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Randomized Trial of Hymovis® *versus* Synvisc® on Matrix Metalloproteinases in Knee Osteoarthritis

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SUMMARY

Background. This study aims to evaluate the effects of HYADD 4-G, a not chemically cross-linked HA (Hyaluronic Acid) derivative, and hylan G-F 20, a cross-linked HA product, on synovial matrix metalloproteinases and cytokines in patients suffering from knee osteoarthritis.

Methods. 31 patients were randomized to receive HYADD 4-G or hylan G-F 20. Synovial fluid was collected before and one week after the first injection. Activity of MMP-3, MMP-2, MMP-13, IL-6 and other cytokines were measured. Changes in synovial fluid neutrophils and lymphocytes were analyzed. The VAS, the WOMAC questionnaire, and a Physician Global Assessment (PhGA VAS) were recorded.

Results. A trend towards a greater decrease in MMP-3 activity was observed in the Hymovis® group. Active MMP-2 and MMP-13 decreased in both groups. IL-6 levels also decreased significantly in both groups. Median change in neutrophils from baseline was significantly different between the treatment groups. No differences between the treatment groups were observed for the WOMAC, VAS and PhGA VAS scores. No serious adverse events were reported.

Conclusions. These findings demonstrate that intra-articular HA injections in patients with knee osteoarthritis tend to protect cartilage structural integrity reducing the activity of key proteolytic enzymes implicated in cartilage degradation as well as inflammatory mediators.

KEY WORDS

Hyaluronic acid; knee; metalloproteinases; osteoarthritis; sodium hyaluronate; cytokines.

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative musculoskeletal disease (1), with an estimated prevalence ranging from 5% for hip OA, 33% for hand OA and up to 50% for knee OA in subjects over 65 years of age (2). Joint tissue integrity can be lost on a traumatic basis or, more frequently, because of chronic inflammation mediated by soluble factors including cytokines, chemokines and degrading enzymes (3). Among the latter, tissue remodeling matrix metalloproteases (MMPs) are key players in OA pathogenesis. They include collagenases (MMP-1, 8, -13), gelatinases A and B (MMP-2 and -9 respectively) and the stromelysins (MMP-3, -10, -11). When activated, MMPs recognize specific sequences in extracellular matrix proteins and mediate their cleavage (4, 5). Collagenases cleave the collagen triple helix, which can be then further degraded by the gelatinases. Stromelysins have broad specificity against non-collagen matrix components such as fibronectin, elastin, laminin, and aggrecan.

All are greatly over-expressed in OA joints. The imbalance between them and their endogenous inhibitors (α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs)), are among the major causes of the degradation of type II collagen in OA (6).

MMP-3 activity is linked specifically to proteoglycan loss. In addition, MMP-3 contributes to the activation of proM-

MP-13, producing an amplification effect. Moreover, MMP-3 expression in synovial tissue of OA patients was positively correlated with the severity of OA, highlighting its contribution in OA pathogenesis (7). OA is also characterized by synovial inflammation (8). Many inflammatory mediators, including IL-1α, TNF-β, and IL-6, are involved in the development and maintenance of OA by inducing the expression of genes encoding for inflammatory proteins and cartilage-degrading enzymes, such as MMPs.

Common OA symptoms include pain and/or stiffness, which in later stages of the disease can be present also at rest. Symptomatic treatment primarily consists of non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections of corticosteroids. However, the short-term effect along with the associated side-effects of these treatments, in particular in an aged population, has generated interest in alternative treatments, such as viscosupplementation through intra-articular hyaluronic acid (HA) injections to restore the viscoelastic properties of synovial fluid (SF) (9-11).

Previous *in vitro* studies have shown that HA exerts an inhibitory effect on several MMPs (12, 13) and HA treatment has been recently shown to reduce MMP-2 levels in SF of patients affected by knee OA (14).

However, the effects of HA preparations may differ depending on method of production, treatment schedule, molecular weight, half-life within the joint, rheological properties and pharmacodynamics (15).

Hymovis®, a CE-marked and FDA-approved viscosupplementation preparation for intra-articular injection in OA patients is a gel made of linear HA partially chemically modified with an alkyl chain. The main component of Hymovis® is the partial hexadecylamide of HA, named HYADD4p5 (16).

