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The effect of hyaluronan injections into human knees on the number of bone and cartilage wear particles captured by bio-ferrography

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ABSTRACT

Osteoarthritis is characterized by degradation of cartilage and subchondral bone, releasing wear particles into the synovial fluid. Intra-articular injections of exogenous hyaluronan are often given to patients suffering from osteoarthritis in order to compensate for the reduction in the level of endogenous hyaluronan and to restore the rheological properties of the synovial fluid. The exact effect of these injections is still ambiguous. In this work bio-ferrography was used to capture magnetically labeled cartilage and bone debris from the synovial fluid in human knees before each of four injections (Euflexxa™). The wear particles were counted and characterized microscopically and chemically. WOMAC, VAS, SF-36 and KS questionnaires indicated significant pain relief during the treatment, but suffered from inconsistency. Bio-ferrography showed a reduction in the concentration of both cartilage and bone particles, with a minimum after the third hyaluronan injection. The advantages of bio-ferrography as a primary assessment tool are discussed. The results indicate that while hyaluronan treatment may temporarily slow the wear rate to an extent beyond a placebo effect, it does not prevent joint degradation altogether.

1. Introduction

Osteoarthritis (OA) is a heterogeneous group of conditions that lead to degradation of cartilage and, in severe cases, to related changes in the subchondral bone [1]. OA is the main cause of disability of elder persons and the major reason for total joint replacement. It is most common in weight-bearing joints, the knee joint being most affected. Its prevalence is expected to continue increasing in the coming years [1,2]. Wear particles, such as bone and cartilage fragments, are released into the synovial fluid (SF) of the affected joint [3,4]. This debris may trigger the release of enzymes, such as collagenase [3], which results in inflammation of the synovial membrane.

SF is an ultra-filtrate of plasma, which has non-Newtonian properties and is thixotropic [5]. It enables efficient movement of the joint, acting as a lubricant, supplying nutrients and removing catabolic products. The synovial membrane secretes and removes SF from the joint space, adjusting both the volume of SF and its macromolecular composition [6]. Endogenous hyaluronan (HA) is the main constituent of SF [7]. This polysaccharide acts as a lubricant during low impact joint movement and as a shock absorber during high impact movement [8]. It is secreted in high concentrations in the extracellular matrix of connective tissues, although its highest concentration is in the SF (2.5–4.0 mg ml^{-1} in normal joints) [6,9]. The molecular structure of HA is shown in Fig. 1a. The polysaccharide chain is made of repeating disaccharide units of N-acetylglucosamine and glucuronic acid. The average molecular weight of healthy human HA is 5000 kDa [9,10]. The viscosity of the SF is affected by the length and conformation of the polymer chains, as well as by interaction between adjacent chains [6].

In arthritic joints the concentration and average molecular weight of HA are lower, at 0.8–2.0 mg ml^{-1} and 3000 kDa, respectively. Consequently, the viscosity of the SF is abnormally low, its functionality is lost and rubbing of the cartilage occurs. Intra-articular knee injection of exogenous HA (a treatment also known as viscosupplementation [9]) has been suggested in order to compensate for the reduction in the concentration of endogenous HA. It was assumed that the exogenous HA could restore the rheological, analgesic and anti-inflammatory effects of SF, which are lost in OA [2,10,11]. The US Food and Drug Administration (FDA) approved exogenous HA injections in 1997, but classified it as a device, not as a medicine. Nevertheless, numerous studies have reported significant pain relief and a better knee function that can persist for up to 6 months due to treatment with three to five weekly intra-articular HA injections [2,10–19]. A few studies have even reported that HA treatment prevents cartilage degradation and release of proteoglycans [7,20–24]. As the half-life of HA is a few days,

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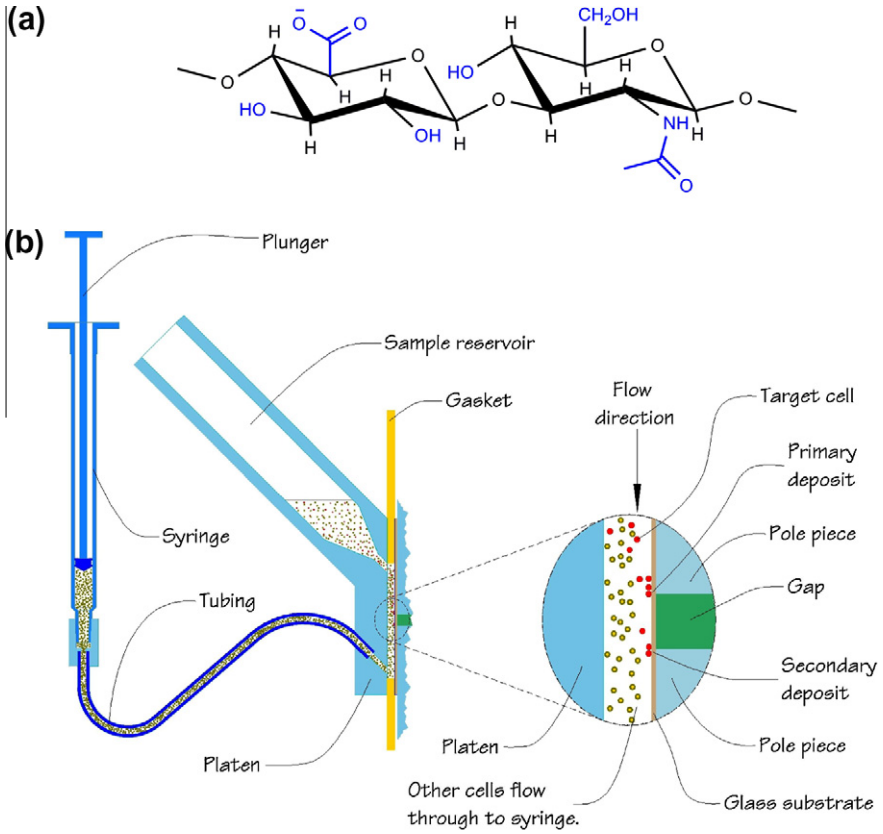

Fig. 1. (a) The repeat disaccharide of hyaluronan: –D-glucuronic acid-β-1,3-N-acetylglucosamine-β-1,4–. (b) The principles of bio-ferrography: the deposition scheme of captured particles on a glass slide [48].

several injections are given to provide sufficient efficacy, although some producers seek to minimize the treatment to one injection only.

