White Paper

A Scientific Review of FlexPro MD®
Comparing *in vitro* & *in vivo* Mouse Study Data
& Human Clinical Trial Results

Background:

Osteoarthritis is characterized by an imbalance between anabolic and catabolic processes in the affected joint. This metabolic dysregulation affects the chondrocytes that produce cartilage but also affects the cells of the synovial membrane across the border of the joint characterized by a loss of viscosity. This results in a loss of synovial fluid lubrication and loss of cartilage with a corresponding lack of joint flexibility and pain.

The development of osteoarthritis (OA) occurs in response to mechanical stress from trauma, obesity, or genetic predisposition. Some common risk factors associated with developing osteoarthritis include age, gender, and family history. Systemic factors include bone density, dietary intake, or estrogen use. Local biomechanical factors include joint laxity and muscle weakness. (1)

OA patients suffer from disability, pain, and a dramatic reduction in the quality of life. Although there is mild, moderate, and severe OA, destruction of joint tissue, synovial membrane, and cartilage inevitably occurs and this leads to aseptic inflammation that prolongs and worsens the disease. (2)

Synovial inflammation has a major role in the progression of OA and is accompanied by macrophage and lymphocyte infiltration and hyperplasia. The macrophages, lymphocytes and chondrocytes produce inflammatory cytokines, including IL-1β, IL-6, and TNF-α, which, in turn, stimulate the production of matrix metalloproteinases (MMPs) that are found in increasing levels in OA joint tissues and synovial fluids. (3)

It has been well-established from 1983 to the present that TNF-α, and interleukins stimulate osteoclast bone resorption in OA joints. (4, 5)

Treatments with non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs) are inadequate in treating the disease due to poor patient-specific adequacy. They often fail to block cartilage degradation. Increasing doses are required to treat OA over time and the ulcerogenic risk of NSAIDs increases the longer they are administered. Corticosteroids are also effective at reducing inflammation and pain in treating OA but their adverse effects, immunosuppression, and the risk of osteoporosis, seriously limit their use. (6, 7)

Lower molecular weight hyaluronic acid (LMW-HA), krill oil and astaxanthin (AST) each have beneficial effects on joint discomfort demonstrated in human clinical trials, animal, and *in vitro* studies. (8, 9, 10, 11, 12, 13, 14, 15, 16, 17)

LMW-HA is a muco-polysaccharide comprised of tandem repeats of D-glucuronic acid and N-acetyl glucosamine. It is present in abundance in the synovial fluid of mammalian joints. At present, intra-articular administration of HA is the treatment of choice for patients with symptomatic knee OA, although meta-analysis of studies has shown that intra-articular knee injections of HMW-HA have a placebo effect identical to aqueous placebo injections and that publication bias in these studies is approximately 37%. (18, 19)

A clinical trial using 200 mg of orally administered HMW-HA was given once daily for 12 months to sixty osteoarthritic subjects who were randomly assigned to an active HA or a placebo group. The subjects in the HA group at the end of the study had better symptom resolution compared to the placebo group. Subjects in both groups conducted quadriceps strengthening exercise everyday as part of the treatment and symptoms were evaluated by the Japanese Knee Osteoarthritis Measure (JKOM) score. The symptoms improved with time in both the HA and placebo groups and the study authors concluded that "Oral administration of HA may improve the symptoms of knee OA in patients aged 70 years or younger when combined with the quadriceps strengthening exercise." (8)

Krill oil is a marine lipid with lower EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) content than ordinary fish oil but these essential fatty acids are bound to phospholipids making them more bioavailable than the triacylglycerides found in fish oils. A randomized, double-blind, parallel-group, placebo-controlled trial of fifty adults 38 to 85 years old with mild knee pain were divided into two groups. The active group was given 2 grams of krill oil or a placebo for 30 days. The study results demonstrated that "krill oil significantly mitigated knee pain in sleeping, standing and the range of motion of both right and left knees compared to placebo." (9, 20)

AST is a xanthophyll carotenoid with unique cell membrane actions and a wide range of clinical benefits. AST quenches free radicals or other oxidants by either accepting or donating electrons, and is a potent scavenger of reactive oxygen species without being destroyed or becoming a pro-oxidant in the process. Its polar-nonpolar-polar molecular design allows it to insert itself into cell and organelle membranes, sometimes spanning the entire width of the membrane. AST can quench free radicals within the hydrophobic interior of membranes and along its hydrophilic outer surfaces. In clinical trials, AST has shown a wide range of benefits and has an excellent safety profile. AST is one of the strongest singlet oxygen quenchers of the carotenoid family and is much more potent in this regard than other common dietary antioxidants.