The polymer structure of this derivative suggests that the alkyl side chain could selectively insert into the hydrophobic pocket of the catalytic site of the MMP and that the HA carboxyl group could act as a zinc-coordinating moiety in the MMP catalytic domain. *In vitro* studies confirmed that Hymovis® exerts an inhibitory effect on several active MMPs (17) and a strong inhibition of MMP activity and expression (*i.e.* MMP-8 and MMP-13) by human OA chondrocytes and synovial fibroblasts has been described (11). These effects have not been yet confirmed in human studies. The aim of the present open-label pilot study was to evaluate the effect of a single intra-articular injection of Hymovis® in comparison with Synvisc®, a gel mixture of cross-linked HA and high molecular weight HA, on local generation of cartilage-degrading MMPs in the synovial fluid of patients with knee OA through the measurement of concentrations of specific metalloproteinases (MMP-2, MMP-3, and MMP-13).

METHODS

Study population

The study was conducted in accordance with the Declaration of Helsinki, the principles of Good Clinical Practice, UNI EN ISO 14155:2011 and was approved by the Ethical Committee of the authors' affiliated institution (Comitato Etico delle Aziende Sanitarie della Regione Umbria, CEAS Umbria obtained on 21st of May 2014). Patients were recruited at a single center and gave their written informed consent.

Inclusion criteria were: age > 50 years, diagnosis of idiopathic knee OA according to the American College of Rheumatology diagnostic criteria (18) at least 1 year before inclusion, presence of osteophytes confirmed by radiography, morning stiffness of ≤ 30 minutes duration or crepitus on motion, Kellgren-Lawrence stage II-IV confirmed by an X-ray performed within 6 months prior to inclusion, presence of knee effusion confirmed by ultrasonography (US) at the time of enrollment, absence of intraarticular corticosteroids for at least 3 months and/or HA for at least 6 months prior to enrolment.

Exclusion criteria were: inflammatory rheumatic disorders, secondary OA, contraindications to intra-articular injections, therapy with systemic NSAIDs or corticosteroid in the week prior first treatment injection, cognitive impairment, hemoglobin levels < 12 g/dL in males and < 11 g/dL in females in the 3 months prior to enrolment, oral anticoagulant therapy, pregnancy, infectious diseases. Rescue therapy was allowed according to the physician's opinion but not within 24 hours prior to any visit.

The sample size for this study was estimated as a minimum of 26 patients to be enrolled, based on the MMP-2 activity levels reported in a previous publication (17), considering a 90% power, a significance level of 0.05, and a 30% drop-out rate.

The study meets the ethical standards of the journal. In particular, all experimental procedures were carried out according to the journal guidelines (19).

Study design

Primary endpoint

The primary endpoint of the study was the effect of a single administration of Hymovis® on the activity of metalloproteinase MMP-3 in the SF of the knee compared to Synvisc®.

Secondary endpoints

Secondary endpoints being measured to determine the effect of one intra-articular injection of Hymovis® were the levels of expression and activity of the metalloproteinase

MMP-3, MMP-2 and MMP-13, as well as cellular count and the expression of pro-inflammatory cytokines in the SF.
To determine the effect of Hymovis® treatment, endpoints being measured were the reduction of pain perceived by the patient, the joint mobility and patient's physical functioning, and treatment safety.

Treatment

Eligible patients were randomized to receive open-label treatment with either Hymovis® (Fidia Farmaceutici SpA, Abano Terme, Italy) or Synvisc® (Sanofi, Bridgewater, NJ, USA). Patients randomized to the Hymovis® treatment arm received two intra-articular (i.a.) injections administered one week apart (days 0 and 7) while patients randomized to the Synvisc® treatment arm received three intra-articular injections administered one week apart (days 0, 7 and 14) (**figure 1**).

Ultrasonography-guided intra-articular injection was performed according to standard techniques. Prior to injection at visit 1 (baseline, day 0) and at visit 2 (day 7) a small sample of SF (3-5 ml) was collected.

Laboratory assessment

SF was collected in sterile Vacutainer tubes containing heparin for MMPs measurement or 0.18% K3EDTA for cell count.
To assess MMP levels, samples were pre-treated using hyaluronidase 250 U/mL at 37 °C for 10 min (5) to reduce sample viscosity and improve homogeneity, centrifuged, and the supernatant was used for MMP testing. If necessary, samples were diluted to fit the standard curve. Three replicates were analyzed for each time point. Active MMP-3 levels were measured using a Sensolyte® 520 fluorimetric kit (Anaspec, Fremont, California, USA; cat. AS-71152)