There are various HA products on the market, which differ with respect to origin, method of production, treatment schedule, molecular weight, half-life within the synovium, rheological properties, pharmacodynamics and cost. Their efficacy has been evaluated using biomarkers of bone and cartilage present in the SF, serum and urine [4,13,25,26], questionnaires [8,13,15–19,27–29], in combination with measuring joint space narrowing [30], and biopsy of cartilage samples [31]. This study used Euflexxa™ (1% sodium hyaluronate, Ferring Pharmaceuticals Inc., Parsippany, NJ) [8,10]. This is the first HA to be produced from a non-avian source (it is bioengineered by biological fermentation of bacteria) and it has one of the highest molecular weights (3000 kDa) of all available HA products [10].

Surprisingly, none of the studies cited above has studied the effect of serial HA injections on the number of cartilage and bone wear particles over time. Hence, the exact effect of HA therapy is still unknown. Most of the existing studies cannot be claimed to be placebo controlled – as soon as a needle is inserted into the joint (either to remove excess SF or to inject saline solution or for active treatment) the patient experiences some relief.

The objective of the current study was to evaluate the effect of serial Euflexxa™ injections on the concentration of cartilage and bone particles in the SF of osteoarthritic human knee joints. Isolation of these wear particles was achieved by means of bio-ferrography. To the best of our knowledge this is the first ever study that has demonstrated the use of this technique in evaluating the efficacy of a drug.

Ferrography is a non-destructive method of particle separation from a suspension onto a glass slide based upon interaction between an external magnetic field and the magnetic moments of the particles [32–34]. This method was developed by Westcott and co-workers in the early 1970s in order to investigate the occurrence of ferrous wear particles in lubricated dynamic components. By determining the number, shape, size, texture and composition of particles on the ferrogram (i.e. a glass slide with wear particles), the origin, mechanism and level of wear can be determined. Bio-ferrography [35–41] is the latest modification of conventional analytical ferrography that was specifically developed to allow magnetic isolation of target cells or tissues. Its strengths of interest for the current study include: (1) the ability to quantify biological matter and, at the same time, analyze its microscopic and chemical features; (2) extremely high selectivity and sensitivity; (3) applicability to any liquid sample; (4) the ability to analyze samples as small as 1 μl and target particles as small as several nanometers; (5) the possibility of simultaneously processing up to five samples within bracketed areas (channels) on a single slide, without cross-contamination.

2. Experimental

2.1. Selection of patients, sample collection and storage

The study protocol and informed consent form were approved by the Helsinki committee of Assaf Harofeh Medical Center. The criterion for patient inclusion was a diagnosis of OA of the knee based on the Kellgren–Lawrence (K-L) grading of radiographs, from 0 (normal) to 4 (most severe) [42]. The exclusion criteria were: (1) patients that suffered from acute septic arthritis; (2) patients that were treated with Coumadin and/or other anti-coagulant drugs; (3) patients that showed mental or physical conditions which

precluded compliance with the study and/or device; (4) patients who had had surgical intervention. 20 patients, 46–80 years old (mean age 66), who fulfilled the inclusion criteria were initially enrolled in this study. However, 8 of these patients were dropped during the study; thus, the results reported in this paper are for 12 patients only (6 males, 6 females, 10 patients with K-L grading 3/4, 2 patients with K-L grading 2).

Samples of SF were aspirated using a sterile needle from symptomatic knee joints. Sample collection was done before the first HA injection, before the third HA injection (i.e. after approximately 2 weeks), before the fourth HA injection (i.e. approximately 3.5 months after the first injection) and before the fifth HA injection (i.e. approximately 6.5 months after the first injection). Each HA injection consisted of a 2 ml dose of 1% sodium hyaluronate. Although four samples of SF were extracted from each patient, some were excluded due to lack of SF effusion, a total volume less than 1 ml or excessive blood in the SF sample. A few of the samples included in this study were not collected exactly on the target day. At least 1 ml of SF was extracted each time and stored in heparinized tubes. Most of the SF samples were characterized by a bright yellow colour, low viscosity and reasonable amounts of debris (turbidity). These are typical of SF in arthritic joints [43]. Several samples also contained macroscopic pieces of tissue, probably synovium.

2.2. Clinical assessment of the HA therapy by questionnaire

Several questionnaires were used at the hospital. The patients graded their subjective weight-bearing pain condition of the knee using a 10 mm Visual Analogue Scale (VAS) questionnaire, in which lower scores indicate less pain [44], before the treatment and after one, two, three and four HA injections. The question the patients had to answer was: "what is your pain level (from 0 to 10) in daily life?" (i.e. ADL, activity in daily life). When the time interval between injections was 1 week this question referred to the pain level during that week. When the time interval between injections was 3 months this question referred to the last 2 weeks. Both the orthopedists and the patients graded the knee condition and the patient's function using the Knee Society Score questionnaire, which consists of both a Knee Score (KS) and a Function Score (FS) (on a scale of 0–100, where a high score indicates improved health) [45], before the treatment and after two, three and four HA injections. The health status and evaluation of the medical effects from the patients' standpoint were assessed initially and at weeks 2, 14 and 28, using the SF-36® score (on a scale of 0–100, where a high score indicates improved health) [46]. The orthopedic surgeon recorded subjective and objective signs of inflammatory and joint reactions. The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) is a widely used self-administered, health status primary measure that asks patients with OA questions concerning the study knee or hip [27,29,47]. It produces an aggregate total score as well as scores for three subscales: pain, stiffness and physical functioning. A lower score for each subscale corresponds to a better condition. In this study a Hebrew version of the WOMAC questionnaire was filled in before the treatment and after two, three and four HA injections. The aggregate total score was calculated using a ProChon Biotech Ltd. (Rehovot, Israel) scale.

2.3. Centrifugation and magnetic labeling of cartilage and bone wear particles

The SFs were centrifuged at 4000 rpm and 4 °C for 15 min in a Magafuge 1.0R to separate the wear particles from the SF (the latter contains collagen fragments which might compete with the collagen in the wear particles for the primary antibody) and were subsequently diluted in 1 ml of distilled water. This procedure was repeated at least three times, reducing the centrifugation time to 10 min. Each of the centrifuged synovial fluid samples was divided into two Eppendorf tubes:

1. *Labeling of osseous particles:* 0.2 ml of sample + 0.8 ml of distilled water + 2 μl of mouse anti-human collagen I antibody (1-8H5) + 10 μl of goat anti-mouse magnetic beads.
2. *Labeling of cartilaginous particles:* 0.2 ml of sample + 0.8 ml of distilled water + 2 μl of mouse anti-human collagen II antibody (RB/RAT) + 10 μl of goat anti-mouse magnetic beads.

