in the elbow, knee and ankle joints[40]. These joints are relatively large joints, and in that respect significantly contribute to circulating and excreted biomarkers. Although other joints, such as the hip, can be involved, this is rather exceptional. In our study, one patient has involvement of other joints than those taken into account in the Pettersson score (hips). Because in general other joints do not interfere, the Pettersson score gives a reliable view on total radiographic joint damage of an individual. In this respect, HA has a significant advantage over diseases including multiple smaller joints such as OA and RA.

Whether and if so which (combination of) biomarkers are predictive of radiographic progression of joint damage remains to be studied. Another interesting issue to investigate is whether joint bleeds affect biomarkers. This is likely, because it has been reported that a single blood exposure results in direct damage of joint cartilage, including enhanced loss of proteoglycans and collagen damage[41]. Perhaps this can aid in monitoring effect of joint bleeding and treatment.

In conclusion, it is shown that cartilage biomarkers, originally studied in OA and RA, can also be evaluated in HA. The specific characteristics of HA might be of use in future evaluation of such cartilage derived biomarkers. Furthermore, the combination of uCTX-II, COMP and CS846 is indicative of the amount of joint damage in patients with haemophilic arthropathy.

ACKNOWLEDGEMENTS

We would like thank all patients that were willing to participate in this study and Drs. M.F. Bakker for her advice regarding statistical analysis.

REFERENCES

1. Sumer EU, Schaller S, Sondergaard BC, Tanko LB, Qvist P. Application of biomarkers in the clinical development of new drugs for chondroprotection in destructive joint diseases: a review. Biomarkers 2006;11:485-506.
2. Henrotin Y, Addison S, Kraus V, Deberg M. Type II collagen markers in osteoarthritis: what do they indicate? Curr.Opin.Rheumatol. 2007;19:444-450.
3. Christgau S, Garnero P, Fledelius C et al. Collagen type II C-telopeptide fragments as an index of cartilage degradation. Bone 2001;29:209-215.
4. Mouritzen U, Christgau S, Lehmann HJ, Tanko LB, Christiansen C. Cartilage turnover assessed with a newly developed assay measuring collagen type II degradation products: influence of age, sex, menopause, hormone replacement therapy, and body mass index. Ann.Rheum.Dis. 2003;62:332-336.
5. Garnero P, Landewe R, Boers M 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. Arthritis Rheum. 2002;46:2847-2856.
6. Jung M, Christgau S, Lukoschek M, Henriksen D, Richter W. Increased urinary concentration of collagen type II C-telopeptide fragments in patients with osteoarthritis. Pathobiology 2004;71:70-76.
7. Reijman M, Hazes JM, Bierma-Zeinstra SM et al. A new marker for osteoarthritis: cross-sectional and longitudinal approach. Arthritis Rheum. 2004;50:2471-2478.
8. Landewe R, Geusens P, Boers M et al. Markers for type II collagen breakdown predict the effect of disease-modifying treatment on long-term radiographic progression in patients with rheumatoid arthritis. Arthritis Rheum. 2004;50:1390-1399.
9. Hedbom E, Antonsson P, Hjerpe A et al. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J.Biol.Chem. 1992;267:6132-6136.
10. Recklies AD, Baillargeon L, White C. Regulation of cartilage oligomeric matrix protein synthesis in human synovial cells and articular chondrocytes. Arthritis Rheum. 1998;41:997-1006.
11. Muller G, Michel A, Altenburg E. COMP (cartilage oligomeric matrix protein) is synthesized in ligament, tendon, meniscus, and articular cartilage. Connect.Tissue Res. 1998;39:233-244.
12. Neidhart M, Hauser N, Paulsson M et al. Small fragments of cartilage oligomeric matrix protein in synovial fluid and serum as markers for cartilage degradation. Br.J.Rheumatol. 1997;36:1151-1160.
13. Clark AG, Jordan JM, Vilim V et al. Serum cartilage oligomeric matrix protein reflects osteoarthritis presence and severity: the Johnston County Osteoarthritis Project. Arthritis Rheum. 1999;42:2356-2364.
14. Young-Min S, Cawston T, Marshall N et al. Biomarkers predict radiographic progression in early rheumatoid arthritis and perform well compared with traditional markers. Arthritis Rheum. 2007;56:3236-3247.
15. Vilim V, Olejarova M, Machacek S et al. Serum levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic progression of knee osteoarthritis. Osteoarthritis.Cartilage. 2002;10:707-713.
16. Sharif M, Kirwan JR, Elson CJ, Granell R, Clarke S. Suggestion of nonlinear or phasic progression of knee osteoarthritis based on measurements of serum cartilage oligomeric matrix protein levels over five years. Arthritis Rheum. 2004;50:2479-2488.
17. Forslind K, Eberhardt K, Jonsson A, Saxne T. Increased serum concentrations of cartilage oligomeric matrix protein. A prognostic marker in early rheumatoid arthritis. Br.J.Rheumatol. 1992;31:593-598.
18. Bonde M, Qvist P, Fledelius C, Riis BJ, Christiansen C. Immunoassay for quantifying type I collagen degradation products in urine evaluated. Clin.Chem. 1994;40:2022-2025.
19. Billinghurst RC, Dahlberg L, Ionescu M et al. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J.Clin.Invest 1997;99:1534-1545.
20. Downs JT, Lane CL, Nestor NB et al. Analysis of collagenase-cleavage of type II collagen using a neoepitope ELISA. J.Immunol.Methods 2001;247:25-34.
21. Poole AR, Ionescu M, Fitzcharles MA, Billinghurst RC. The assessment of cartilage degradation in vivo: development of an immunoassay for the measurement in body fluids of type II collagen cleaved by collagenases. J.Immunol.Methods 2004;294:145-153.

22. Rizkalla G, Reiner A, Bogoch E, Poole AR. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. J.Clin.Invest 1992;90:2268-2277.
23. Lohmander LS, Ionescu M, Jugessur H, Poole AR. Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. Arthritis Rheum. 1999;42:534-544.
24. Mansson B, Carey D, Alini M et al. Cartilage and bone metabolism in rheumatoid arthritis. Differences between rapid and slow progression of disease identified by serum markers of cartilage metabolism. J.Clin.Invest 1995;95:1071-1077.
25. Raffini L, Manno C. Modern management of haemophilic arthropathy. Br.J.Haematol. 2007;136:777-787.
26. Roosendaal G, van Rinsum AC, Vianen ME et al. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-153.
27. Rodriguez-Merchan EC. The destructive capabilities of the synovium in the haemophilic joint. Haemophilia. 1998;4:506-510.
28. Roosendaal G, Mauser-Bunschoten EP, de Kleijn P et al. Synovium in haemophilic arthropathy. Haemophilia. 1998;4:502-505.
29. 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;80:540-545.
30. Roosendaal G, Lafeber FP. Blood-induced joint damage in hemophilia. Semin.Thromb.Hemost. 2003;29:37-42.
31. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12 Suppl 3:117-121.
32. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 1998;47:477-486.
33. Garnero P. Noninvasive biochemical markers in osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg VM, eds. Osteoarthritis.: Wolters Kluwer Lippincott Williams & Wilkins; 2008:215-232.
34. Pettersson H GM. Musculoskeletal and Other Hemorraghic Complications. Diagnostic imaging in hemophilia.: Springer-Verlag; 1985:28-54.
35. Pettersson H, Ahlberg A, Nilsson IM. A radiologic classification of hemophilic arthropathy. Clin.Orthop.Relat Res. 1980153-159.
36. Rosenquist C, Fledelius C, Christgau S et al. Serum CrossLaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. Clin.Chem. 1998;44:2281-2289.
37. Andersson ML, Petersson IF, Karlsson KE et al. Diurnal variation in serum levels of cartilage oligomeric matrix protein in patients with knee osteoarthritis or rheumatoid arthritis. Ann.Rheum.Dis. 2006;65:1490-1494.
38. Kong SY, Stabler TV, Criscione LG et al. Diurnal variation of serum and urine biomarkers in patients with radiographic knee osteoarthritis. Arthritis Rheum. 2006;54:2496-2504.
39. Fischer K, van Hout BA, van der Bom JG, Grobbee DE, van den Berg HM. Association between joint bleeds and Pettersson scores in severe haemophilia. Acta Radiol. 2002;43:528-532.
40. Arnold WD, Hilgartner MW. Hemophilic arthropathy. Current concepts of pathogenesis and management. J.Bone Joint Surg.Am. 1977;59:287-305.
41. Roosendaal G, Tekoppele JM, Vianen ME et al. Blood-induced joint damage: a canine in vivo study. Arthritis Rheum. 1999;42:1033-1039.

Chapter 9

Digital scoring of haemophilic arthropathy using radiographs is feasible

Accepted for publication provided adequately revised
Haemophilia

N.W.D. Jansen[1,2]
K.L. Vincken[3]
A.C.A. Marijnissen[1]
B. Lundin[4]
L. Heijnen[2,5]
G. Roosendaal[2]
H. Pettersson[4]
F.P.J.G. Lafeber[1]

1. Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands
2. Hematology and Van Creveld Clinic, University Medical Center Utrecht, The Netherlands
3. Image Sciences Institute, University Medical Center Utrecht, The Netherlands
4. Radiology, Lund University Hospital, Sweden
5. Rehabilitation Center De Trappenberg, Huizen, The Netherlands

ABSTRACT

Background

Radiographs are important tools to evaluate structural changes in many joint diseases. In case of haemophilic arthropathy (HA), the Pettersson-score is widely used. The rising of digital radiography enables evaluation of these structural changes in a more quantitative and detailed manner, potentially improving diagnosis and follow-up. The aim of this study is to evaluate whether digital image analysis in case of HA is feasible, using a presently available method for radiographic changes in knee osteoarthritis (OA), Knee Image Digital Analysis (KIDA).

Methods

62 knee radiographs were scored according to Pettersson and with KIDA, each by two independent observers. Inter-observer variations and correlations between the two scoring methods were determined.

Results

The inter-observer variation was smaller for KIDA than for Pettersson and for KIDA not significantly different from evaluation of OA joints. Good correlations were found for the two methods where comparison of parameters was appropriate. Importantly, for each of the parameters within one point in the ordinal Pettersson-score, a large window still existed in the continuous KIDA-grading.

Conclusion

Digital analysis of radiographs to quantify joint damage in HA is feasible. The use of continuous variables, as used in a digital method such as KIDA has the advantage that it enables objective and much more sensitive detection of small changes than by use of an ordinal analogue method such as the Pettersson-score. Based on the present results, it would be worthwhile to adapt the KIDA method for the specific characteristics of HA and to extend the method to elbow and ankle radiographs.

INTRODUCTION

Joint destruction due to recurrent haemarthroses is the main cause of morbidity in patients suffering from haemophilia[1]. Destruction of joints because of joint bleeds involves subchondral changes, enlargement of the epiphysis, changes at the joint margins, narrowing of the joint space and ultimately joint deformity[2-6]. This haemophilic arthropathy (HA) can be visualized using various imaging techniques, including radiography. At present, the Pettersson scoring system[7;8] is the most widely used method to quantify structural changes in joints of patients with haemophilia with the use of radiographs. This scoring system was first published in 1980 and since then has been used extensively in various types of studies. It uses radiographs of the most affected joints in haemophilia: the knees, elbows, and ankles[9], which are scored for 8 features of HA: osteoporosis, enlarged epiphysis, irregular subchondral surface, narrowing of joint space, subchondral cyst formation, erosions of joint margins, gross incongruence of articulating bone ends, and deformity (table 1). This score is on an ordinal scale (0 – max 2, for each item), and gives a maximum score for each joint of 13, yielding a maximum patient-score of 78, including all six index joints.