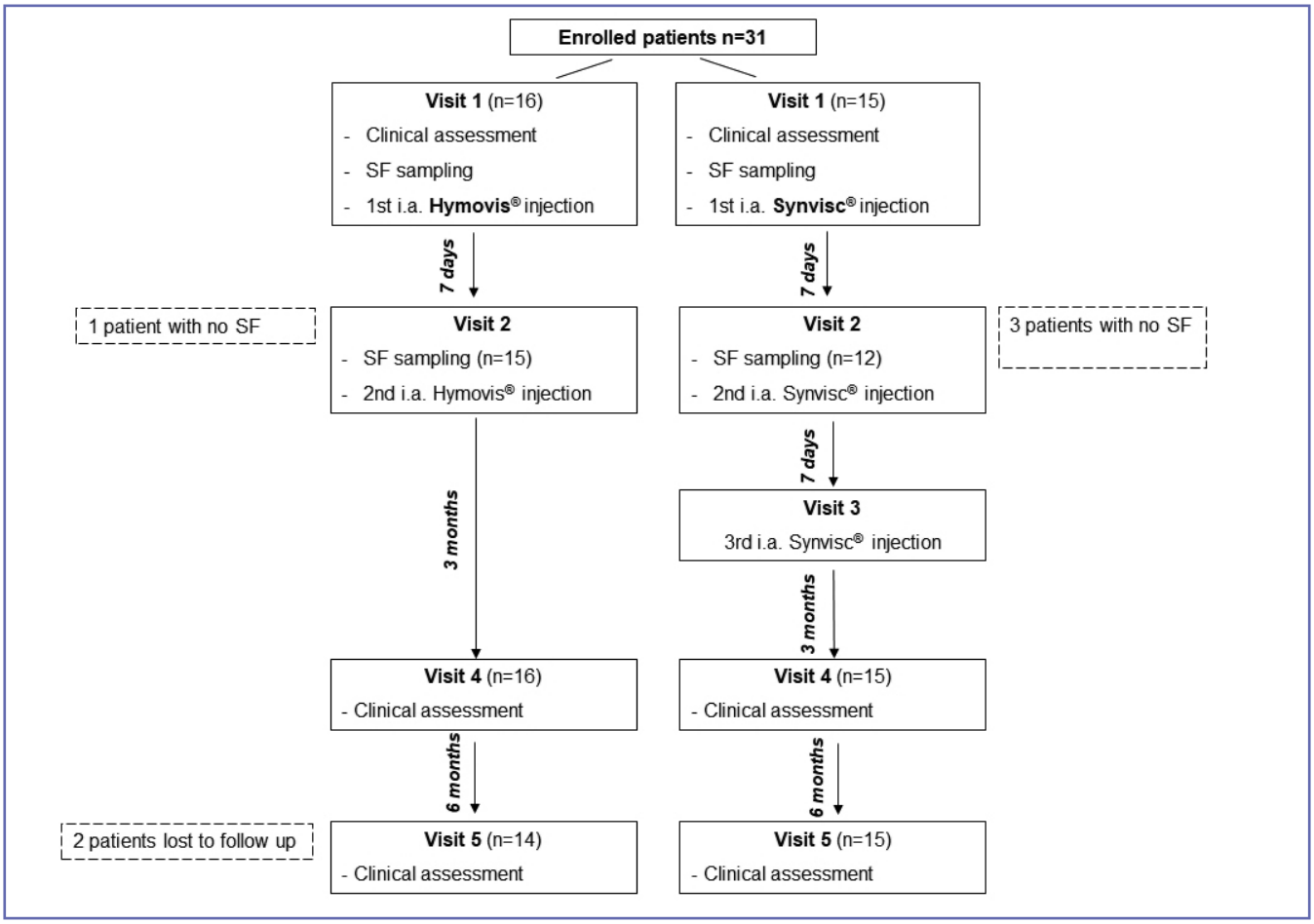

Figure 1. Flow chart of the study. Sixteen patients were treated with Hymovis® and 15 with Synvisc®. Reassessment followed after 1 week. Two patients in the Hymovis® group were lost to follow up after the 3-month follow-up visit.

that uses a 5-FAM (fluorophore) and QXL®520 (quencher) labelled fluorescence resonance energy transfer (FRET) peptide substrate for measurement of enzyme activity, showing high specificity for MMP-3. Fluorescence recovery upon cleavage of the FRET peptide by MMP-3 was monitored at excitation/emission = 490 nm/520 nm. Active MMP-3 was quantified against a standard curve built using human MMP-3 enzyme, pre-activated with 4-aminophenylmercuric acetate (APMA).