The mouse monoclonal primary anti-collagen antibodies were from MP Bio-Medicals. The goat anti-mouse IgG MACS MicroBeads solution was from Miltenyi Biotec. The beads in this solution are 50 nm in diameter. This solution together with a secondary antibody was added to the other two components following a 1 h incubation and the three-component cocktail was further incubated for 30 min prior to being run on the Bio-Ferrograph.

2.4. Isolation of cartilage and bone wear particles by bio-ferrography (BF)

The Bio-Ferrograph 2100 system from Guilfoyle Inc., which was used in this work, is a benchtop cytometry-based instrument. It utilizes a magnetic field that has maximum field strength across an interpolar gap, where the collection of magnetically susceptible particles takes place. The maximum magnetic field strength across the gap is 1.8 T. However, the gradient in the field is maximal at the edges of the gap, where deposition is concentrated (Fig. 1b [48]), thus forming a rectangular deposition band. In order to minimize contamination of samples from the environment, the Bio-Ferrograph was placed in a biological safety cabinet (ADS Laminar's Optimale 12). Several controls were evaluated and reported in a previous publication [49].

The capture flow rate (i.e. towards disposable syringes) was fixed at 28 μl min^{-1}, which had been found to be sufficiently slow to ensure effective adsorption of the antibodies and full recovery of the particles. Capture of cartilage and bone particles was carried out in two different channels on the ferrogram, each running one of the two cocktails described above. In all cases a washing step with distilled water was included to remove sample fluid residues from the cassette. The fragile glass substrate (ferrogram) was separated from the remaining parts of the cassette by means of a dedicated vacuum hold-down unit. Finally, it was allowed to dry for several minutes inside the safety cabinet.

2.5. Microscopic and chemical characterization of wear particles

All particles larger than 5 μm within the deposition band in each of the two channels (captured with anti-collagen I and anti-collagen II, respectively) were counted manually and characterized morphologically and chemically by environmental scanning electron microscopy (ESEM) (Quanta 200 FEG from FEI) equipped with an energy dispersive X-ray spectroscopy (EDS) detector. Most particles smaller than 5 μm were not characterized due to limitations of the sampling volume ("onion") in EDS analysis. Most ferrograms were analyzed in high vacuum mode because preliminary tests showed that this mode resulted in less background noise from biological matter and enabled quicker identification of particles, at higher numbers, compared with the low vacuum mode. Working in high vacuum mode requires the ferrogram to first be coated with a conductive coating. Copper was selected because, in contrast to gold, it does not mask the peaks for sulfur and phosphorus [49]. A 15–20 nm thick coating was obtained by evaporation under a pressure of 3×10^{-6} Torr at a deposition rate of 2.5 nm s^{-1}. Other

operating conditions included a high voltage of 20 kV, a spot size of 3 and a working distance of 10 mm, a secondary electron detector for imaging and a 50 s acquisition time for EDS.

The concentration of the wear particles in the SF (N) was calculated as:

$$N = X/V \ [1/\mathrm{ml}] \quad (1)$$

where X is the number of particles counted manually in a specific channel on the ferrogram after normalization to sample dilutions and V is the volume of the SF as obtained from the hospital.

The origin of each particle was determined based on its chemical composition and the primary antibody which captured it, as follows [49]:

1. *Particles rich in Ca and in collagen I:* subchondral bone fragments.
2. *Particles rich in Ca and in collagen II:* the interface between subchondral bone and calcified cartilage.
3. *Particles rich in S and in collagen II:* articular cartilage fragments.
4. *Particles rich in S and in collagen I:* repaired cartilage, calcified cartilage, degenerate cartilage, synovium or meniscus fragments. These particles were referred to as "cartilaginous particles".
5. *Particles containing neither Ca nor S (typically consisting of only Mg, P or C):* uncertain origin.

Calcium and phosphorous are both present in apatite, the primary inorganic constituent of all mammalian skeletal tissues (Ca/P = 1.67 in stoichiometric hydroxyapatite and around 1.60 in biological apatite [50]). Hence, they may be used to identify bone particles. Cartilage, on the other hand, contains appreciable amounts of sulfur, due to the proteoglycan constituent aggrecan. The zone of calcified cartilage can be recognized by high concentrations of calcium, phosphorous and sulfur. It should be noted that while most of the collagen in uneroded hyaline cartilage is of type II, osteoarthritic cartilage may also contain some collagen I, which is derived from chondrocytes [6]. Regarding the particles in category 2, Oegema et al. [51] suggested that the biomineralization process is accompanied by a significant drop in the content of proteoglycans, which is expected to result in a decrease in the concentration of S compared with Ca. Mendel et al. [49] found the minimal Ca/S mass ratio of an unambiguously identified bone particle to be 4.08 (where the mass of P and Mg were also taken into account). Cartilage particles were identified by a mass ratio of Ca/S < 1.0.

2.6. Biostatistics

The non-parametric Wilcoxon's test for paired samples was used to evaluate a single stage of the treatment in comparison with other stages (such as the initial condition). The Friedman test for several related samples (evaluating the whole treatment process) was also employed. Data were considered statistically significant at $P \leqslant 0.05$. In both cases SPSS ver. 15 software was used.

3. Results and discussion

3.1. Clinical assessment by questionnaire

To avoid bias the questionnaires were filled in and analyzed at the hospital, independently of the bio-ferrography work, which was carried out at the university. Fig. 2 shows the biostatistical analysis of the data in different questionnaires. Fig. 2a shows the analysis of the VAS data. With the exception of one, all patients reported pain relief (reflected by a lower grade of VAS) at some point during the HA treatment, compared with the initial condition. Fig. 2a shows that there was continuous pain relief during the HA treatment. The mean VAS grading values were 7.1, 6.6, 6.2, 4.8 and 4.0 before the treatment and after one, two, three and four HA injections, respectively. The Friedman test revealed that the results after four HA injections were significantly different ($P = 0.004$, $n = 11$).