There is a rise of novel imaging techniques, such as Magnetic Resonance Imaging (MRI) and ultrasonography (US), and development of methods for standardized assessment based on these techniques is in progress. However, the practical value of such new methods is not settled. Radiography is well established and still remains the golden standard for structural assessment of haemophilic arthropathy. Radiography is, at least when compared to MRI, relatively cheap, fast, and available in almost all hospitals, even in most developing countries.

There is a shift in radiography from analogue to digital images in radiography. The advantage is that such radiographs are directly visible on computer screens and can as such be used directly for digital analyses. This led to the development of several digital evaluation methods for joint degeneration as seen in frequently occurring joint diseases such as osteoarthritis (OA)[10-15]. Digital evaluation has two major advantages: it is more objective and it uses continuous variables instead of ordinal variables as in many analogue scoring methods.

Structural changes in HA match in many aspects the changes seen in OA[16]. Amongst others, subchondral and marginal bone changes, narrowing of the joint space, and the centrifugal pattern of cartilage damage are observed in HA as well as in OA, and contrast those seen in e.g. rheumatoid arthritis.

Recently, a novel digital method to analyze standard knee radiographs was developed and validated for OA: Knee Image Digital Analysis (KIDA)[17]. KIDA is an interactive computer program enabling objective quantification of joint space width, subchondral bone density, osteophyte area, and deviation of the joint angle. KIDA was shown to be a reliable method to quantify and document the individual radiographic parameters of knee OA with a small inter- and intra-observer variation

and to be able to distinguish in detail healthy from OA joints. A great advantage of this method is the fact that the technique uses continuous variables, enabling a more precise distinction of changes than on the basis of a ordinal scale, in case of OA the frequently used Kellgren & Lawrence (K&L) grading[18].
In this study, it is evaluated whether digital image analysis in case of HA is feasible by using a presently available method for radiographic changes of the OA knee (KIDA) by comparing the KIDA-outcome with outcome parameters of the most frequently used Pettersson scoring system.

MATERIALS AND METHODS

Radiographs and scoring

Radiographs of 62 knees of 31 successive patients suffering from either haemophilia A or B visiting the outpatient clinic of the Van Creveld Clinic of the University Medical Center Utrecht were used. The radiographs were taken according to standard procedures. In short, semi-flexed, posterior-anterior (PA) radiographs of the tibia-femoral joint were taken under full weight bearing according to the protocol of Buckland-Wright[19;20]. Radiographs were taken in the presence of an aluminium step wedge on the lateral side of the knee, against the detector (film) within the field of exposure, in order to quantify bone density changes in time and correct for possible magnification of the radiograph. In principle, these are standard UMC Utrecht weight bearing radiographs with the addition of a density reference wedge. The method for OA knees has been described in detail by Marijnissen *et al.*[21].
The digital radiographs were scored according to Pettersson[7;22] (table 1) independently by LH at the UMC Utrecht and by BL in Lund, Sweden (the latter educated in scoring by Pettersson). This method evaluates the following parameters: osteoporosis, enlarged epiphysis, irregular subchondral surface, narrowing of joint space, subchondral cyst formation, erosions of joint margins, gross incongruence of articulating bone ends, and deformity (angulation and/or displacement between articulating bones), by use of an ordinal scale from 0 to max 2 per parameter. The maximum score per joint is 13.

Table 1: Pettersson-scoring

Radiologic change	Finding	Score
Osteoporosis	Absent	0
	Present	1
Enlarged epiphysis	Absent	0
	Present	1
Irregular subchondral surface	Absent	0
	Partially involved	1
	Totally involved	2
Narrowing of joint space	Absent	0
	Joint space > 1mm	1
	Joint space ≤ 1mm	2
Subchondral cyst formation	Absent	0
	1 cyst	1
	> 1 cyst	2
Erosions of joint margins	Absent	0
	Present	1
Gross incongruence of articulating bone ends	Absent	0
	Slight	1
	Pronounced	2
Deformity	Absent	0
(angulation and/or displacement between	Slight	1
articulating bones)	Pronounced	2

Additionally the radiographs were evaluated by two independent experienced observers by use of KIDA. KIDA is a software application for interactive analysis of radiographs of the knee, based on ImageXplorer, developed at the Images Sciences Institute, Utrecht, The Netherlands (KV)[23] for digital image analysis of radiographs of OA knees. In six consecutive steps, which take between 5 and 10 minutes per joint, digital radiographs are analyzed. Outcome parameters include continuous data on mean and minimum joint space width (JSW) (mm), subchondral bone density (mm Alu Eqs.), reference values for bone density of femur and tibia, osteophyte area (mm^2), and the angle (°) and breadth (mm) of the joint (see figure A).

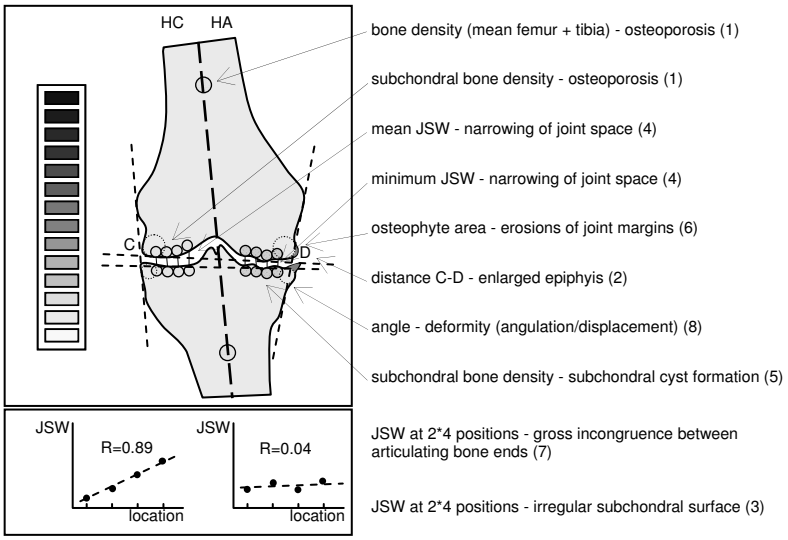

Figure A: The KIDA program
Schematic representation of KIDA. Lines and circles are interactively indicated. On the left the aluminium bone density standard is represented. The 16 circles at the bone ends are used to determine both JSW as well as subchondral bone density. The correlation-coefficient of the JSW (lower panel) is calculated from the 2*4 locations along the joint. The average of the correlation-coefficients of the medial and lateral compartment is used. The comparisons made between KIDA and Pettersson are indicated on the right (with the numbers referring to the items in the Pettersson score as well as the corresponding figures). HC = healthy control; HA = haemophilic arthropathy.

Evaluation and statistical analysis

Inter-observer variation:

Two experienced observers performed the Pettersson-scoring and two observers evaluated the radiographs using KIDA. The observers were blinded to patient-related information. Statistical analysis to compare the inter-observer variation for both Pettersson and KIDA were performed according to Bland and Altman[24]. Briefly, the inter-observer variation was determined by plotting the difference between the scores of the first and second observer against the mean of the two scores. The distance between the mean of measurement differences and zero indicate the bias. 95% confidence intervals (C.I.) were calculated. Assuming no systematic bias (mean of differences equals 0), 1.96 times the standard deviation (SD) defines the smallest detectable difference (SDD). To compare inter-observer variations between both scoring methods and between KIDA performed on radiographs of OA joints *versus* HA joints, the SD of the mean difference was normalized to the mean of all observations, because values between groups were different in magnitude. For the comparison with the inter-observer variations of the OA joint, the original data as described by Marijnissen *et al.* were used[25].

Correlation between the outcome of both methods:
Comparison of the Pettersson parameters with KIDA outcome, using the mean of both observers, was done using Spearman's correlation analysis with SPSS 12.0.1 software. P-values ≤ 0.05 were considered statistically significant. The following correlations, assuming to be appropriate, were made:

1) Pettersson "osteoporosis" was compared to KIDA mean bone density of femur and tibia and to the mean subchondral bone density of femur and tibia (figure 1a and b). A negative correlation was anticipated, because the more osteoporosis, the higher the Pettersson score, the lower the KIDA bone density measurements.
2) "Enlarged epiphysis" was compared to the distance between the edges of the femur (distance CD; figure 2). A positive correlation was expected.
3) "Irregular subchondral surface" was compared to the mean calculated correlation coefficient of the medial and lateral JSW, each obtained from the four locations in both joint compartments (figure 3 and figure A). The more irregular the subchondral surface, the lower the correlation (compare in figure A the lower right panel, representing an irregular surface, to the lower left panel, representing a regular surface in which the JSW gradually increases from lateral to medial). A negative correlation was expected.
4) "Narrowing of joint space" was compared to the mean JSW and to the minimum JSW (figures 4a and b). A high Pettersson score for JSW narrowing will correlate with a small joint space width, expected to result in a negative correlation.
5) "Subchondral cyst formation" was compared to the mean subchondral bone density of femur and tibia (figure 5). A negative correlation was expected, because the more cyst formation, the lower the bone density.
6) "Erosions of joint margins" were compared to the mean osteophyte area (figure 6). A positive correlation was expected because the area below the developing osteophyte is often erroneously scored as erosion in the Pettersson score (LH). The other way around, in case of erosion the preserved bone on top is often erroneously scored as an osteophyte (BL).
7) "Gross incongruence of articulating bone ends" was compared to the above described (ad 3) mean calculated correlation coefficient of the JSW (figure 7). A negative correlation was anticipated on.
8) "Deformity (angulation and/or displacement of articulating bones)" was compared to the angle of the joint (figure 8) from which a positive correlation was expected.

RESULTS

Inter-observer variations of Pettersson and KIDA

The inter-observer variations according to Bland and Altman[26] of the Pettersson parameters are shown in table 2. The inter-observer variations of parameters obtained with KIDA from the same radiographs are shown in the upper panel of table 3. In general, there was no structural difference in the scoring of the two observers, neither for Pettersson, nor for KIDA, as can be concluded from the mean differences, for most variables being close to zero. The absolute values in both scoring systems vary, therefore, to compare the inter-observer variation between the two methods, the SD of the mean difference was normalized to the mean value of all observations (ΔSD/Mean in %). When comparing these values, it is clear that in general the inter-observer variation of the KIDA parameters is smaller than the inter-observer variation in Pettersson.

Parameter	Mean ± SD	Δ Mean ± SD	ΔSD/Mean (%)	Range	95% C.I.
Osteoporosis					
	0.26 ± 0.44	0.10 ± 0.35	134	-1 - 1	-0.59; 0.78
Enlarged epiphysis					
	0.27 ± 0.45	0.00 ± 0.48	177	-1 - 1	-0.94; 0.94
Irregular subchondral surface					
	0.48 ± 0.82	-0.03 ± 0.18	37	-1 - 0	-0.38; 0.32
Narrowing of joint space					
	0.43 ± 0.73	-0.11 ± 0.37	85	-1 - 1	-0.83; 0.61
Subchondral cyst formation					
	0.51 ± 0.86	0.08 ± 0.42	82	-1 - 2	-0.74; 0.90
Erosions of joint margins					
	0.17 ± 0.38	-0.02 ± 0.22	130	-1 - 1	-0.45; 0.42
Gross incongruence of articulating bone ends					
	0.25 ± 0.52	-0.15 ± 0.36	142	-1 - 0	-0.84; 0.55
Deformity					
	0.18 ± 0.40	-0.03 ± 0.54	301	-2 - 1	-1.10; 1.03

Table 2: inter-observer variations of Pettersson
Inter-observer variations according to Bland and Altman. Mean ± SD depicts the mean actual value of each parameter of all radiographs. Δ Mean ± SD = mean difference ± SD between the first and second observation of all radiographs; ΔSD/Mean = SD of difference between the two observers, divided by the mean value; range = range of difference between the first and second observation; 95% C.I. = mean difference ± 1.96 times the SD.