Total MMP-3 levels were assessed using a Sensolyte® MMP-3 ELISA kit (Anaspec, cat. AS-72103) and quantified against a standard curve built using human MMP-3.

The active and total MMP-2 and MMP-13 were measured using the Sensolyte® Plus 520 MMP-2 fluorimetric kit (Anaspec, cat. AS-72224) and Sensolyte® Plus 520 MMP-13 fluorimetric kit (Anaspec, cat. AS-72019), respectively. Both kits (ELISA assay), use a specific monoclonal anti-human MMP antibody to pull down MMP-2 or MMP-13, respectively, from the mixture. MMP activity is then quantified by a 5-FAM/QXLTM 520 FRET peptide. Total MMP-2 and MMP-13 in synovial fluid was quantified against a standard curve built using human MMP-2 or MMP-13 respectively, pre-activated using APMA. A validation of the recovery of MMP-13 activity, total MMP-13 level, and active and total MMP-2 activity was performed after hyaluronidase treatment, because a specific validation of this kit in synovial fluid has not been reported.

Cytokine levels (IL-1β, IL-6, IL-8, TNFα and RANTES) were measured by flow cytometry using a FlowCytomix kit (Bender MedSystems,Vienna, Austria), according to the manufacturer's instructions.

For cell count, after hyaluronidase incubation (250 U/ml at 37 °C for 10 min) (20), 10 µL SF was added to 190 µL of Stromatol® solution (Mascia Brunelli Srl, Milan, Italy) and total cell number was counted by optical microscopy using a Bürker chamber (Neuroprobe Inc, Gaithersburg, Maryland, USA) with a 40x objective. Differential count was determined in smears of cytocentrifuged, hyaluronidase-treated SF after May-Grünwald staining. SF samples were also analyzed by flow cytometry (FC 500, Beckman Coulter, Miami, Florida, USA) for platelet and platelet-leukocyte aggregates counts.

Clinical assessment

At baseline (visit 1, day 0), visit 2 (day 7), visit 4 (month 3) and visit 5 (month 6) patients self-reported knee pain on a 100 mm visual analogue scale (VAS) with 0 mm indicating no pain and 100 mm unbearable pain and answered the 24-item Western Ontario and McMaster Universities Arthritis Index (WOMAC) questionnaire. This consists of three subscales: pain (five questions), stiffness (two questions), and physical function (17 questions). The WOMAC 3.1 Index measures the patient's response to each of the 24 questions on a 5-point Likert scale with higher scores indicating greater symptom severity (0=none to 4=extreme). The WOMAC normalized subscale and total scores were then calculated. Finally, the physician global assessment (PhGA) of how the treated knee affected the patient's status at the above time points was also assessed on a 0-100 mm VAS scale where 0 indicated "not at all" and 100 indicated 'extremely'.

Statistical methods

The analysis included data from all patients who received at least one injection of the study products and for whom a sample of synovial fluid at visit 1 (day 0) and visit 2 (day 7) was collected.

Descriptive statistics for changes and percent changes from baseline were used to compare treatment groups and pre and post treatment values. Non-parametric tests were used to make comparisons between treatment groups (the Mann-Whitney U test) and to compare values between baseline and after treatment within treatment group (the Wilcoxon signed rank test). Comparison against baseline at all time points was adjusted as per the Bonferroni method to 0.05/2=0.025. To minimize the high variability of the percent changes from baseline of active MMP-3, the Chauvenet's criterion was adopted. A Mann-Whitney U test was performed to compare treatment groups without detected outliers.

VAS, PhGA and WOMAC scores were analysed using the same non-parametric tests mentioned above. Data are expressed as mean ± standard error of the mean (SEM). A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Thirty-one patients (10 males, 21 females, age 72.6 ± 1.8 years, range 53-88) were recruited (**table I**). Of these, 16 were allocated to receive Hymovis® and 15 Synvisc®. Two patients in the Hymovis® group were lost to follow up after the 3-month follow-up visit. In addition, synovial fluid assessment (specifically for active MMP-3 levels) was not performed in 4 patients at visit 2 (1 Hymovis® and 3 Synvisc®) due to lack of a sufficient amount of US detectable SF. Therefore, 15 patients were included in the Hymovis® group and 12 patients in the Synvisc® group for the assessment of laboratory data.