Fig. 2b and c show the analysis of the KS and FS data, respectively. With the exception of one, all patients reported an improvement in KS at some point during the HA treatment, compared with the initial condition (reflected by a higher grade of KS). From Fig. 2b it is evident that the median KS increased during HA treatment. The mean KS values were 42.8, 47.4, 54.1 and 57.1 before the treatment and after two, three and four HA injections, respectively. The Wilcoxon test showed that the KS values after two HA injections were significantly higher than in the initial condition ($P = 0.02$, $n = 12$), but were not significantly different than after three HA injections ($P = 0.101$, $n = 11$). Compared to the initial condition, the KS results after either three or four HA injections were significantly different ($P = 0.014$, $n = 11$; $P = 0.0045$, $n = 11$). The results after three HA injections were not significantly different from after four HA injections ($P = 0.142$, $n = 11$). According to the Friedman test the KS values during the HA treatment were significantly higher ($P = 0.009$, $n = 11$). It may thus be concluded that, based on the KS questionnaire, the HA injections resulted in a significant improvement in pain relief, range of motion and stability of the knee. Fig. 2c shows that the median FS value was minimal after two HA injections. The mean FS values were 57.5, 62.5, 62.5 and 66.4 before the treatment and after two, three and four HA injections, respectively. The Wilcoxon test showed that, compared with the initial condition, the differences in FS values after two and three HA injections were not significant ($P = 0.124$, $n = 12$; $P = 0.176$, $n = 11$), but became significant after four HA injections ($P = 0.024$, $n = 11$). The FS values after three HA injections were not significantly different from after two injections ($P = 0.333$, $n = 11$). Comparison between the FS values after four and three injections also revealed an insignificant difference ($P = 0.098$, $n = 11$). According to the Friedman test the FS values during the HA treatment did not become significantly higher ($P = 0.108$, $n = 11$). Thus, although the increase in mean FS value during the HA treatment may indicate an improvement in the knee condition, the other statistical analyses do not enable a definite conclusion to be drawn. In addition, no correlation was observed between the KS and FS data in this study.

Fig. 2d shows the analysis of the overall SF-36 score. An analysis of specific components of this score, such as average pain, average general health, average physical role limit, average energy and average emotional well-being, was also carried out, but is not presented in this paper. According to Fig. 2d the minimal median of the SF-36 data was after two HA injections, while after three and four HA injections it was higher than in the initial condition. The mean values of the overall SF-36 score were 56.6, 56.1, 68.2 and 70.3 before the treatment and after two, three and four HA injections, respectively. The Friedman test revealed a limited difference after four HA injections ($P = 0.052$, $n = 11$).

Fig. 2e shows the analysis of the WOMAC aggregate total score. Most patients (8 out of 12) reported an improvement in health at some point during the HA treatment compared with the initial condition (reflected in a lower WOMAC aggregate total score). From Fig. 2e it is evident that the median WOMAC value decreased during the HA treatment, with a minimum after the third injection. The mean aggregate total score decreased continuously from 4.6 before treatment to 4.4, 2.75 and 2.6 after two, three and four HA injections, respectively. The Wilcoxon test showed that the WOMAC values after two HA injections were not significantly higher than in the initial condition ($P = 0.346$, $n = 12$), but were significantly different from after three HA injections ($P = 0.033$,