For the comparison with the inter-observer variations of the OA joint, the original data as described by Marijnissen *et al.* were used[27]. In general the inter-observer variation of KIDA in case of HA is comparable to the variation in KIDA in case of OA (table 3; lower panel), except for the mean osteophyte size, for which the inter-observer variation is smaller in OA.

HA

Parameter	Mean ± SD	Δ Mean ± SD	ΔSD/Mean (%)	Range	95% C.I.
Mean bone density (mm Alu Eq.)					
	32.43 ± 4.44	0.22 ± 1.77	5	-1.19 -12.41	-3.25; 3.69
Mean subchondral bone density (mm Alu Eq.)					
	34.32 ± 3.96	0.00 ± 0.48	1	-0.37 - 2.44	-0.94; 0.94
Distance CD (mm)					
	84.09 ± 4.72	0.66 ± 1.10	1	-1.72 - 5.89	-1.49; 2.80
Correlation coefficient JSW					
	0.83 ± 0.17	-0.01 ± 0.18	22	-0.59 - 0.48	-0.36; 0.33
Mean JSW (mm)					
	5.32 ± 2.01	-0.34 ± 0.39	7	-1.04 - 0.96	-1.10; 0.42
Min JSW (mm)					
	3.45 ± 1.88	-0.90 ± 1.06	31	3.57 - 0.52	-2.97; 1.17
Mean osteophyte (mm^2)					
	3.56 ± 6.47	1.04 ± 2.69	76	-2.42 - 16.17	-4.23; 6.32
Angle joint (°)					
	1.69 ± 1.49	0.02 ± 0.49	29	-1.55 - 1.26	-0.93; 0.98

Table 3: inter-observer variations of KIDA

Inter-observer variations according to Bland and Altman. Mean ± SD depicts the mean actual value of each parameter of all radiographs. Δ Mean ± SD = mean difference ± SD between the first and second observation of all radiographs; ΔSD/Mean = SD of difference between the two observers, divided by the mean value; range = range of difference between the first and second observation; 95% C.I. = mean difference ± 1.96 times the SD. Upper panel: HA; lower panel: OA[28].

OA

Parameter	Mean ± SD	Δ Mean ± SD	ΔSD/Mean (%)	Range	95% C.I.
Mean bone density (mm Alu Eq.)					
	28.64 ± 4.21	-0.07 ± 0.47	2	-1.21 - 2.04	-0.98; 0.84
Mean subchondral bone density (mm Alu Eq.)					
	30.22 ± 4.38	-0.09 ± 0.47	1	0.87 - 2.11	-1.00; 0.82
Distance CD (mm)					
	81.75 ± 7.55	0.24 ± 1.07	1	1.57 - 6.14	-1.86; 2.34
Correlation coefficient JSW					
	0.83 ± 0.15	-0.02 ± 0.13	16	-0.37 - 0.34	-0.28; 0.24
Mean JSW (mm)					
	5.14 ± 1.08	-0.05 ± 0.52	10	-2.48 - 2.03	-1.08; 0.97
Min JSW (mm)					
	2.90 ± 1.65	-0.11 ± 0.52	18	-2.45 - 1.12	-1.12; 0.90
Mean osteophyte (mm^2)					
	6.21 ± 4.76	0.82 ± 2.26	36	-8.03 - 6.01	-3.61; 5.24
Angle joint (°)					
	3.02 ± 2.05	-0.08 ± 1.10	36	4.41 - 2.99	-2.24; 2.09

Correlations between Pettersson- and KIDA-parameters

In figure 1-8, the correlations are shown between each individual Pettersson-parameter with one or two appropriate KIDA-parameters. The mean of the two observers was taken for both scoring systems.

- Figure 1a and b: Correlation with osteoporosis was observed for both the mean bone density of the reference value of femur and tibia (R= minus 0.44; p= 0.01) and for the mean subchondral bone density of femur and tibia (R= minus 0.27; p=0.04).
- Figure 2: For the enlarged epiphysis as measured with the Pettersson score, the breadth of the femur, measured between points C and D by use of KIDA correlated with R= 0.32; p=0.01.
- Figure 3: Irregular subchondral surface correlated very well with the mean correlation coefficient of the JSW, calculated from the JSW measurements at the four locations in both medial and lateral in the joint (R= minus 0.59; p<0.001).
- Figure 4 a and b: KIDA has two important parameters for JSW: the mean and the minimum JSW. The mean JSW shows a correlation coefficient of R= minus 0.64; p<0.001 and the minimum JSW R= minus 0.71; p<0.001 with the narrowing of joint space as obtained by the Pettersson method.
- Figure 5: Subchondral cyst formation showed a correlation of R= minus 0.34; p= 0.01 with the mean subchondral bone density of femur and tibia.
- Figure 6: Erosions of joint margins correlate with the mean of the osteophyte size as measured with KIDA (R= 0.63; p<0.001).
- Figure 7: Gross incongruence of articulating bone ends correlated well with the mean correlation coefficient of the JSW: R= minus 0.53; p<0.001.
- Figure 8: There was no statistically significant correlation between deformity as measured by Pettersson and angle of the joint, measured by KIDA (R= 0.06; p=0.65).

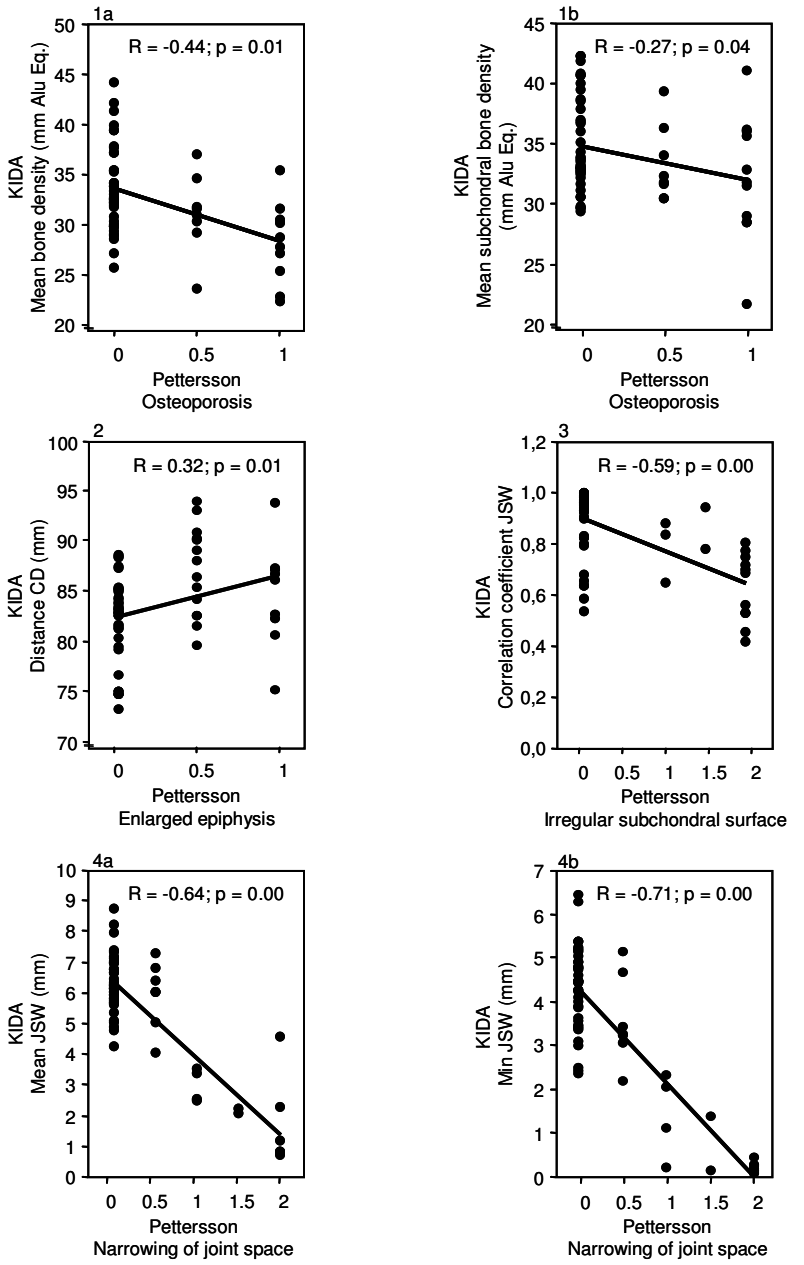

Figure 1-4: Correlations between Pettersson- and KIDA-parameters
Mean Pettersson-parameters were plotted against the mean KIDA-parameters as determined by two observers for each method. **1:** Osteoporosis; **2:** Enlarged epiphysis; **3:** Irregular subchondral surface; **4:** Narrowing of joint space

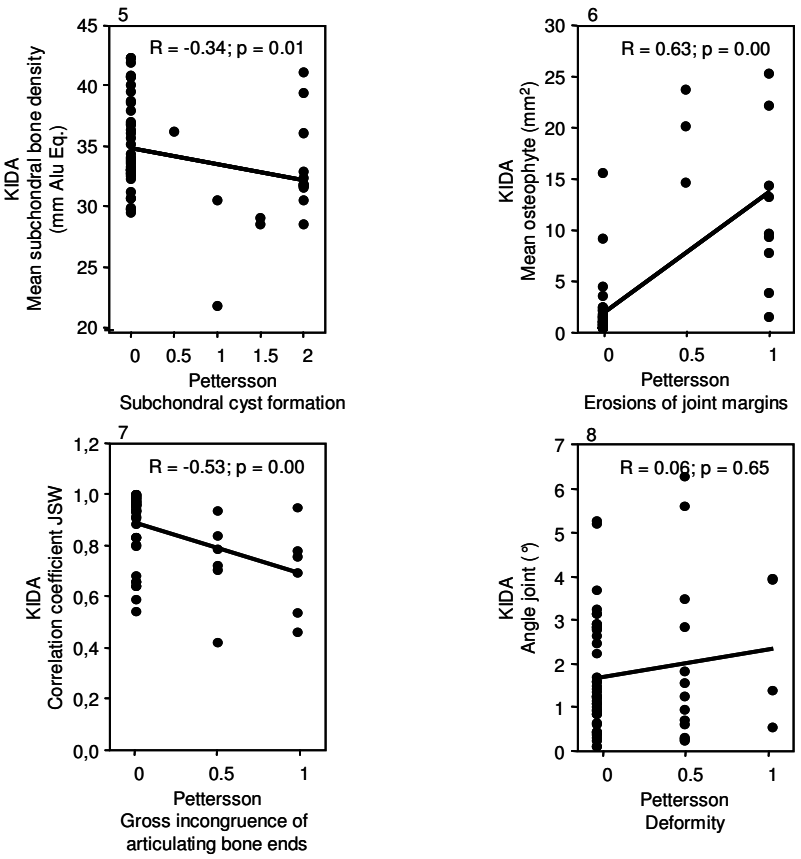

Figure 5-8: Correlations between Pettersson- and KIDA-parameters
Mean Pettersson-parameters were plotted against the mean KIDA-parameters as determined by two observers for each method. **5:** Subchondral cyst formation; **6:** Erosions of joint margins; **7:** Gross incongruence of articulating bone ends; **8:** Deformity (angulation and/or displacement between articulating bones).