Mean duration of OA was 5.7 ± 0.9 years. At baseline, 74% of patients had Kellgren-Lawrence grade 3, 16% grade 2

Table I. Baseline characteristics of patients included in the study.

	Hymovis® (n=16)	Synvisc® (n=15)	All patients (n=31)
Mean age (years)	73.9 ± 2.7	71.2 ± 2.4	72.6 ± 1.8
Sex:			
Male	7 (43.7%)	3 (20.0%)	10 (32.3%)
Female	9 (56.3%)	12 (80%)	21 (67.7%)
Mean body weight (kg)	67.0 ± 2.7	67.5 ± 2.5	67.3 ± 1.8
Mean BMI (kg/m^2)	24.9 ± 0.9	24.7 ± 0.6	24.8 ± 0.5
Mean duration of OA (years)	6.2 ± 1.5	5.2 ± 1.1	5.7 ± 0.9
Kellgren-Lawrence:			
Grade 2	3 (18.8%)	2 (13.3%)	5 (16.1%)
Grade 3	11 (68.8%)	12 (80.0%)	23 (74.2%)
Grade 4	2 (12.4%)	1 (6.7%)	3 (9.7%)
Mean interval between treatments 1 and 2 (days)	8.1	7.0	7.6

OA osteoarthritis; BMI body mass index. Data are expressed as mean ± SEM.

and 10% grade 4. 97% had crepitus on motion, 74% had clinically evident swelling and 68% morning stiffness.

During the study, 5 patients received prohibited medications, 2 in the Hymovis® group (steroid injection and NSAIDs) and 3 in the Synvisc® group (2 patients NSAIDs and one patient oxycodone). In all cases, medications were administered after receiving the last study injection.

Synovial fluid biomarkers

Platelet count and platelet-leukocyte aggregates, as well as total cell count, did not change significantly after treatment with Hymovis®. At microscopic analysis, a significant decrease in percentage of neutrophils (20.9 ± 2.2% *vs* 14.6 ± 2.9%, p=0.046) was observed following Hymovis® treatment while not in the Synvisc® group. The median change from baseline was significantly different between the treatment groups (Hymovis® -7.0% *vs* Synvisc® -3.0%, p=0.024).

Table II shows changes in total and active MMP-2, MMP-3 and MMP-13 levels for both treatment groups. No significant difference was observed for median active MMP-3 levels: percent change from baseline was -5.3% (range: -61.7% to +100.9%) for Hymovis® and +8.8 (range: -48.1% to +82.9%) for Synvisc®. A trend towards a greater decrease in MMP-3 activity was observed in the Hymovis® group, as active MMP-3 decreased in 9/15 and increased in 6/15 Hymovis® patients, compared with a decrease in 4/12 and increase in 8/12 Synvisc® patients. Reductions in active MMP-2 and active MMP-13 were observed in both groups. Overall there were no significative differences in total MMP-2, MMP-3 or MMP-13 between the treatment groups or within each treatment group.

Active MMP-2 levels tended to decrease in both treatment groups (**table II**), while active MMP-3 level decreased, although not significantly, in Hymovis® group and not in Synvisc® group. This trend was also confirmed when analyzing the percent variation against baseline adopting the Chauvenet's criterion, which detected one patient in the Hymovis® group as an outlier. After excluding this patient, a significant difference between treatment groups was evidenced (p=0.042) in favor of Hymovis® where a median decrease in activity of -10.6% was observed.

Moreover, the active MMP-3/pro-MMP-3 and the active MMP-2/pro-MMP-2 ratio, an index of MMP activity, tended to decrease (p=0.09 and p=0.14, respectively) after Hymovis® treatment (**figure 2**) while it did not change in the Synvisc® group (p=0.24 and p=0.62, respectively).

For MMP-13, no difference in active form and in active MMP-13/pro-MMP-13 ratio was observed in the two treatment groups (**table II**).

A significant decrease in IL-6 levels was observed at visit 2 compared to baseline in both groups, without difference between the two treatments (Hymovis® group: 3866 ± 1162 *vs* 1321 ± 387 pg/mL; Synvisc® group: 3908 ± 1045 *vs* 1849 ± 332 pg/mL). IL-6 levels decreased from baseline in 10/15 Hymovis® patients, and in 10/12 Synvisc® patients. None of the other inflammatory cytokines measured, IL-1β, TNF-α, IL-8 and RANTES, showed difference between treatment groups or when comparing post-treatment values to baseline within each treatment group.