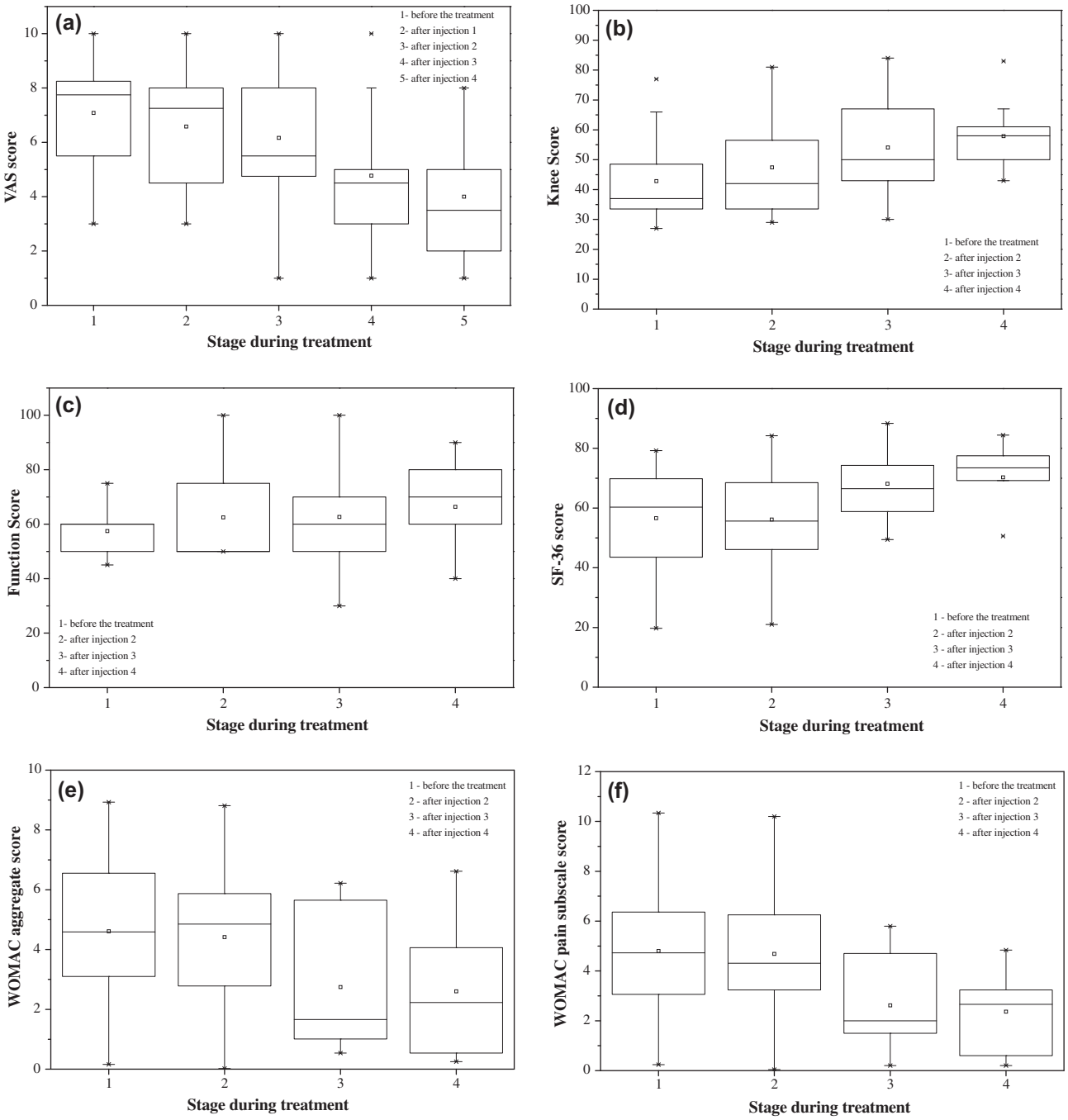

Fig. 2. Biostatistical analysis of the questionnaires filled in the hospital. The horizontal line and the square within the box indicate the median and mean, respectively, while the borders of each box indicate the lower and upper quartiles. (a) Visual Analogue Scale (VAS); (b) Knee Score (KS); (c) Function Score (FS); (d) overall SF-36 score; (e) WOMAC aggregate total score; (f) WOMAC pain subscale score.

$n = 11$). Compared with the initial condition, the WOMAC results after three HA injections were not significantly different ($P = 0.075$, $n = 11$), although there seems to have been a trend towards improvement. Compared with the initial condition, the WOMAC results after four HA injections were not significantly different ($P = 0.066$, $n = 9$), but there still seemed to be a trend towards improvement. The results after three HA injections were not significantly different from after four HA injections ($P = 0.767$, $n = 9$). According to the Friedman test the WOMAC scores during the HA treatment were not significantly lower ($P = 0.392$, $n = 9$).

Fig. 2f shows the analysis of the WOMAC pain subscale score. A significant pain relief after three and four HA injections is evident, in comparison with the initial condition ($P = 0.005$ and 0.004, respectively). Furthermore, there was significant pain relief after three HA injections in comparison with after two HA injections ($P = 0.041$). The mean pain score before treatment was 4.8, decreasing to 2.62 after three HA injections and to 2.37 after four HA injections. The Friedman test showed a significant difference after four HA injections ($P = 0.014$).

The clinical assessment by questionnaires obtained in this study is in accordance with many other studies that have reported significant pain relief and improved knee function, which can persist for up to 6 months, due to HA injections [15,16,18,27,28]. There are several limitations regarding the use of questionnaires that should be taken into account. First, in several comparisons the data was not statistically significant (see above). Second, the correlation be-

tween the trends observed in different questionnaires was not perfect. For example, the trends in the mean scores for WOMAC aggregate total, WOMAC pain subscale, VAS, KS and FS all showed continuous improvements in knee condition, while SF-36 showed the worst condition after the second HA injection. The trends in the median scores for VAS, KS and FS all showed continuous improvement in knee condition, while SF-36 showed the worst condition after the second HA injection and the WOMAC aggregate total and pain subscale scores showed the best condition after the third HA injection. Third, although WOMAC has been widely used as a primary outcome measure for OA joints, it should be borne in mind that in order to enable comparison between WOMAC assessments in different countries it has been found necessary to make adaptations for use in different cultures [52]. Moreover, cases have been reported where the WOMAC aggregate total score did not correlate with radiographic changes due to OA [52]. Indeed, the orthopedists participating in the present study have found that it is not always the patients with severe pain who actually have the most severe OA. Pain is a subjective criterion which depends on patient activity and does not always correlate with objective clinical findings. In addition, Karlsson et al. [27] found that the WOMAC scores could not differentiate between HA treatment and a placebo during the first 26 weeks of their study.

One of the drawbacks of the current study was that most patients reported that they were using analgesics during the HA treatment. In addition, the placebo effect was not controlled. Hence, the pain relief reported in the questionnaires may not be related to the HA treatment per se. An extension of this study may include the addition of control groups to subtract the placebo effect and the selection of only those patients that avoid analgesics while receiving the HA treatment. Finally, it should be borne in mind that the patients in this study referred to different time intervals when scoring their pain level; initially, the time intervals between HA injections were shorter. Thus, a further extension of this study may be the injection of HA at constant time intervals, thus eliminating any "memory" effect on the patient grading.

3.2. The shape and chemical composition of wear particles

Fig. 3 shows several examples of the shapes of cartilage and osseous wear particles captured by bio-ferrography, as imaged by ESEM. Similar shapes have recently been documented in OA joints not treated with HA [49]. This may indicate that the same mechanism of wear operates both in the absence and in the presence of HA treatment. While a lamellar shape was most common for cartilage particles throughout the treatment, osseous particles most commonly had a chunky shape. The shape of cartilage particles may be related to their origin within the tissue [49,53]. Numerical analysis of the shape of wear particles (not presented herein) did not reveal any effect of the HA treatment, possibly because of the small sample size. In 5 patients the mean length of cartilage particles was 31–78 μm, while their mean area was 410–2752 μm^2. In these patients the mean length of osseous particles was 14–39 μm, while their mean area was 18–1764 μm^2.

Fig. 4 demonstrates typical EDS spectra for cartilage (Fig. 4a) and osseous (Fig. 4b) particles. High S and Ca contents are evident for the cartilage and osseous particles, respectively. Almost all particles analyzed in this study exhibited a C peak, which may be ascribed to organic matter such as collagen. Many particles exhibited a Mg peak (see Supplementary results), while several particles exhibited high levels of oxygen. Mendel et al. [49] reported particles rich in Mg, C and O. Magnesium particles, for example, may play a pathological role in arthritis [54–56]. Proteoglycans, on the other hand, contain oxygen and carbon.

3.3. Change in the concentration of wear particles during the HA treatment

A macroscopic view of a ferrogram of a patient for whom the effect of HA injections on the concentration of wear particles was significant is shown in Fig. 5. The direction of fluid sample flow is shown by an arrow. A cocktail containing anti-collagen I in channels 1 and 3 was run, while a cocktail containing anti-collagen II was run in channels 2 and 4. These channels are marked in the figure "osseous" and "cartilaginous", respectively. The deposition bands of SF collected before the HA treatment, which contain the wear particles, are clearly evident in channels 1 and 2. As expected, the deposition band of cartilage particles is denser than that of osseous particles. After two HA injections the deposition bands in channels 3 and 4 are hardly evident, thus reflecting a significant reduction in the concentrations of both cartilage and bone particles.