Astaxanthin is:

14 times stronger than vitamin E
18 times stronger than pine bark extract
21 times stronger than synthetic astaxanthin at quenching free radicals *in vitro*
54 times stronger than beta carotene
65 times stronger than vitamin C

(21, 22, 23)

A Detailed Review of a Clinical Trial of a Multi-component, FlexPro MD®, Containing Zanthin® Astaxanthin, LMW-HA and Krill Oil:

Although tested individually in clinical trials with promising results for the treatment of joint discomfort, a combination of krill oil, AST and LMW-HA had never previously been tested as a multi-component formula. There is also no human data on such a multi-component formula for treating reduced strength, flexibility, mobility, and discomfort of surrounding supportive tissues of the knee and/or hip in subjects who were not previously diagnosed with OA or RA.

A clinical trial testing a proprietary multi-component formula of krill oil, AST and LMW-HA recruited 140 subjects from 18 to 80 years old with non-arthritic arthralgia (joint pain). The subjects had never previously been diagnosed with osteoarthritis or rheumatoid arthritis. However, they did suffer from reduced strength, mobility, flexibility, and generalized temporary or recurring discomfort of the surrounding supportive tissues of the knee and/or hip. (37)

The study design was an IRB-approved, double-blind, placebo-controlled, three-arm, 56-day informed consent trial to evaluate the effects of the multi-component formula. Of the 140 subjects, 107 completed the trial—32 subjects in Group A, 40 subjects in Group B, and 35 subjects in Group C.

Group A subjects were administered a commercially-available chondroitin-glucosamine supplement. Group B subjects were administered the "Joint Health" product and Group C was given a palm kernel oil placebo.

Primary outcomes included: comparison of changes in range of motion using a standard Goniometer, the six-minute walk test, Beighton joint flexibility test, WOMAC and VAS.

WOMAC and VAS

A further primary outcome variable was to detect any changes in joint pain using the Western Ontario and McMaster Universities Osteoarthritis Index™© pain assessment and pain intensity rating scale (WOMAC). The WOMAC pain intensity rating scale has been tested for validity. WOMAC subscales (pain, stiffness, and physical function) were previously shown to be internally consistent with Cronbach's coefficient alpha of 0.91, 0.81, and 0.84, respectively. Test–retest reliability was satisfactory with ICCs of 0.86, 0.68, and 0.89, respectively. (48) The validity and statistical significance of WOMAC confirms that it is a robust and appropriate assessment of pain used in the present study. (49)

Additionally, a primary objective outcome variable was measured by subjects answering a daily on-line Visual Analogue Scale (VAS) pain-assessment. The Visual Analog Pain-Assessment Scale has been well-validated in clinical trial reviews of chronic musculoskeletal pain, knee osteoarthritis pain and hip pain. (50, 51, 52) The validity of the written version compared to a computerized version entitled WOMAC VAS 3.0 was assessed and considered a valid alternative to the written version. (52) In patients with chronic inflammatory or degenerative joint pain, the pain VAS has previously demonstrated sensitivity to changes in pain assessed hourly for a maximum of 4 hours and weekly for up to 4 weeks following analgesic therapy. The sensitivity to changes in pain using the Visual Analog Pain-Assessment Scale have been validated in studies going back to 1975. (53)

The primary outcome variables were tested at baseline (day 0) and on days 14, 28 and day 56, with an additional Daily Diary VAS assessment on Day 7. The secondary objective of the study was to assess the safety of the product in comparison to the glucosamine-chondroitin group and the placebo group taking palm kernel oil. Another secondary objective was to evaluate the safety of the test product and to note if there were any adverse events.

To assess the safety of the 'Joint Health' test product, blood samples were taken at the beginning and at the end of the 56-day trial, including a clinical chemistry profile that included a lipid profile, total cholesterol, lipoproteins, triglycerides, and a high sensitivity C-Reactive protein test. A measure of RBC omega-3 fatty acids and a gene expression evaluation. Adverse events were self-reported, recorded and then evaluated by the clinical staff.

Subject compliance was excellent. Very little rescue medication was requested and the few adverse events (AE's) that were reported were minor (mainly headaches) and were probably not attributable to the 'Joint Health' product.

During the 56-day study, the participants periodically visited the clinic to replenish the study product; for joint flexibility/mobility measurement using the Beighton Joint Flexibility Test; for participation in a six-minute walk test; and to have their pain levels measured by the Western Ontario and McMaster University Osteoarthritis Index™© pain assessment and pain intensity rating scale (WOMAC). In addition, the participants had to answer daily an on-line Visual Analog Scale (VAS) pain assessment.

Results Assessment

The subjects in the active Group B who were administered the "Joint Health" product had lower scores at every time point measured, i.e., on days 15, 29 and day 57 using the WOMAC pain intensity rating scale; Group B also had lower scores on days 7, 14, 28, 35, 42 and day 56 using the VAS self-assessed pain intensity rating scale when compared to the subject scores in Group A given the glucosamine-chondroitin product and Group C given the palm oil placebo.