DISCUSSION

In this study it is shown that digital analysis of radiographs of knees of patients with a variety in severity of haemophilic arthropathy is feasible. The inter-observer variation is in general smaller for the digital KIDA method with continuous variables than for the analogue Pettersson score with ordinal variables. Moreover, the inter-observer variation of KIDA used for HA was not significantly different from that obtained with OA knee joints, for which KIDA was originally developed. Supportive in this respect was a recent publication by Silva and colleagues, concluding that the currently available scoring systems for HA (including the Pettersson score), have low inter- and intra-observer reliability rates[29] and that improvements are needed in this respect.

For the Pettersson parameters irregular subchondral surface, narrowing of joint space, erosions of joint margins, and gross incongruence of articulating bone ends, related KIDA parameters with good correlations ($R \geq 0.5$) were available. For osteoporosis, enlarged epiphysis and subchondral cyst formation, fairly good correlations ($0.3 \geq R \leq 0.5$) with KIDA parameters were found. These parameters, and especially osteoporosis and enlarged epiphysis, are difficult to evaluate for the radiologist and scoring of these items is rather subjective. The lower correlation does not necessarily mean that KIDA is not suited – it could very well be the other way around – KIDA working better than the subjective scoring and in that respect being more correlated to “true” pathology than the human reading.

Although the angle of the joint is measured in KIDA, this did not show a good correlation with the Pettersson parameter joint deformity, which is probably due to the fact that angulation is just one of the components of this joint deformity, besides displacement of articulating bones.

The use of digital analysis, in this study KIDA, has several benefits compared to the Pettersson scoring system. First, and also most important, the use of the continuous scale in KIDA *versus* the ordinal scale in Pettersson, yields the ability to detect smaller changes by use of KIDA, as can easily be seen in the figures 1-8, where there is a large variation of values of KIDA within one whole point in Pettersson. Second, as mentioned above, the inter-observer variation for KIDA is smaller than that of Pettersson. Furthermore, the scoring by use of KIDA can easily be learned to a non-academic (within one hour[30]), while for Pettersson-scoring an experienced physician/radiologist is needed. Another great advantage of KIDA is the possibility for easy digital storage of data for long-term follow-up for both research and use in clinical practice. This information can easily be exchanged with other hospitals.

A disadvantage of this method is the necessity of the wedge, since it is needed for both the bone density measurements as well as for the correction of possible magnification of the radiographs, of influence on measurements of linear characteristics such as JSW and breadth of the joint. The duration of the scoring

procedure takes somewhat more time with KIDA (5-10 minutes per radiograph) than with Pettersson. This can be a limitation for clinical practice, but this can be overcome by the fact that the scoring can be performed by somebody else than the physician.

Although in the western world there is a rise of novel imaging techniques, such as Magnetic Resonance Imaging (MRI) and accessory scoring methods, radiography is still the golden standard in the imaging of joints, since the technique is cheap, fast, and available in almost all hospitals, even in most developing countries. This means that radiographic scores remain important in the evaluation of joint damage in haemophilia.

It is obvious that although HA and OA have much in common[31], the KIDA scoring system as developed for OA characteristics needs adaptation for the specific characteristics of HA. For example, the cyst formation, erosions, displacement between the articulating bones and enlargement of the epiphysis are not optimally covered by KIDA in its present form. However, such adaptations can easily be made and need subsequent validation. Moreover, a comparable digital evaluation should be developed for the elbow and the ankle. The study by Marijnissen *et al* [32] of the OA ankle (AIDA; Ankle Image Digital Analysis) could be a basis for a new method specific for the HA ankle. For the elbow, a new program needs to be developed as no digital methods for evaluation of radiographs of this joint are available.

Taken together, we have shown that digital image analysis of radiographs of knees of patients suffering from HA is feasible and has less variation than the currently used Pettersson-scoring system. Therefore, we think it is worthwhile to adapt the KIDA-method more specific for HA, and expand it to elbow and ankle, and thereby developing a new radiographic scoring system for HA.

ACKNOWLEDGEMENTS

We thank Mrs M. Lafeber and Drs. M. Kinds for analyzing the radiographs by the use of KIDA.

REFERENCES

1. Raffini L, Manno C. Modern management of haemophilic arthropathy. Br.J.Haematol. 2007;136:777-787.
2. Luck JV, Jr., Silva M, Rodriguez-Merchan EC et al. Hemophilic arthropathy. J.Am.Acad.Orthop.Surg. 2004;12:234-245.
3. Madhok R, Bennett D, Sturrock RD, Forbes CD. Mechanisms of joint damage in an experimental model of hemophilic arthritis. Arthritis Rheum. 1988;31:1148-1155.
4. Pettersson H, Ahlberg A, Nilsson IM. A radiologic classification of hemophilic arthropathy. Clin.Orthop.Relat Res. 1980153-159.
5. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12 Suppl 3:117-121.
6. Stein H, Duthie RB. The pathogenesis of chronic haemophilic arthropathy. J.Bone Joint Surg.Br. 1981;63B:601-609.
7. Pettersson H GM. Musculoskeletal and Other Hemorraghic Complications. Diagnostic imaging in hemophilia.: Springer-Verlag; 1985:28-54.
8. Arnold WD, Hilgartner MW. Hemophilic arthropathy. Current concepts of pathogenesis and management. J.Bone Joint Surg.Am. 1977;59:287-305.
9. Marijnissen AC, Vincken KL, Viergever MA et al. Ankle images digital analysis (AIDA): digital measurement of joint space width and subchondral sclerosis on standard radiographs. Osteoarthritis.Cartilage. 2001;9:264-272.
10. Lynch JA, Buckland-Wright JC, Macfarlane DG. Precision of joint space width measurement in knee osteoarthritis from digital image analysis of high definition macroradiographs. Osteoarthritis.Cartilage. 1993;1:209-218.
11. Marijnissen AC, Vincken KL, Vos PA et al. Knee Images Digital Analysis (KIDA): a novel method to quantify individual radiographic features of knee osteoarthritis in detail. Osteoarthritis.Cartilage. 2008;16:234-243.
12. Conrozier T, Lequesne M, Favret H et al. Measurement of the radiological hip joint space width. An evaluation of various methods of measurement. Osteoarthritis.Cartilage. 2001;9:281-286.
13. Conrozier T, Vignon E. Quantitative radiography in osteoarthritis: computerized measurement of radiographic knee and hip joint space. Baillieres Clin.Rheumatol. 1996;10:429-433.
14. Vignon E, Piperno M, Le Graverand MP et al. Measurement of radiographic joint space width in the tibiofemoral compartment of the osteoarthritic knee: comparison of standing anteroposterior and Lyon schuss views. Arthritis Rheum. 2003;48:378-384.
15. Roosendaal G, van Rinsum AC, Vianen ME et al. Haemophilic arthropathy resembles degenerative rather than inflammatory joint disease. Histopathology 1999;34:144-153.
16. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann.Rheum.Dis. 1957;16:494-502.
17. Buckland-Wright JC, Wolfe F, Ward RJ, Flowers N, Hayne C. Substantial superiority of semiflexed (MTP) views in knee osteoarthritis: a comparative radiographic study, without fluoroscopy, of standing extended, semiflexed (MTP), and schuss views. J.Rheumatol. 1999;26:2664-2674.
18. Buckland-Wright JC, Ward RJ, Peterfy C, Mojcik CF, Leff RL. Reproducibility of the semiflexed (metatarsophalangeal) radiographic knee position and automated measurements of medial tibiofemoral joint space width in a multicenter clinical trial of knee osteoarthritis. J.Rheumatol. 2004;31:1588-1597.
19. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307-310.
20. Silva M, Luck JV, Jr., Quon D et al. Inter- and intra-observer reliability of radiographic scores commonly used for the evaluation of haemophilic arthropathy. Haemophilia. 2008

Chapter 10

Summary and general discussion

Parts of this chapter will be included in an invited review in the British Journal of Haemophilia, entitled: " What do we know and what do we need to know of haemophilic arthropathy?"

The aim of this thesis was to gain more insight in various aspects of *blood-induced joint damage*, and to gain knowledge of how these mechanisms can be translated to, and be of value for, clinical practice; *from mechanisms to clinical practice*. Several human *in vitro* and animal *in vivo* studies, as well as studies involving patients with haemophilia, have been performed in light of this aim. The results of these studies are summarized in this chapter, followed by an integrated discussion of these results.

SUMMARY

Section I: Mechanism

It is known that blood has devastating direct effects on cartilage. Apoptosis of chondrocytes, induced by radicals, is involved in this and leads to long-lasting adverse effects after only a short exposure. This knowledge is mainly based on *in vitro* studies in which healthy human cartilage tissue explants are exposed for 4 days to 50% volume/volume (v/v) blood[1-4]. Despite rather detailed insight in the mechanism of blood-induced cartilage damage, little was known about e.g. the minimum blood load needed to induce the harmful effects and whether impaired cartilage is more susceptible to exposure to blood than healthy cartilage is.

Chapter 2: A blood load of 50% v/v for 4 days was primarily based on clinical experience in case of an isolated joint bleed in haemophilia patients and on a rabbit *in vivo* study that suggested a natural evacuation time of at least 4 days[5]. Upon testing a range of concentrations and exposure times, it is now demonstrated that a 2-day exposure of cartilage to 10% v/v blood *(in vitro)* leads to prolonged biochemical impairment of joint cartilage. A 1-day exposure to 50% v/v and a 4-day exposure to 5% v/v on the other hand, lead to transient impairment of joint cartilage. Herewith the treshold of the blood load leading to prolonged cartilage damage *in vitro* is determined. Although these results are *in vitro* findings, they suggest that aspiration of blood from a joint as soon as possible, but at least within 48 hours after a joint haemorrhage, is to be considered. Aspiration under these conditions would potentially prevent or diminish long-lasting impairment of cartilage tissue. The load (time and/or dose) dependency of several of the adverse effects as described in chapter 2 suggests that the sooner aspiration takes place, the better results can be obtained.

Chapter 3: The use of healthy cartilage tissue in experiments mimics a first joint bleed. But in haemophilia and due to repeated sports traumas, recurrent haemarthroses (can) occur. The first joint bleed(s) compromise(s) the cartilage and also in joints affected by e.g. a process of osteoarthritis, cartilage is impaired. This impaired cartilage is likely to have limited repair capacity and thereby can possibly be

more susceptible to blood-induced damage. In chapter 3 it is demonstrated that impaired cartilage is at least as susceptible to damage induced by a 2-day exposure to 10% v/v blood as healthy cartilage is. From this, it is concluded that at all times, joint bleeds should be prevented as good as possible. Irrespectively, because complete prevention of the occurrence of joint bleeds is not realistic, research must be performed to develop proper treatment of joint bleeds.

Section II: Treatment

The most comprehensible example of blood-induced joint damage is seen in haemophilia. The repeated joint bleeds lead to joint damage within several years. This haemophilic arthropathy due to recurrent haemarthroses is the main cause of morbidity in patients suffering from haemophilia. Even despite the significant improvement of treatment with clotting factor, joint bleeds still occur. It will be impossible, due to high costs, to treat all patients with haemophilia in such a way (complete clotting factor substitution) that joint bleeds will be banned completely. Therefore, a search for treatment modalities to prevent blood-induced arthropathy is reasonable.

Chapter 4: Chondrocytes have to maintain cartilage matrix integrity on their own and more or less have to do that for a life time, since they hardly proliferate. As mentioned, DNA fragmentation of chondrocytes, and hence cell death, due to oxidative agents is responsible for the prolonged harmful effects to cartilage when exposed to blood. This tempted us to study the poloxamer P188 as a potential treatment modality in case of blood-induced cartilage damage. P188 is a tri-block copolymer of two hydrophilic sites with in-between a hydrophobic site enabling insertion into membrane pores that can be present due to a variety of traumas. In this way, P188 provided survival of many different cell types, amongst them chondrocytes[6-8]. However, as described in chapter 4, P188 was not able to prevent the adverse changes in proteoglycan turnover that are induced by exposure to blood. Based on the first exploratory experiments it was concluded that a more detailed survey of the potential effects of P188 is not worthwhile.