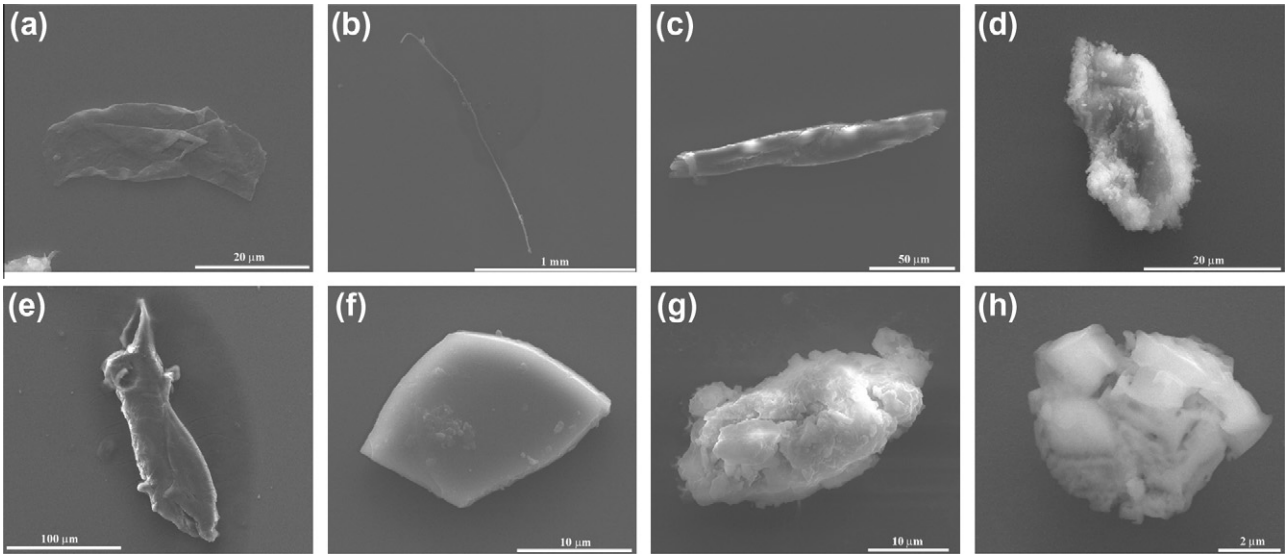

Fig. 3. Selected wear particles captured by bio-ferrography and their various shapes. (a) Lamellar, (b) fibrous, (c) rod-like, (d) chunky and (e) irregular shaped cartilage particles. (f) Lamellar, (g) chunky-spongy surface and (h) chunky-angular surface osseous particles.

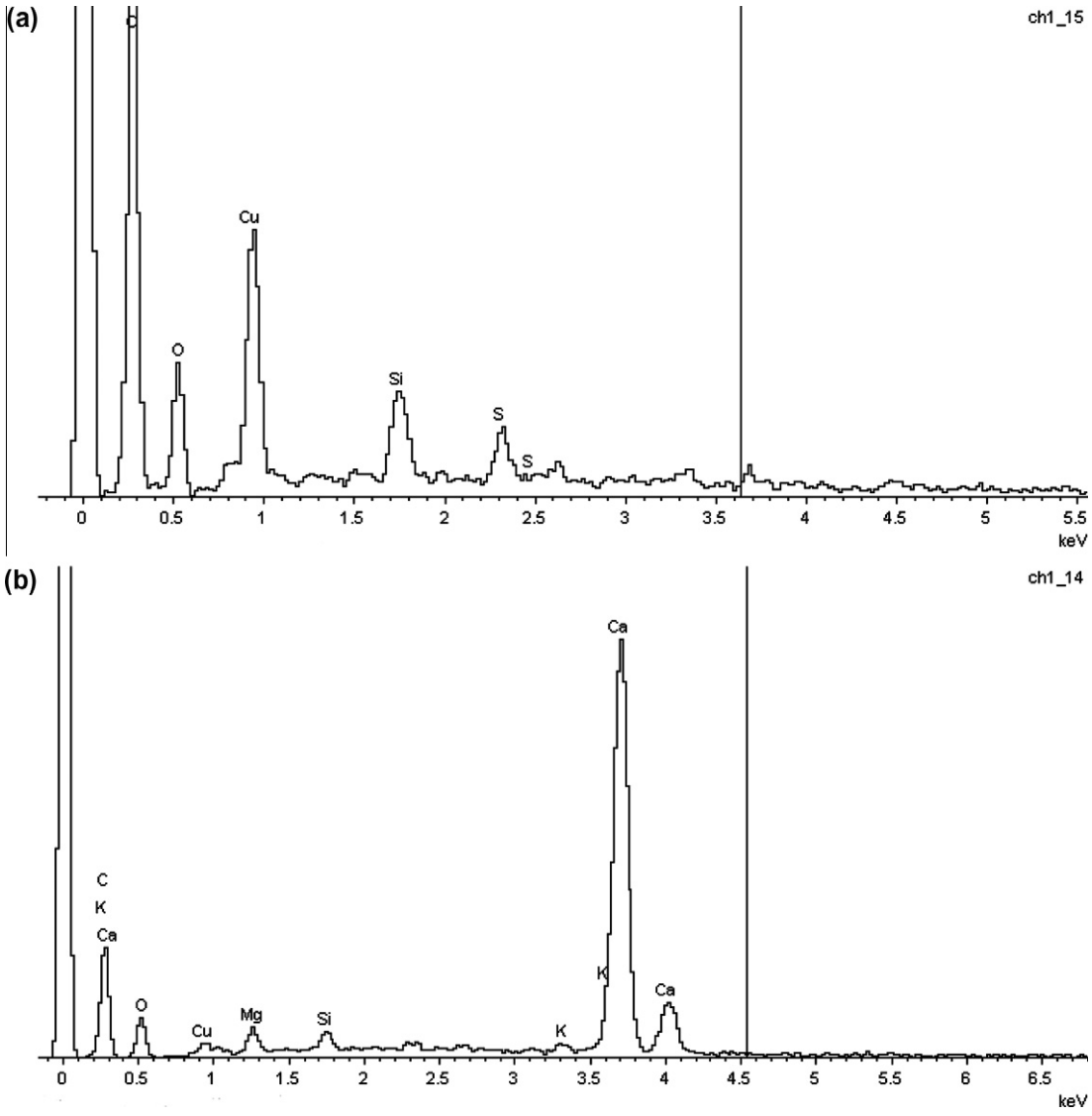

Fig. 4. Typical EDS spectra of (a) cartilage and (b) osseous wear particles.