The data showed a highly statistically significant pain reduction effect in Group B given the "Joint Health" product over the entire course of the study, with a 35% pain reduction after 14 days treatment, increasing to a 44% reduction after 28 days and a 55% reduction after 56 days of treatment.

Figure 1.

VAS Percent Improvement Joint Comfort

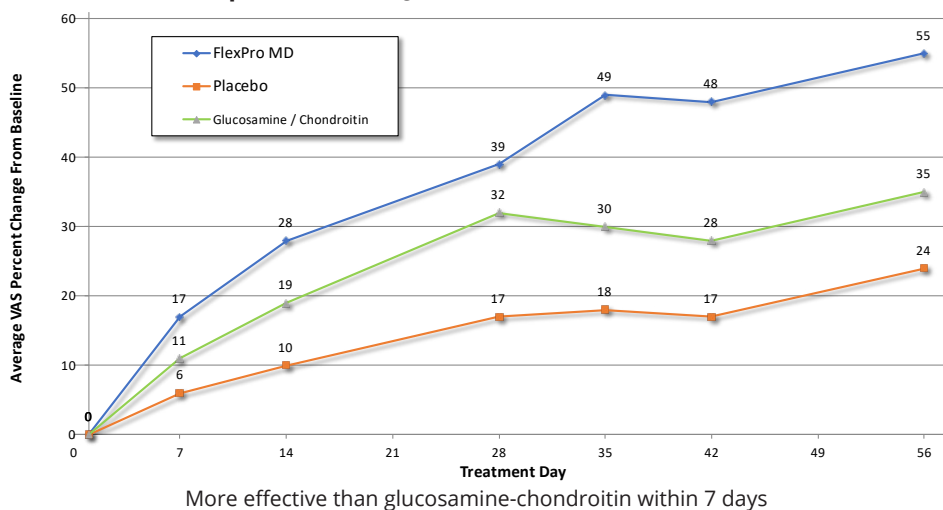More effective than glucosamine-chondroitin within 7 days

Figure 2. Visual Analog Score Test
Percentage of Subjects achieving Optimal Joint Comfort, "back to normal"

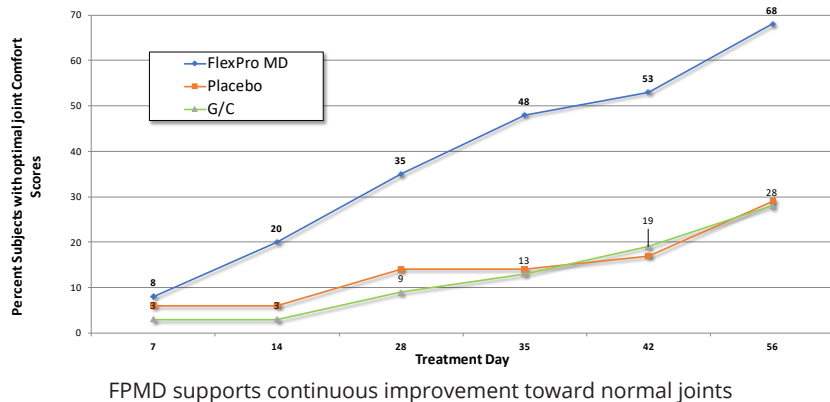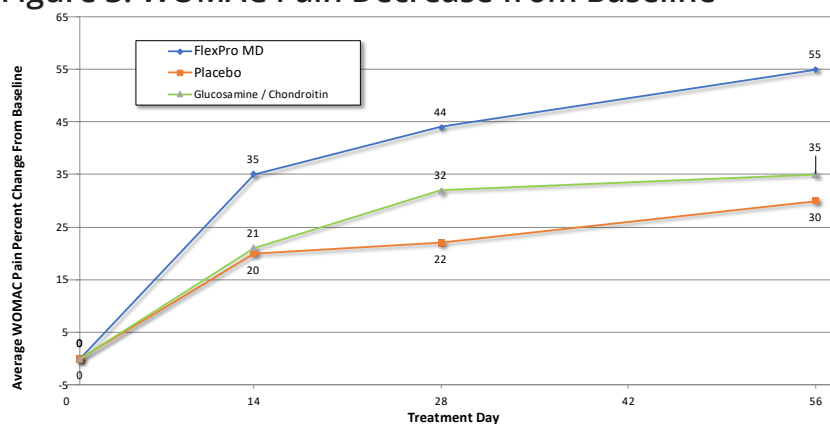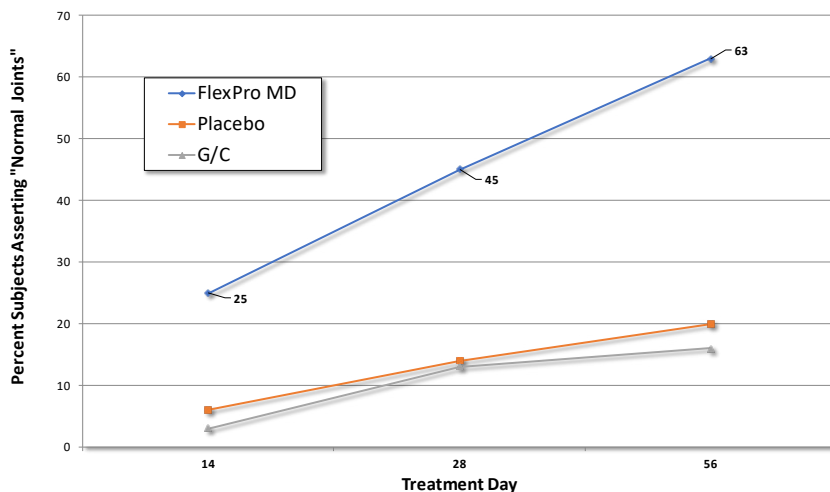FPMD supports continuous improvement toward normal joints