Chapter 5: Blood-induced joint damage includes both synovial inflammation-mediated mechanisms as well as cartilage-mediated processes. In literature, IL-10 was described as a candidate in treatment of chronic inflammatory joint diseases and was reported to have direct beneficial effects on cartilage. In case of blood-induced joint damage, IL-10 might have multiple targets to modulate this joint damage. As is shown in chapter 5, IL-10 was indeed able to diminish the adverse effects of blood on the cartilage matrix turnover of both healthy and impaired cartilage. Moreover, the matrix turnover of haemophilic cartilage, frequently exposed to blood *in vivo*,

improved upon addition of IL-10. Also the production of the pro-inflammatory and tissue-destructive cytokines IL-1β and TNFα by haemophilic synovial tissue decreased in the presence of IL-10. It was concluded that the observed beneficial effects warrants further studies to evaluate IL-10 as a candidate in treatment of tissue damaging effects of joint haemorrhages.

Section III: Translation

An issue of concern in translation of results to clinical practice is the discrepancy in the magnitude of the effect of blood-exposure between *in vitro* and animal *in vivo* experiments obtained thus far. The effects in the canine *in vivo* situation were, although harmful as well, from a lesser magnitude than those seen *in vitro*. Insight in the factors contributing to this discrepancy is a prerequisite for the development of a suited model enabling translation of results from *in vitro* studies, via animal *in vivo* studies to the human clinical situation.

Chapter 6: In previous canine *in vivo* studies two injections of blood in the knee joint with a two-day interval were assumed to mimic a blood-exposure of 4 days to ~50% v/v. However, surprisingly, blood is cleared very rapidly from the canine knee joint as is described in chapter 6. Within 48 hours, the blood load was reduced to ~5%. Since blood-induced cartilage damage *in vitro* is time- and dose-dependent (chapter 2), this rapid clearance can play a role in the discrepancy between long-term *in vitro* and *in vivo* effects of blood-induced joint damage. Nevertheless, despite the quick clearance, both cartilage and synovial tissue were already adversely affected by this short blood-exposure.

Chapter 7: Another important factor possibly contributing to the difference between the *in vitro* and *in vivo* effects, is the fact that in the *in vitro* explant culture system, cartilage is exposed at all sides, since cartilage tissue explants are fully submerged in a certain concentration of blood. In contrast, in the *in vivo* situation cartilage is exposed to blood at the articular surface only. Exposure of cartilage to the articular surface results in less severe damage than when cartilage is fully submerged in blood as is described in chapter 7. This observation may contribute to the explanation of the discrepancy between long-term *in vitro* and *in vivo* effects of blood-induced joint damage. Nevertheless, although less outspoken, also after articular surface exposure alone, blood has harmful effects on articular cartilage.

Section IV: Clinical practice

In clinical practice in care of patients with haemophilia, there is a need for sensitive measures of joint damage. There is hardly knowledge of how the arthropathy develops and progresses, what the actual effect of a single joint bleed is, and what the effects of treatment with clotting factor concentrate are on the development and progression of arthropathy. Furthermore, proper tools will not just be of value for clinical practice, but may be of great value in clinical research as well.

Chapter 8: In this chapter biomarkers of bone and cartilage turnover, as evaluated in rheumatoid arthritis and osteoarthritis, showed to correlate with the currently available and recommended radiographic score of haemophilic arthropathy, the Pettersson score. The combination of the cartilage biomarkers urinary CTX-II, serum COMP, and serum CS846 correlates best with the degree of arthropathy. Interestingly, no biomarker of bone turnover was found to correlate with the arthropathy. It was concluded that a combination of biomarkers relate significantly better to severity of joint damage as seen in patients with haemophilic arthropathy than individual biomarkers. Furthermore, it was concluded that because of its specific characteristics, haemophilic arthropathy may be of use in future studies to identify and evaluate biomarkers of cartilage and bone turnover.

Chapter 9: The Pettersson score is presently the golden standard to asses the degree of joint damage in patients with haemophilia. This method however does not enable the detection of small changes. Digital image analysis enables more precise and objective measurement of several features of joint damage as seen on radiographs, such as joint space width narrowing, subchondral changes, and osteophyte formation. Availability of such analysis in case of haemophilic arthropathy would be of great value. In chapter 9 it is shown, using a presently available method for digital image analysis of knee radiographs in case of osteoarthritis, that digital image analysis in case of haemophilic arthropathy is feasible. Therefore it is concluded that it is worthwhile to develop digital image analysis software specific for features of blood-induced arthropathy for the specific joints involved in haemophilia arthropathy.

GENERAL DISCUSSION

To reach the ultimate goal of prevention and treatment of blood-induced arthropathy, to which this thesis is aimed to contribute, appropriate research tools must be available. These include *in vitro* and animal *in vivo* models that reflect the human *in vivo* situation, as well as sensitive outcome parameters in clinical studies with patients.

In vitro studies

In vitro models will never be able to fully reflect the *in vivo* situation, due to practical limitations and the absence of a complete context with all factors, tissues, and processes possibly involved. These models however, are necessary in research. Therefore, *in vitro* models need to be optimized in such a way that they reflect the *in vivo* situation as good as possible, always considering the balance between possibilities and limitations.

Regarding the *in vitro* model of blood-induced cartilage damage, a step has been made towards a model that probably mimics the *in vivo* situation better. In this newly developed culture system, cartilage biopsies are exposed to the articular surface only, and not to all cutting edges as well, as is the case in the conventional explant culture system. It appeared that the effects of blood exposure to the articular surface alone led to less severe effects than exposure to all sides. But nonetheless prolonged adverse effects were induced.

The effects of blood on already affected cartilage as described in chapter 3 might be different from healthy cartilage when just the articular (damaged) surface is exposed. Full exposure could have shaded possible differences between intact and damaged articular surfaces, because the articular surface only forms 1/6 of the entire surface exposed. For the other 5/6 of the surface area the explants of degenerated and healthy cartilage probably do not differ greatly. Also the effect of IL-10 as described in chapter 5 might be less profound when only the articular surface is exposed, although IL-10 is likely to be able to pass the articular surface easily, with full depth penetration, because of its size. It can also not be excluded that the threshold of the blood load that is needed to induce long-lasting cartilage damage (as described in chapter 2) is shifted to higher concentrations and/or duration when just the articular surface is exposed to blood.

However, this is all speculative, and therefore this novel culture system needs further study. Not only with respect to blood-induced damage, but also with respect to cartilage cultures assessing the effect of other compounds/treatment modalities in general. A practical limitation in the use of this novel culture system is the fact that it requires large pieces of cartilage and these are not always easily available. Therefore, adaptations of this culture system might be needed, making it less

demanding regarding the size of the cartilage samples. This would facilitate its implementation in research.
Nonetheless, the original explant system did (as yet) not result in false outcomes. The effects of blood exposure were only more outspoken. For other conditions, as discussed above, it remains to be evaluated whether there are differences between these *in vitro* systems regarding its outcome. Therefore the conventional cartilage culture system is still useful, even though the results have to be interpreted with caution and a verification of outcome using the novel approach might be warranted.
As mentioned above, it has to be kept in mind that *in vitro* tissue culture systems in general have their limitations. There are always many parameters that will be different from *in vivo*. However this does not qualify the cartilage culture system (conventional and newly developed system) as inferior, on the contrary. It provides the opportunity to evaluate detailed biochemical processes independent from adjacent tissues such as bone and synovial tissue. Moreover, it enables optimizing the condition to be tested before translation to animal *in vivo* studies (as we learned from chapter 2 and 6) and thereby limiting unnecessary or unjustified use of animals.
The use of animal *in vivo* studies in haemophilia research remains inevitable, not only because the *in vitro* systems never fully reflect the *in vivo* situation, but also because in clinical studies the relation between clinical outcome and disease processes in the tissues involved can not easily (or even not at all) be made.

Animal *in vivo* studies

The canine *in vivo* model of blood-induced joint damage as described in this thesis is unique in its kind. A recent publication by Monahan on animal studies of relevance to haemophilia[9] clearly demonstrates that there are not many models of haemophilic arthropathy. There is just one other model that needs to be mentioned in this respect; a mouse model developed by the group of Valentino[10]. Both this model and our model have their pros and cons. Although the mouse model is a true haemophilia model in the sense that these mice have less factor VIII activity, spontaneous joint bleeds do not occur. Joint bleeding in these mice has to be triggered by trauma, but this can lead to additional joint damage. Moreover, the model is primarily inflammation-driven. It is also a very small animal, limiting the possibilities of detailed evaluation. Our model is not a true haemophilia model, since coagulation is not disturbed in these animals. The advantage of our model is that the bleeding is controlled, with respect to the moment and volume of the blood-injections, without possible additional joint injury. Moreover, the size of the animals enables detailed (biochemical) evaluation of the joint.
The importance of the difference in coagulation conditions in both models remains to be debated. Although we do not use anti-coagulation factors and the dogs do not have a coagulation disorder, in all our previous and presently described studies (chapter 6), hardly any coagulation was found in the joint (personal observation and

personal communications). Apparently the condition in the joint, protects the blood from clotting, even in healthy (non-haemophilic) conditions. Moreover, there is no difference in the severity of the effects of exposure of cartilage to coagulating *versus* non-coagulating blood as determined *in vitro* (chapter 2). These observations support the usefulness of our canine model of blood-induced arthropathy.

An important question in the use of this canine *in vivo* model is the actual blood load needed to mimic the human situation. In previous canine *in vivo* studies, the effect of haemarthroses was less outspoken than those after *in vitro* blood exposure. Part of this discrepancy can be attributed to fact that *in vivo* just the articular surface is exposed as discussed above. The extremely quick clearance of blood from the canine knee joint (as described in chapter 6) might be an additional explanation. This rate of clearance was far higher than originally anticipated on, and seems to be much quicker than it is observed in humans. However, the latter needs thorough study, because it is only based on experience in clinical practice and never has been quantified. Studies using frequent ultrasound monitoring of the joint after a bleed to determine the rate with which the effusion subsides might be helpful in this respect. But assuming the clearance rate in humans is less quick than observed in the canine knee joint, multiple injections of blood with a short period in between (perhaps even daily injections of blood) might be needed in the canine model to imitate the human clearance rate better. It is worthwhile to study the actual joint damage under these conditions.

In future studies it might be helpful to evaluate biomarkers of cartilage and bone turnover in this model as well. In chapter 8 it is described that a certain combination of commercially available biomarkers of cartilage turnover correlate very well with radiographic joint damage in human haemophilic arthropathy. Whether these markers also reflect the joint damage in the canine model, which would be of use in studies on possible treatments, remains to be tested. In support of this, in canine models of osteoarthritis, uCTX-II is elevated compared to controls[11].

After further perfection of this canine *in vivo* model of blood-induced joint damage, the protective effects of IL-10 might be subject of *in vivo* evaluation, based of the promising effect of IL-10 *in vitro* (chapter 5).

Another possible treatment option to study in this *in vivo* model is arthrocentesis. Quick clearance of blood from the joint has shown to reduce the effect of blood exposure, both *in vitro* (chapter 2) and *in vivo* (chapter 6). This can be accomplished by aspiration of the joint after a haemarthros. Whether this indeed has regulating effects on blood-induced joint damage can be studied in the canine *in vivo* model. Furthermore, the possible additional effect of lavage, or concomittant administration of IL-10 for instance, can be evaluated. If proven beneficial in such studies, a prospective clinical trial can be performed. However, representative and sensitive outcome measures to asses its effects are prerequisites for such a clinical trial.