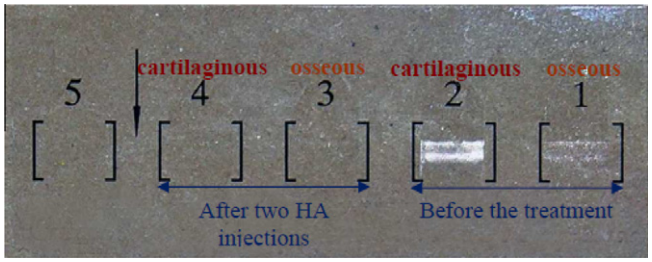

Fig. 5. A macroscopic view of a ferrogram of a patient for whom the effect of HA injections on the concentration of wear particles was significant. The direction of flow is marked by an arrow. A cocktail containing anti-collagen I was run in channels 1 and 3, while a cocktail containing anti-collagen II was run in channels 2 and 4. Before HA treatment rectangular deposition bands containing the wear particles are clearly evident in channels 1 and 2.

In all cases a reduction in the concentration of cartilage particles was measured after two HA injections compared with the initial condition (before HA treatment); the percentage reduction varied between 6% and 86% for different patients. After three HA injections a reduction in the concentration of cartilage particles was also observed in most cases, within the range 38–98%. However, in two cases an increase (3% and 51%) in the concentration of cartilage particles compared with the initial condition was found. In four patients a minimum in the concentration of cartilage particles was monitored after two HA injections, while in another four patients the minimum occurred after three HA injections. In most cases the concentration of cartilage particles increased after four HA injections compared with the concentrations after two and three injections; in one case it was even higher (441%) than that before treatment. It should be noted that the number of valid samples for analysis decreased from 12 to 11, 9 and 4 for the initial condition, after two HA injections, after three HA injections and after four HA injections, respectively. As cartilage degradation is the main process related to OA, these results indicate that the HA injections actually affected the wear rate to an extent that was beyond a placebo effect.

With respect to osseous particles, in most cases a reduction in their concentration was measured after two HA injections compared with the initial condition; the percentage reduction was between 30% and 91% for different cases. However, in two cases an increase (36% and 85%) in the concentration of osseous particles compared with the initial condition was measured after two HA

injections. After three HA injections a reduction (7–98%) in the concentration of osseous particles compared with the initial condition was measured. However, in three cases the concentration increased (by 21%, 22% and 24%). After four HA injections in two cases a reduction (by 65% and 82%) in the concentration of osseous particles compared with the initial condition was also measured, but in another two cases an increase (by 184% and 319%) was measured. In four cases the minimum in the concentration of osseous particles was after two HA injections, while in three cases it was after three HA injections. A correlation seemed to exist between the trends of the curves of cartilage particle concentration and of osseous particle concentration over time.

The statistics for the variations in the concentrations of cartilage and osseous particles during treatment are shown graphically in Fig. 6a and b, respectively. Two non-parametric tests were applied, the Wilcoxon signed ranks test and the Friedman test. The first test was carried out in order to evaluate the efficacy of the second and third HA injections compared with the initial condition (before HA treatment) and to estimate the change between these two injections. The second test was carried out in order to evaluate the efficacy of the treatment in the long term. Fig. 6a shows that the median cartilage particle concentration decreased after two and three HA injections and increased after the fourth HA injection. In addition, there was a slight reduction in the cartilage particle concentration after the third HA injection in comparison with the second HA injection. The mean cartilage particle concentration in the initial condition was 193 particles ml^{-1}, which decreased to 69 and 49 particles ml^{-1} after two and three HA injections, respectively. After the fourth HA injection, however, it increased to 157 particles ml^{-1}. Despite this increase, the mean was still not as high as that before treatment. The Wilcoxon test showed that, compared with the initial condition, the results after two and three HA injections were significantly different ($P = 0.0015$ and 0.019, respectively), while after four HA injections they were not significantly different ($P = 0.2325$, $n = 4$). Comparing the condition after two and three HA injections, the results were not significantly different ($P = 0.287$). According to the Friedman test the results after three HA injections were significantly different ($P = 0.03$).

Fig. 6b shows that the median osseous particle concentration decreased after two and three HA injections and increased after the fourth HA injection. In addition, there was a slight reduction in the osseous particle concentration after the third HA injection in comparison with the second HA injection. The mean osseous particle concentration in the initial condition was 152 particles ml^{-1}, which decreased to 41 and 27 particles ml^{-1} after two and three HA injections, respectively. However, it increased after the fourth HA injection to 83 particles ml^{-1}. Despite this increase, the mean was still not as high as that before treatment. The Wilcoxon test showed that, compared with the initial condition, the results after two HA injections were significantly different ($P = 0.013$), while after three and four HA injections they were not significantly different ($P = 0.055$ and 0.2325, respectively). Comparing the condition after two and three HA injections, the results were not significantly different ($P = 0.2875$). According to the Friedman test the results after three HA injections were not significantly different ($P = 0.197$).

From Fig. 6 and the data provided above it is evident that the reduction in cartilage wear particle concentration after two and three HA injections was more significant than that of osseous particles. It is also evident that while HA treatment may temporarily slow the wear rate, it does not prevent joint degradation altogether. It is worth noting that the highest reduction in overall wear particle concentration was in the two patients with K-L grading 2 (see Section 2.1). It may thus be thought that HA treatment would be most effective in patients with low grade OA. However, this study must be extended to a much larger patient population in order to prove this unambiguously and with statistical significance.