Table II. Matrix Metalloproteinase (MMP) levels (ng/ml) at baseline and 7 days after treatment with Hymovis® or Synvisc®.

	Hymovis® (n=15)			Synvisc® (n=12)		
	baseline	Visit 2	p value (change within each group)	baseline	Visit 2	p value (change within each group)
MMP-2						
Active MMP-2	13.5 ± 1.2	10.0 ± 1.5	*0.084*	13.7 ± 1.7	9.4 ± 2.5	*0.26*
Pro MMP-2	636.5 ± 82	700 ± 107	*0.63*	553 ± 60	585 ± 73.5	*0.63*
Active/pro	0.025 ± 0.003	0.018 ± 0.003	*0.14*	0.016 ± 0.03	0.007 ± 0.037	*0.30*
MMP-3						
Active MMP-3	105.1 ± 8.2	84.9 ± 7.8	*0.25*	86.3 ± 7.1	91.7 ± 6.7	*0.42*
Pro MMP-3	1429 ± 332	1621 ± 303	*0.49*	1589 ± 521	1371 ± 425	*0.33*
Active/pro	0.13 ± 0.026	0.07 ± 0.01	*0.09*	0.09 ± 0.01	0.11 ± 0.01	*0.24*
MMP-13						
Active MMP-13	21.7 ± 2.3	20.9 ± 1.6	*0.97*	26.4 ± 2.8	22.3 ± 1.6	*0.23*
Pro MMP-13	50.9 ± 4.7	60.1 ± 6.1	*0.072*	47.4 ± 4.4	47.9 ± 4.7	*0.93*
Active/pro	0.47 ± 0.06	0.41 ± 0.06	*0.30*	0.59 ± 0.07	0.52 ± 0.06	*0.51*

Data are expressed as mean ± SEM.