Outcome parameters

Possible treatment modalities that evolve from *in vitro* and animal *in vivo* studies need to be tested in clinical trials. This requires proper outcome parameters. Moreover, such outcome measures may add to our knowledge of the actual effects of joint bleeds (and treatment) in patients. Sensitive measures of structural changes in the joint due to haemarthroses are needed in this respect. Two measures have been evaluated in this thesis; biomarkers of cartilage and bone turnover, and digital analysis of radiographs. Surprisingly, although these measures have been subject of study for years in the field of osteoarthritis and rheumatoid arthritis, they have both never been studied for evaluation of haemophilic arthropathy.

Biomarkers of cartilage and bone turnover are speculated to be a reflection of processes taking place in the body. Therefore they might even be able to predict radiological progression. Although very promising, this thesis is the first to describe the evaluation of biomarkers of cartilage and bone turnover in case of haemophilic arthropathy. Questions that remain to be answered are whether a haemarthros leads to direct joint damage that can be evaluated by these biomarkers, and whether these biomarkers can predict the rate of progression. If indeed there are biomarkers that reflect the effect of a haemarthros, it would be of interest to determine whether an effect of clotting factor administration also can be evaluated by these means. Also effects of potential treatment modalities such as aspiration (chapter 2) and IL-10 (chapter 5) might be monitored in more detail by the use of biomarkers. Moreover, haemophilic arthropathy, due to its unique characteristics as discussed in chapter 8, might become a tool to validate newly developed biomarkers, that can subsequently be tested for the more complex patient-populations such as osteoarthritis and rheumatoid arthritis.

While biomarkers are measures of the current ongoing processes, radiographs reflect the cumulative result of the past processes. Radiographs are important tools to assess the severity of joint damage and the change in joint damage over time. In case of haemophilia, the Pettersson scoring method is widely used. But, with all respect, this Pettersson score, developed in the 1980s, has become outdated. It yields a score on an ordinal scale from 0 to max 2 for 8 different parameters of the six index joints. This makes the Pettersson scoring unsuitable for detailed sensitive evaluation of joint degeneration. At present, multiple digital methods to analyse different aspects of joint degeneration are developed and used, a possibility that is facilitated by digitalization of the radiographic procedures. Digital evaluation has the advantage that it yields information on a continuous scale, enabling detection of smaller changes. Moreover, they are objective and do not need clinical knowledge of the disease process; they are just a mathematical tool. From chapter 9 in this thesis it appeared that the use of such a digital evaluation of the knee is feasible and provides the possibility to describe the radiographic damage objectively and in detail. This finding warrants future studies on the adaptation of the presently used method for the

knee for the specific characteristics of haemophilic arthropathy in addition to the development of such programs for ankles and elbows. As such, digital analyses of the radiographic features specific for haemophilic arthropathy would be an important improvement for evaluation of joint damage in research, like for instance studies on biomarkers, and in clinical practice.

Overall, the most important conclusion from the research described in this thesis, is that blood exposure, irrespective under what condition, either *in vitro* or *in vivo*, either for short or long period of time, either to high or low amounts of blood, either on healthy or degenerated cartilage, clearly has adverse effects on the joint. This emphasizes that prevention of joint bleeds is of great importance, and since complete prevention is probably not possible, good interventions must be developed, to avoid blood-induced joint damage.

REFERENCES

1. Hooiveld M, Roosendaal G, Wenting M et al. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. Am.J.Pathol. 2003;162:943-951.
2. 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;42:784-790.
3. 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;24:1350-1354.
4. Roosendaal G, Vianen ME, Marx JJ et al. Blood-induced joint damage: a human in vitro study. Arthritis Rheum. 1999;42:1025-1032.
5. Muirden KD. Clearance of Fe59-labelled erythrocytes from normal and inflamed rabbit knee joints. I. Relationship to the anaemia of rheumatoid arthritis. Ann.Rheum.Dis. 1969;28:548-551.
6. Phillips DM, Haut RC. The use of a non-ionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants. J.Orthop.Res. 2004;22:1135-1142.
7. Rundell SA, Baars DC, Phillips DM, Haut RC. The limitation of acute necrosis in retro-patellar cartilage after a severe blunt impact to the in vivo rabbit patello-femoral joint. J.Orthop.Res. 2005;23:1363-1369.
8. Baars DC, Rundell SA, Haut RC. Treatment with the non-ionic surfactant poloxamer P188 reduces DNA fragmentation in cells from bovine chondral explants exposed to injurious unconfined compression. Biomech.Model.Mechanobiol. 2006;5:133-139.
9. Monahan PE. Experimental animal use in the study of haemophilic bleeding. Haemophilia. 2008;14:112-116.
10. 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;10:280-287.
11. Intema F, Sniekers Y, Weinans H et al. Regulation of sunchondral bone and cartilage changes in two canine models of osteoarthritis. Manuscript in preparation 2008

Bloed-geïnduceerde gewrichtsschade:
Van mechanismen naar klinische praktijk

Dit proefschrift behandelt verschillende aspecten van bloed-geïnduceerde gewrichtsschade: schade aan gewrichten die ontstaat na één of meerdere bloedingen in het gewricht. Deze gewrichtsbloedingen kunnen optreden in gezonde mensen als gevolg van een letsel, zoals het scheuren van gewrichtsbanden of een botbreuk in het gewricht. Daarnaast kunnen gewrichtsbloedingen ook spontaan optreden bij mensen die aan een stollingsstoornis lijden. Bij mensen met de stollingsstoornis hemofilie komt gewrichtsschade dan ook vaak voor.
Dit hoofdstuk bevat een samenvatting van de resultaten van het in dit proefschrift beschreven onderzoek, voorafgegaan door een korte uitleg over gewrichten, hemofilie en bloed-geïnduceerde gewrichtsschade zoals in **hoofdstuk 1** is beschreven.

Gewrichten

Belangrijke onderdelen van gewrichten, zoals knieën, enkels en schouders, zijn kraakbeen en synovium. Kraakbeen is de bekleding van beide botuiteinden die het gewricht vormen. Synovium is de binnenbekleding van het gewrichtskapsel dat de beide botuiteinden met elkaar verbind. Samen met de gewrichtsbanden en spieren zorgen deze weefsels ervoor dat gewrichten soepel kunnen bewegen. Het synovium doet dat door het produceren van een stroperige vloeistof (synoviale vloeistof), waarmee de gewrichtsholte gevuld is. Deze synoviale vloeistof is een smeermiddel voor het gewricht en verzorgt de voeding van kraakbeen.
Kraakbeen is een bijzonder soort weefsel, dat hard en stug lijkt, maar in werkelijkheid heel veerkrachtig en elastisch is. Het dempt de krachten die op een gewricht komen te staan tijdens belasting van het gewricht. Kraakbeen is een bindweefsel-matrix met daarin maar één soort cel: de chondrocyt. Deze chondrocyten onderhouden de grote hoeveelheid matrix waaruit kraakbeen bestaat. Deze matrix bestaat voornamelijk uit collagenen en proteoglycanen, die een soort sponsachtige structuur vormen waarin water kan worden opgenomen, maar ook uit kan worden geperst. Door deze bijzondere structuur kan het kraakbeen zijn functie uitoefenen.

Hemofilie

Hemofilie is een erfelijke ziekte. Mensen die aan deze ziekte lijden hebben te weinig van een bepaald soort stollingsfactor in hun bloed. Hierdoor stolt hun bloed minder snel dan bij gezonde mensen. Als gevolg hiervan stopt het bloeden na een verwonding minder snel. Bovendien kunnen bij mensen met hemofilie spontane spier- en gewrichtsbloedingen optreden. Gewrichtsschade als gevolg van gewrichtsbloedingen is de meest voorkomende complicatie bij mensen met hemofilie. Deze gewrichtsschade heet hemofilie arthropatie (HA).
Er is geen genezing mogelijk van hemofilie. De behandeling bestaat uit het toedienen van de ontbrekende stollingsfactor. Omdat dit niet volledig kan door de hoge kosten van deze stollingsfactoren kan het optreden van gewrichtsbloedingen niet volledig

worden voorkomen. Bovendien heeft wereldwijd niet iedere hemofiliepatiënt de beschikking over deze kostbare stollingsfactoren. Dus ondanks de beschikbaarheid van stollingsfactoren blijft hemofilie arthropatie vooralsnog een probleem.

Bloed-geïnduceerde gewrichtsschade

De aanwezigheid van bloed in een gewricht heeft directe effecten op zowel het synovium als het kraakbeen. Het synovium is betrokken bij het verwijderen van het bloed, maar door het verwijderen van grote hoeveelheden bloed raakt het synovium ontstoken. Dit kan resulteren in een chronische ontsteking van het synovium. Door deze ontsteking raakt de structuur van het synovium beschadigd en komen er allerlei schadelijke stoffen terecht in de synoviale vloeistof die het kraakbeen aantasten.

Het directe effect van bloed op kraakbeen is het verstoren van de aanmaak en afbraak van de bestanddelen (proteoglycanen en collagenen) van kraakbeen. De aanmaak van de proteoglycanen vermindert en de uitscheiding van deze proteoglycanen wordt vergroot, waardoor het gehalte van deze proteoglycanen verlaagd wordt. Bovendien worden er allerlei enzymen geactiveerd die collagenen en andere eiwitten in kraakbeen afbreken. Dit tezamen verslechtert de structuur van kraakbeen, welke juist zo belangrijk is voor het uitoefenen van de functie. Een belangrijke oorzaak voor deze schade is het feit dat blootstelling van kraakbeen aan bloed leidt tot dood van de chondrocyten: de cellen van het kraakbeen die de kraakbeenmatrix maken en onderhouden. Deze cellen vermenigvuldigen zo goed als niet in volwassen kraakbeen en daarom is het verlies van deze cellen zo goed als onomkeerbaar.

De uitscheiding van afbraakproducten van kraakbeen in de synoviale vloeistof kan ook de ontsteking van het synovium verder aanwakkeren. Bloed heeft dus directe effecten op het synovium en op het kraakbeen en deze effecten beïnvloeden elkaar ook nog eens.

Uiteindelijk kan dit alles leiden tot ernstige gewrichtsschade, die invaliderend kan zijn. Deze gewrichtsschade heeft kenmerken van zowel artrose (‘gewrichtsslijtage’) als van artritis (‘reuma’) en net als bij deze ziekten is uiteindelijk het vervangen van het gewricht door een prothese (een kunstgewricht) vaak nodig voor het behoud van functie. Bij patiënten met hemofilie is dit vaak al op relatief jonge leeftijd noodzakelijk.

Het doel van het onderzoek dat beschreven is in dit proefschrift, is om meer inzicht te krijgen in aspecten van bloed-geïnduceerde gewrichtsschade en middelen om deze kennis te kunnen inzetten in de klinische praktijk, in de behandeling van gewrichtsbloedingen in het algemeen en hemofilie arthropatie in het bijzonder. Vandaar de titel van dit proefschrift:

Bloed-geïnduceerde gewrichtsschade: van mechanismen naar klinische praktijk.