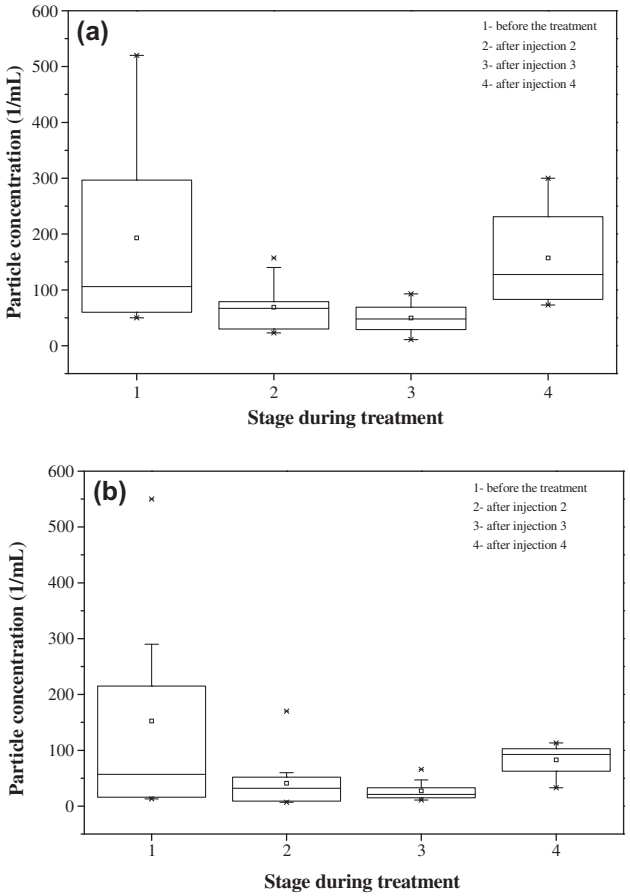

Fig. 6. The concentration (particles ml^{-1}) of (a) cartilage and (b) osseous wear particles as measured by bio-ferrography before and during HA treatment.

The success rate of any drug may be significantly increased if, for example, bio-ferrography is to be found sensitive enough to identify early damage to the joint while the latter is still classified "normal" by the orthopedist. Samples of SF were not extracted after the first HA injection because, in most cases, there was no SF renewal at that time. It has often been observed by orthopedists that the volume of SF is increased in response to irritation caused by wear particles. Hence, it is possible that poor SF renewal resulted from a significant reduction in the concentration of wear particles after the first HA injection. Alternatively, poor SF renewal may be related to a placebo effect or to the need for an extended timeframe to form it. Thus, this study does not enable any conclusions to be drawn as to whether the reduction in wear particle concentration after two HA injections was a result of the first injection, second injection or a combination of the two. The change in concentration of both cartilage and osseous particles during treatment may not be related exclusively to changes in the efficacy of HA, but also to the different time intervals between injections (see Section 2.1). Moreover, some of the SFs were not aspirated exactly on the predetermined date, which might have some effect on the results. In general, an increase in the concentration of wear particles during treatment may also be due to: (1) poor effectiveness of the HA injection after three months and/or for patients with severe OA (K-L grading 3/4); (2) more use of the joint after HA treatment than before HA treatment due to the analgesic effect of the treatment; (3) the HA treatment having only a palliative effect on the joint. Hence, a possible extension of the present study could be to determine the optimal time interval between HA injections with respect to the concentration of wear particles in the SF in a pre-

cisely controlled manner. It could be that the number of HA injections can be reduced, at least to three, thus making the treatment less expensive and eliminating some discomfort to the patient. Another important extension of this work would be to add at least one more sampling point, some time after the last HA injection, and to compare the results of bio-ferrography with those of the questionnaires at that time point. This may reveal, for example, whether there is a time lag between the evolution of wear particles and patient pain.

Another issue is that of the "baseline" concentration of wear particles in the SF of healthy, or near healthy, knees. On the one hand, typical concentration ranges for different elements in the SF have been reported for both healthy and OA joints. These concentrations may be somehow related to the volume of wear particles in the SF. On the other hand, it may be reasonably argued that there is no such thing as "normal" concentrations of wear particles in the SF, as these concentrations probably depend on a variety of factors, such as physical activity, age and genetics. Yet, based on previous work [49], it may be expected that there would be no osseous particles in most healthy joints. With respect to cartilaginous particles, Evans et al. [57] injected saline into the knee cavity of healthy or near healthy patients (11–14 years old) and captured wear particles by conventional ferrography, using $ErCl_3$ as the magnetizing agent. Different numbers of lamellae of superficial cartilage were found in the three patients. However, not only were the techniques of magnetization and capture different in that study compared with the present study, no concentrations of wear particles were reported. Nowadays, it is unlikely that Helsinki approval to draw SF, inject saline into the knee cavity or obtain synovial tissue from healthy donors in order to establish a baseline for wear particle concentration could be obtained. Hence, the focus should be on within patient change in the number of wear particles over the course of treatment.

The histology of wear particles isolated from diseased joints can be used to further support the differentiation between their anatomic origins. This study could be further improved by using more specific biomarkers, such as matrix metalloproteinases, interleukins, prostaglandins, telopeptides, cartilage oligomeric matrix protein (COMP) and neo-epitopes that would allow more precise identification of the wear particles.

To conclude, among the various questionnaires used in this study to assess the efficacy of HA injections, WOMAC (medians of both the aggregate total score and the pain subscale score) correlated best with the wear particle concentrations determined by bio-ferrography, indicating the best knee condition after the third injection. However, given the limitations of the questionnaires discussed in this paper, we believe that bio-ferrography is the only objective, reliable, sensitive and cost-effective tool to evaluate the effect of treatment, using intra-articular debris as an outcome measure. Obviously, more work is required to validate this technique for use in orthopedics.

4. Conclusions

This research demonstrates the effect of serial hyaluronan (Euflexxa™) injections on the concentration of cartilage and bone wear particles in osteoarthritic knee joints over time. For the first time bio-ferrography was used to evaluate the efficacy of a drug. A reduction in the concentration of both cartilage and osseous particles was seen, with minimal cartilage concentrations after two and three hyaluronan injections. It is suggested that hyaluronan treatment reduces the rate of joint degradation, but does not prevent it altogether. Hyaluronan therapy was found to be extremely efficient for patients with low grades of osteoarthritis. The evaluation of joint degradation by bio-ferrography was found to correlate well with WOMAC assessments. Bio-ferrography may thus become a routine diagnostic, prognostic and monitoring technique, aiding in the determination of osteoarthritis class in an objective manner, due to the high sensitivity and specificity inherent in the use of specific monoclonal antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2010.08.030.

Appendix B. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1 and 5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.08.030.

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