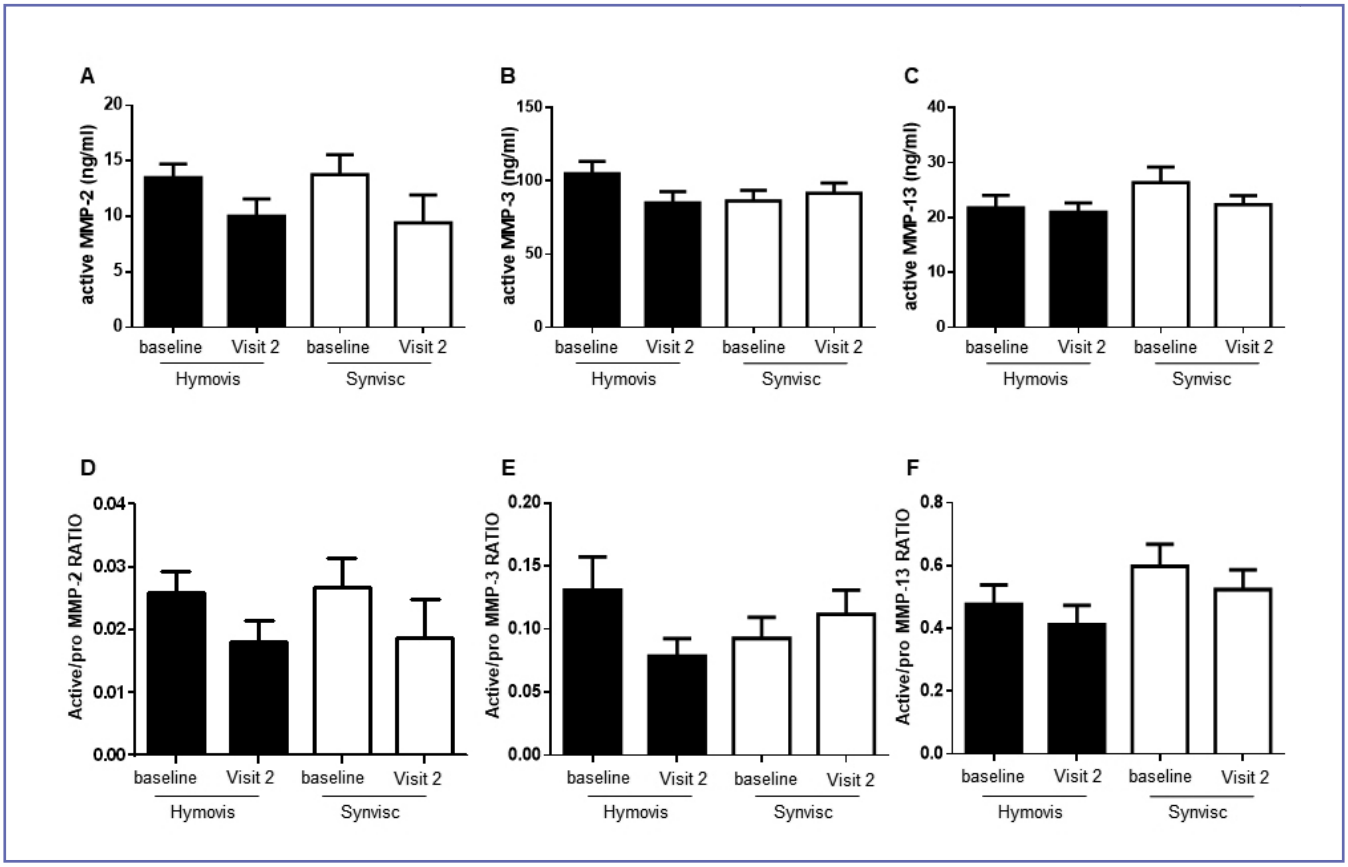

Figure 2. Levels of metalloproteinases after Hymovis® and Synvisc® injection compared to baseline. Active MMP-2 (A), MMP-3 (B), MMP-13 (C) levels and their ratio *versus* proenzyme form (D, E, F). Data are reported as means ± SEM.

Clinical assessment

No differences between the treatment groups or after treatment in the treatment arms were observed for the WOMAC scores.

The percentage of Hymovis® treated patients with increased or decreased VAS perceived pain values at 3 and 6 months as well that concerning PhGA are shown in **figure 3.**

No serious adverse events, adverse device effects or device deficiencies were reported. There were 4 adverse events, 3 patients reported increased knee pain which was considered possibly related to administration (1 on Hymovis®, 2 on Synvisc®) and one patient in the Hymovis® group reported flu symptoms which were not considered to be related to treatment.

DISCUSSION

Viscosupplementation with HA-based formulations was shown to have variable degrees of efficacy in the therapy of knee OA (21-24). HA suppressed interleukin IL-1β-induced MMP activity in OA synovial tissue explants *in vitro* (25) and decreased the levels of MMP-2 in synovial fluid of OA patients (20). This finding suggested that viscosupplementation with HA could provide clinical benefit by blocking MMP activity in the OA joint. Hymovis®, an intra-articular formulation of HA, is a valid therapeutic option in the treatment of osteoarthritis based on its viscosupplementation properties, displaying a beneficial effect for up to 12 months (26). The current study aimed to investigate the effects of a single intra-articular injection of Hymo-