Sectie I: Mechanisme

In **hoofdstuk 2** is de minimale hoeveelheid bloed en de duur van de blootstelling van kraakbeen aan dit bloed die tot langdurige beschadiging van het kraakbeen leidt, bestudeerd. Dit is gedaan door *in vitro* ('in glas', dus in het laboratorium, in kweek) kraakbeen bloot te stellen aan variërende hoeveelheden bloed gedurende een variërend aantal dagen. De hierdoor opgetreden kraakbeenschade is gemeten aan de hand van de aanmaak en afbraak van de proteoglycanen van het kraakbeen en de activiteit van kraakbeen afbrekende enzymen. Het bleek dat een relatief korte blootstelling van 2 dagen aan een zeer lage concentratie van 10% bloed al leidt tot langdurige schade aan het kraakbeen. Deze kennis is van belang in de discussie over de vraag of het verwijderen (puncteren en aspireren) van bloed uit een gewricht na een gewrichtsbloeding moet worden uitgevoerd om schade op termijn te voorkomen. De resultaten uit deze studie geven aan dat dit zinvol lijkt en dat dit in ieder geval binnen 2 dagen na de bloeding gedaan zou moeten worden wil het schade kunnen voorkomen.

De vraag in **hoofdstuk 3** is of kraakbeen dat reeds beschadigd is gevoeliger is voor bloed-geïnduceerde schade dan gezond kraakbeen. Om deze vraag te kunnen beantwoorden is het effect van een 2-daagse blootstelling aan 10% bloed (dus de minimale blootstelling die tot langdurige schade leidt van gezond kraakbeen) vergeleken met het effect van een dergelijke blootstelling van beschadigd kraakbeen. Twee verschillende gradaties kraakbeenschade zijn daarbij gebruikt. Uit deze studie bleek, dat het milde en ernstig beschadigde kraakbeen tenminste net zo gevoelig is voor de blootstelling aan bloed als het gezonde kraakbeen. Dus ook bij mensen met al beschadigde gewrichten blijft het voorkomen van gewrichtsbloedingen erg belangrijk. Mocht een bloeding toch optreden, dan kan behandeling, ook als er al kraakbeenschade is, verdere schade voorkomen.

Sectie II: Behandeling

Omdat gewrichtsbloedingen niet volledig voorkomen kunnen worden, is het belangrijk om onderzoek te doen naar mogelijke behandelingen die bloed-geïnduceerde gewrichtsschade kunnen verminderen en het liefst zelfs kunnen voorkomen.

Zoals hierboven beschreven zijn de chondrocyten in kraakbeen in belangrijke mate verantwoordelijk voor het in stand houden van kraakbeen. Blootstelling van kraakbeen aan bloed leidt helaas tot de dood van deze cellen. Van een bepaalde stof, het polymeer pluronic (ook wel poloxamer P188 genoemd) is aangetoond dat het de dood van verschillende soorten cellen, waaronder ook chondrocyten, kan verhinderen. Helaas bleek dat P188 niet in staat is om *in vitro* veroorzaakte bloed-

geïnduceerde kraakbeenschade te voorkomen (**hoofdstuk 4**). Klaarblijkelijk spelen andere mechanismen een rol in bloed-geïnduceerde dood van chondrocyten die niet door P188 kunnen worden voorkomen; daarmee is verder onderzoek naar deze stof niet zinvol.

Een andere stof die kan inspelen op verschillende processen die een rol spelen bij bloed-geïnduceerde gewrichtsschade is interleukine-10 (IL-10). Interleukines (ook wel cytokinen genoemd) zijn eiwitten, die 'boodschappers' zijn in het immuunsysteem. Ze stimulerende of remmen bepaalde activiteiten van cellen. IL-10 is een interleukine dat ontsteking kan remmen, maar een stimulerende werking heeft op chondrocyten. Daarom is onderzocht of IL-10 mogelijk een remmende werking op bloed-geïnduceerde gewrichtsschade kan hebben. Uit het onderzoek beschreven in **hoofdstuk 5** bleek dat wanneer IL-10 aanwezig is tijdens de blootstelling van kraakbeen aan bloed, het schadelijke effect hiervan minder wordt. Anders gezegd: IL-10 beschermt tegen bloed-geïnduceerde kraakbeenschade *in vitro*. Bovendien bleek dat wanneer IL-10 wordt toegevoegd aan een kweek van beschadigd kraakbeen van patiënten met hemofilie arthropatie, dit een gunstige werking op de aanmaak van de kraakbeenmatrix heeft. Het synovium van gewrichten met hemofilie arthropatie produceert factoren zoals IL-1 en TNFα, die ontsteking stimuleren en het gewricht beschadigen. In deze studie bleek dat IL-10 de productie van deze factoren door synovium van gewrichten met hemofilie arthropatie vermindert. IL-10 lijkt dus een goede kandidaat voor de behandeling van bloed-geïnduceerde gewrichtsschade en zou als zodanig verder onderzocht moeten worden.

Sectie III: Vertaling

Met het woord vertaling wordt hier de vertaling van de *in vitro* situatie, de laboratoriumsituatie, naar de *in vivo* ('in leven') situatie bedoeld. In onderzoek in het algemeen en zeker ook voor onderzoek naar bloed-geïnduceerde gewrichtsschade, blijft het gebruik van proefdieren helaas noodzakelijk. Alleen in een volledig organisme kunnen alle effecten en hun onderlinge beïnvloeding die bijdragen aan een ziekte en de behandeling daarvan, getest worden. Alleen op deze manier kan het onnodig en mogelijk gevaarlijk testen van behandelingen op mensen worden voorkomen. Uit voorgaand onderzoek bleek dat de effecten van experimentele gewrichtsbloedingen in honden minder waren dan op basis van de *in vitro* resultaten werd verwacht. In dit proefschrift zijn twee studies beschreven naar mogelijke verklarende factoren hiervoor.

Als eerste mogelijke verklaring is in **hoofdstuk 6** de snelheid onderzocht waarmee bloed uit het kniegewricht van de hond door het synovium wordt verwijderd. Het bleek dat de snelheid waarmee bloed uit de gewrichtsholte verdween veel hoger is

dan aanvankelijk gedacht. Binnen 48 uur is ~95% van het bloed uit de gewrichtsholte verdwenen. Hierdoor wordt de uiteindelijke blootsteling minder langdurig en minder heftig. Zoals uit hoofdstuk 2 is geleerd zal dit tot minder schade leiden dan oorspronkelijk werd verwacht. Ondanks de snelle klaring waren al wel schadelijke effecten op het kraakbeen en het synovium waarneembaar. Hoe deze snelle klaring wordt bereikt is echter nog onduidelijk en moet verder onderzocht worden.

Een ander verschil tussen de *in vitro* en *in vivo* situatie is het feit dat *in vivo* alleen de zijde die grenst aan de gewrichtsholte (de articulaire zijde) wordt blootgesteld aan bloed, terwijl in de *in vitro* situatie het kraakbeen ook wordt blootgesteld aan de kanten waarmee *in vivo* het kraakbeen verbonden is aan het bot en aan de andere snij-oppervlakken. Daarom is, zoals beschreven in **hoofdstuk 7**, een *in vitro* kweeksysteem ontwikkeld waarin het kraakbeen ook *in vitro* aan alleen de articulaire zijde kan worden blootgesteld aan bloed. De effecten van blootstelling aan bloed in dit systeem zijn minder heftig dan de effecten van blootstelling aan alle zijden. Dit verschil speelt dus mogelijk ook een rol in het verschil tussen de *in vitro* en *in vivo* resultaten. Hoe dan ook, de effecten van bloed op kraakbeen, *in vitro* en *in vivo,* zijn schadelijk.

Sectie IV: Klinische praktijk

Om uiteindelijk de vertaling van de *in vitro* en dier-experimentele *in vivo* bevindingen naar de mens te kunnen maken is er behoefte aan gevoelige uitkomstmaten voor gewrichtsschade in de mens. Uitkomstmaten die de schade kunnen beschrijven en het effect van behandeling kunnen volgen. Deze uitkomstmaten zijn dan ook van grote waarde voor onderzoek naar gewrichtsschade bij hemofiliepatiënten. Twee van dit soort mogelijke maten, die al uitvoerig bestudeerd worden bij patiënten met gewrichtsaandoeningen als artrose en artritis, zijn onderzocht wat betreft de toepasbaarheid voor hemofilie arthropatie.

In **hoofdstuk 8** zijn biomarkers van kraakbeen en bot bestudeerd. Biomarkers zijn stoffen die meetbaar zijn in bijvoorbeeld de bloedvloeistof (serum) en urine. Het gaat vaak om afbraakproducten die vrijkomen bij processen die zich afspelen in het lichaam. Momenteel wordt veel onderzoek gedaan naar biomarkers van kraakbeen- en botafbraak en -aanmaak. Een verscheidenheid aan kraakbeen- en botbiomarkers, beschreven voor artrose en artritis, is vergeleken met de ernst van de gewrichtsschade bij hemofiliepatiënten. Deze schade wordt uitgedrukt in de Pettersson-score. Het bleek dat de combinatie van 3 soorten kraakbeen biomarkers (CTX-II in urine, en COMP en CS846 in serum) een hoge mate van samenhang (correlatie) heeft met de Pettersson-score. Deze biomarkers zijn dus mogelijk bruikbaar om de ernst van de gewrichtsschade vast te stellen. Het moet verder

onderzocht worden of deze biomarkers ook bruikbaar zijn om de verergering (progressie) van de gewrichtsschade te voorspellen en of ze het effect van bloedingen en behandeling kunnen bepalen.

Naast biomarkers kunnen ook röntgenfoto's een bijdrage leveren aan het goed in beeld brengen van de gewrichtsschade. De Pettersson-score is een score die gemaakt wordt aan de hand van röntgenfoto's van de knieën, enkels en ellebogen, omdat vooral deze gewrichten aangedaan zijn bij patiënten met hemofilie. De aan- of afwezigheid van specifieke kenmerken van gewrichtsschade worden gescoord. Dat wil zeggen, er wordt een getal (0, 1 of 2) toegekend aan deze kenmerken. Dit is een relatief grove maat. Met een digitale analyse kan een veel preciezer getal voor gewrichtsschade worden verkregen. In **hoofdstuk 9** is bestudeerd of het mogelijk is om röntgenfoto's van hemofiliepatiënten digitaal te analyseren met behulp van een computerprogramma. Voor de knie is deze analyse ontwikkeld voor het meten van gewrichtsschade als gevolg van artrose. Het bleek dat deze methode voor artrose ook geschikt is voor hemofilie arthropatie. Het gebruikte programma kan met eenvoudige aanpassingen nog beter geschikt gemaakt worden voor de specifieke kenmerken van hemofilie arthropatie. Daarnaast zullen er programma's ontwikkeld moeten worden voor het meten van de elleboog en de enkel. Geconcludeerd kan worden dat digitale analyse van röntgenfoto's van gewrichten van hemofiliepatiënten mogelijk is en een verbetering is in het meten van gewrichtsschade.