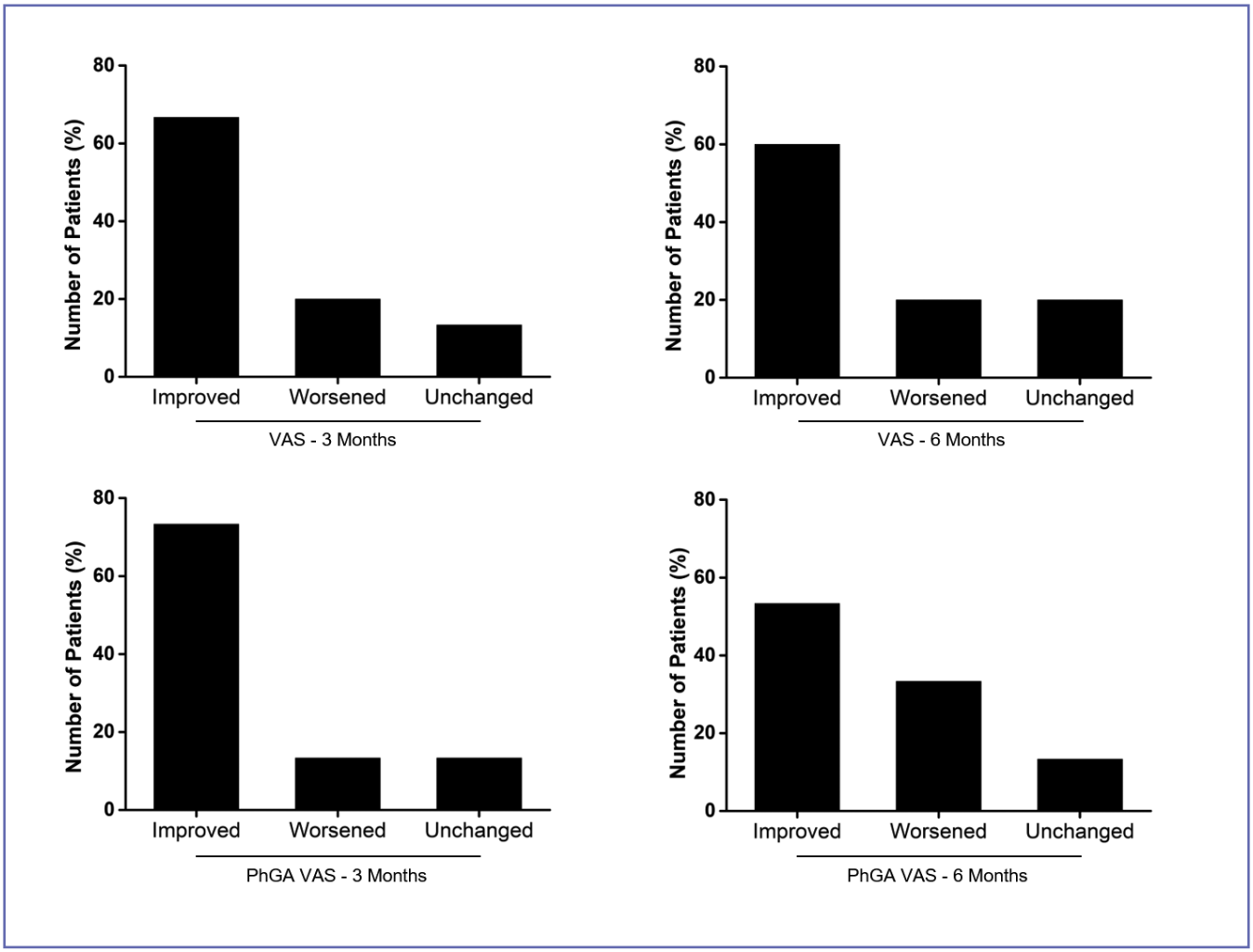

Figure 3. Number of patients (%) considered improved, worsened or unchanged in visual analogue scale (VAS) and physician global assessment (PhGA) at 3 and 6 months from baseline. VAS scores were compared using Wilcoxon pair data test.

vis® on the expression and activity of metalloproteinases in the synovial fluid of patients with OA of the knee and demonstrated that an i.a. injection of Hymovis® may have both symptom and chondroprotective effects in OA by decreasing MMPs. 60% of Hymovis® treated patients, in fact, had a decrease in active MMP-3 levels one week after the first intra-articular injection. Although not statistically significant, this trend was confirmed when excluding an outlier patient in the Hymovis® group using Chauvenet's method. Hymovis® treated patients also showed a reduction in active MMP-2 and active MMP-13 levels (67% and 53% of patients respectively), as well as a reduction of the active MMP-2/pro-MMP-2 ratio and the active MMP-3/pro-MMP-3 ratio indices of MMP activity. The dynamic balance of active-pro MMPs is the crucial factor in the regulation of MMPs' enzymatic activity, as the role of MMPs in pathophysiologic conditions does not depend on the absolute concentration of MMPs, but also on the active/proMMPs ratio.

These results are consistent with a previous *in vitro* investigation that demonstrated that HYADD-4G, a lead compound in a class of alkyl derivatives of HA, showed the highest inhibition potency towards all human MMPs and especially against MMP-13 and MMP-8 (17).

The significant reduction in neutrophils and IL-6 levels observed in the Hymovis® group may indicate a reduction in the inflammatory state in the synovial fluid of these patients.

Following treatment, there were no significant differences in OA related symptoms between the treatment groups. No serious adverse events, adverse device effects or device deficiencies were reported.

Our findings suggest that viscosupplementation using Hymovis® reduces pain in patients with knee osteoarthritis and protects structural integrity of cartilage by decreasing MMPs. A first limitation of the present study consists in the relatively small number of individuals being studied. Yet, our findings are consistent with previous reports, adding strength to our conclusions. Secondarily, the study was open-label, and this could have introduced a bias. The mechanism as well as the potential therapeutic value of HA suppression of MMPs and the chondroprotective effects of viscosupplementation remain to be elucidated by further preclinical and clinical data.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests. NG is an employee of Fidia Farmaceutici SpA. All the authors state, however, that Fidia Farmaceutici SpA did not participate in the decision to submit this manuscript for publication.

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