Met het onderzoek beschreven in dit proefschrift (samengevat en bediscussieerd in **hoofdstuk 10**) zijn belangrijke stappen gezet gericht op het uiteindelijke doel van onderzoek naar bloed-geïnduceerde gewrichtsschade, namelijk het voorkomen van deze schade. Hiervoor zijn goede *in vitro* en *in vivo* modellen nodig, evenals gevoelige meetmethoden voor studies met patiënten. Het huidige onderzoek heeft bijgedragen aan al deze drie vereisten: er is een stap gemaakt in het ontwikkelen van een beter *in vitro* model, het *in vivo* model is verder gekarakteriseerd, en analyse van biomarkers en röntgenfoto's als meetmethoden in de klinische praktijk zijn onderzocht voor wat betreft de toepasbaarheid. Bovendien zijn er basale vragen, zoals de tijdsduur- en concentratie-afhankelijkheid van bloed-geïnduceerde kraakbeenschade en de gevoeligheid van beschadigd kraakbeen voor bloed beantwoord en zijn mogelijke therapeutische toepassingen onderzocht, waarvan IL-10 veelbelovend lijkt.
Uiteraard is verder onderzoek noodzakelijk. Het nieuwe *in vitro* kweeksysteem moet geoptimaliseerd worden zodat dit systeem de *in vivo* situatie nog beter nabootst. Het diermodel moet dusdanig aangepast worden, zodat de gewrichtsbloedingen beter vergelijkbaar zijn met gewrichtsbloedingen in de humane situatie. Met de ontwikkeling van een dergelijk model kan dan het effect van mogelijke therapeutische

toepassingen, zoals IL-10 of aspireren, worden onderzocht. Biomarkers en digitale röntgenanalyse moeten worden geoptimaliseerd en worden gevalideerd voor het meten van gewrichtsschade bij hemofilie arthropatie.
Al met al is het belangrijk om te concluderen dat bloed, in alle gevallen, duidelijk een schadelijk effect heeft op gewrichten, en dat daarom het voorkomen van gewrichtsbloedingen erg belangrijk is. Omdat het volledig voorkomen van gewrichtsbloedingen niet mogelijk zal zijn, blijft ook de ontwikkeling van behandelmethodes van belang.

Dankwoord

Dankbaar ben ik voor het feit dat ik hier mijn dank uit kan spreken aan hen die hebben bijgedragen aan dit proefschrift, om wat voor reden of op welke wijze dan ook.

Uiteraard de heren promotoren en co-promotoren:
Hooggeleerde Prof.dr. Bijlsma, bedankt voor de ruimte die u gaf om dit onderzoek uit te voeren.
Hooggeleerde Prof.dr. Theobald, ik hoop dat ik u heb kunnen laten zien, dat ook processen zonder T-cellen interessant en belangrijk kunnen zijn.
Zeergeleerde Dr. Lafeber, beste Floris, zonder jou was dit niet geweest wat het nu is. Een extra dankzegging voor het feit dat je de eindsprint mogelijk hebt gemaakt. Op naar het volgende proefschrift!
Zeergeleerde Dr. Roosendaal, beste Goris, heerlijk hoe je me wist te motiveren en zoals het een goede coach betaamt soms probeerde te overtuigen dat ik soms ook rustig aan moest doen. Ik zag dat anders. Jij had gelijk.

De basis van mijn onderzoek:
Enorme dank aan alle hemofiliepatiënten die deel wilden nemen aan de studies die in dit proefschrift beschreven zijn. Een extra bedankje aan hen, die ik een periode maandelijks bezocht heb.

Aad de Ruiter, Frank Bernhard en Martijn van Osch. Duizend maal dank voor jullie medewerking en gezelligheid. Appeltaartje?

Uiteraard kon ik mijn *in vitro* proeven niet doen zonder, naast het kraakbeen, een grote hoeveelheid aan buisjes bloed. Als een ware vampier ging ik soms door het lab. Dank aan allen die bloed hebben gegeven voor alle *in vitro* studies. De taart voor de donor met de meeste donaties volgt!

De bloeddonoren waren meestal de mensen van het reumalab:
Alle AIO's: Sarita (met jou zit ik als langst, bedankt dat je er voor me was als ik ff moest razen), Femke (roomie tijdens de OARSI), Marije (mag ik dat SPSS boek nog éven lenen?), Tineke (ik weet wat het is, die ex vivo's), Jos (nespresso, dokter J?), Margot (saunaatje?), Angela (het is: Goris, en Nathalie, niet: Gorres en Natte Alie…), Erwin (welkom in de wondere wereld van de biomarkers): bedankt voor alle gezelligheid tijdens de AIO-etentjes en –uitjes en ik hoop, zij het clandestien, nog wel eens mee te mogen doen. Ik wens jullie allemaal stuk voor stuk succes met het afronden van jullie promoties en hoop van alle getuige te kunnen zijn.

Marieke, geloof het nou, jij bent mijn kraakbeenkoningin!
Marion, de natuur is mooi en de waarheid ligt onder onze ogen (onder de microscoop). Nu nog zien.
Kim, is de r is nog in de maand?
Arno, mooi hè, synovium?

Simon, zoals je zelf schreef, van artrose naar hemofilie arthropatie is soms maar een kleine stap. Bedankt dat ik deze stap heb kunnen maken. En ja, van de volgende stap ga je ook getuige zijn. Op naar de postdoc diners?
Anne Karien, dank voor je hulp met de METC-aanvragen. En dat digitale röntgenanalyse maar een grote toepassing zal krijgen! Aan ons zal het niet liggen!
Joel, IL-10 rocks!

Joline, roomie, zij het van het laatste uur. Wel heel bijzondere uren voor mij. Dank dat je me (figuurlijk!) de ruimte hebt gegeven om die uren ten volste te kunnen benutten.
Janet, het was fijn dat ik bij jou soms stoom mocht afblazen en gezellig over kindjes kon kletsen.

Siska, echt naaste collega's zijn we niet geweest. Desondanks leefde je met me mee. Super! Het stokje geef ik graag weer verder door.

Tevens wil ik graag mijn overige collega's van de afdeling Reumatologie & Klinische Immunologie bedanken voor de samenwerking tijdens de afgelopen jaren.

Mijn "tweede" werkplek: de Van Creveldkliniek:
Ik wil jullie allen heel hartelijk bedanken voor alle gezelligheid en aandacht die er altijd voor me was.

Evelien, dank voor het includeren van patiënten in de biomarkerstudie.
Mevrouw Heijnen, bedankt voor uw bereidheid om de röntgenfoto's te scoren.
Kathelijn, de gesprekken die we hadden waren bijzonder. Ik hou je op de hoogte.
Meneer Van Rinsum, Stresa was mooi hè? Bedankt voor al het patiëntenmateriaal.
Gerda, Hannie, Nanda en Els, dank voor alle bloedjes en plasjes!
Jenneke, Bertie, Patricia, Sylvia, dank voor alle logistiek!
Monique, Ingrid, Petra en Dietje, collega onderknuppels, ook al zijn jullie collega's op afstand, het is er niet minder gezellig om.
Frank, (je hoort voor mij toch nog een beetje onder dit kopje) bedankt voor alle gezelligheid op reis, ook al duurde die soms iets langer dan gepland. Bijzondere dank voor de uren die je hebt gestoken in het maken en bewerken van de foto's van

het beeldje, waardoor mijn proefschrift de omslag kreeg, die ik al jaren voor ogen had.
Piet, ook jij bedankt voor de gezelligheid op reis (en de whiskey)! Jouw lach is de mooiste!

Verder wil ik graag bedanken:
Michel Hooiveld, dank voor het werk dat ik mocht voortzetten.
Koen Vincken, dank voor al je hulp met de röntgenfoto's. Ik hoop dat we een goede nieuwe score voor HA kunnen neerzetten.
Jeroen de Groot en Benno van El van TNO: dank voor de gastvrijheid en de mogelijkheid tot het bepalen van MMP-activiteit ten gevolge van blootstelling van kraakbeen aan bloed. Iets zegt mij dat dit een vervolg moet krijgen.
Björn Lundin, thank you for your interest and input in the two papers that you co-authored.
Mensen van het GDL, Romy, Helma, Wendy, super hoe mijn studie op korte termijn en adequaat bij jullie kon plaatsvinden. Hartelijk dank voor jullie medewerking.

Rest mij nog hen die niet direct zakelijk gezien, maar wel degelijk van betekenis zijn geweest voor mij en mijn onderzoek.

Meneer Nevenzel, dat u mijn stem toch verkoos om lang na mijn vertrek van het Staring College nog jaarlijks door het klaslokaal te horen schallen… Ik begrijp het nog steeds niet. En ik vrees velen met mij… Maar ongetwijfeld heeft het mijn interesse in de biologie vergroot. Evenals uw eigen enthousiasme dat heeft gedaan. Bedankt voor de basis, het fundament. En uw nimmer aflatende interesse. Op naar dat staminee!

Bastiaan: het is af! Zoals beloofd heb ik gezorgd dat ik er sta. Jij ook. Geweldig!

Jocea, dank voor onze vele gesprekken waarin je mijn gevoelens en ideeën vaak bevestigde en even helder onder woorden wist te brengen. Dank voor je wijze raad en dat je er voor me was, van het begin tot aan het eind. Ik zeg: white lady? Madelon, doe je mee? Hoeveel citroenen zullen we kopen?

Lieve Cora, in hemofilie worden de antilichamen die gevormd kunnen worden ten gevolge het toedienen van de ontbrekende stollingsfactor inhibitors genoemd. Jij bent al jaren mijn antibody, maar zeker geen inhibitor! On the contrary! Zeker in de laatste fase, waarin we tegelijk zaten, werkten onze gesprekken juist motiverend.

Als het hora est voor ons beiden heeft geklonken, gaan we weer een interactie aan. Maar wat is het nou: biotine of horse radish peroxidase…?

Lieve Renée, wat kan ik anders zeggen dan: thanks for your support?! Je bent bijzonder. Voor je eerlijkheid en het feit dat je toch altijd achter me staat (nu zelfs lettterlijk), ondanks dat je mijn beslissingen (als ik ze al eens neem) vaak niet begrijpt of goedkeurt, is een simpel bedankt niet genoeg.

Lieve, mooie Leonore, in vind het fijn je te hebben leren kennen. Dat je mijn paranymf wil zijn waardeer ik enorm. Dank dat je me zo laat genieten van die kleine ventjes van je. Wat is het leven toch mooi en eigenlijk zo eenvoudig! Zullen we het nog eens proberen? Zeeland en 2x4 wieltjes…?

Mijn gekke ‘gezinnetje’, Sus Yvette & Harrie, Hendrik Jan & Marijke en nu ook die mooie Felix, Iris & Leo, Jasmijn & Albert: we zijn allemaal anders en toch horen we bij elkaar. Ik ben blij jullie als familie te hebben.

Papa, dit heb je meegemaakt van a tot z. Dank voor je luisterend oor en belangstelling. Je verdient een ster.

Mama, dank dat je me laat gaan, ook al ga ik steeds verder weg. Maar al worden kindjes nog zo groot…

Curriculum Vitae

Nathalie Jansen was born on the 10th of January 1980 in Apeldoorn, the Netherlands. In 1998 she finished secondary school at the 'Staring College' in Lochem.

From 1998 until 2003 she studied Biomedical Sciences at the University of Utrecht. A major internship on the development of non-viral gene therapy for phenylketonuria was executed at the department of Metabolic Disorders at the Wilhelmina Children's Hospital in Utrecht under the supervision of I.E.T. van den Berg, PhD and R. Berger, PhD.
A literature study on proteins involved in spindle formation during the cell cycle and the consequences of failure of proper functioning of these proteins was written under the supervision of D.R. Gutknecht, PhD, from the department of Reproductive Medicine at the University Medical Centre Utrecht (UMCU).
The minor internship was performed at the department of Rheumatology & Clinical Immunology in the UMCU under the supervision of S.C Mastbergen, PhD and F.P.J.G. Lafeber, PhD, during which the effect of selective inhibition of cyclooxygenase-1 and -2 in human articular cartilage tissue was studied.

From March 2004 research for this thesis was performed at the department of Rheumatology & Clinical Immunology of the UMCU under the supervision of F.P.J.G. Lafeber, PhD, and G. Roosendaal, MD, PhD, working at the department of Hematology and Van Creveldkliniek. In May 2007 she received the Horoszowski-award of the World Federation of Hemophilia for best original paper (chapter 5).
From March 2008 she continued her work on blood-induced joint damage at the same departments.