Figure 3. WOMAC Pain Decrease from Baseline

2 times more effective than glucosamine-chondroitin in reducing joint discomfort

Figure 4. WOMAC Pain Score Test
Percentage of Subjects Asserting "Normal Joint Comfort"

3 times more effective than glucosamine-chondroitin in restoring normal joint comfort

The Group B subjects reported pain relief as early as 7 days from the onset of treatment. By day 56, Group B reported a 55% reduction of pain from their baseline levels. Groups A and C at day 56 reported lower pain reductions of 35% and 30%, respectively, compared to their baseline levels. A further analysis of the pain data involved quantization of the number of subjects that joint comfort on that day and continued to experience joint comfort until the conclusion of the study. Having "total joint comfort" was defined as having a sum total WOMAC or VAS level of 9 or less on the day queried and all subsequent days, for the five questions posed. Figures 1-4 show the trend of subjects experiencing relief through the trial. On day 56, the percentage of subjects that experienced joint comfort determined by WOMAC and VAS were 63% and 68%, respectively, whereas less than 30% of the subjects in Group A and C experienced joint comfort.

The knee range of motion (ROM) data was not definitive using the Beighton Flexibility test or Goniometer measurements at the end of the study due to confounding results. The subjects in Group A given glucosamine-chondroitin had a greater maximum change in range of motion between the two tests at the end of the study. Also, the subjects in Group A had a greater initial change in range of motion between the two tests at the end of the study.

The mean triglyceride values of the three groups were in the normal range when measured on the two test days during the trial. No effects were observed in the study either because the krill oil dose was considerably lower than the one to three grams needed for hypocholesteremic effects seen in human trials (54, 55), or simply due the fact that the subjects in the study had relatively normal serum lipid levels to begin with. The mean VLDL levels in the three groups was in the normal range at day 0 and day 56 when tested; the mean HDL levels for all groups was above 39 mg/dl on both test days; the mean LDL-Cholesterol levels averaged above 99 mg/dl at the commencement of the study and remained above this level throughout the study because of higher values in many of the study participants, but this was not due to any aberrant data points. We conclude that there was no significant effect on serum lipids in the 'Joint Health' group, the glucosamine-chondroitin-group or in the palm oil placebo group.

High Sensitivity C-Reactive protein (hs-CRP) levels were measured over the course of the trial. A large majority of the study participants had normal hs-CRP throughout the study. However, some subjects had periodic 'flare-ups' of their inflammation, and these higher levels skewed the group's averages on certain days, however of the few recorded hs-CRP flare-ups all resolved between test points.

Omega-3 fatty acid levels increased an average of 20.14% in the blood of the subjects given the 'Joint Health' product. 20 of the 23 fatty acids measured showed an increase in the active 'Joint Health' group.

Post Study Questionnaire Results

The results of a final questionnaire were very positive in favor of the active 'Joint Health' group, who perceived the product to have "positive effects" and they expressed a high level of "intent to buy".

Safety Assessment

All products tested were determined to be safe in this study as demonstrated by clinical blood analyses, which included lipid profiles. The vital signs, self-reported adverse events (AE's) and physical examinations also demonstrated the safety of the 'Joint Health' product, the glucosamine -chondroitin product, and the palm kernel oil placebo in the present study. No changes in blood parameters were seen in any of the study participants in the three groups during the study.

Validation of Lipopolysaccharide (LPS)-induced *in vivo* Arthritis Mouse Model

To validate the mechanisms of FlexPro MD®'s in the above human trial, we used a well-established lipopolysaccharide (LPS-induced arthritis model) in male C57BL/6 mice who were 8 to 10 weeks old. The LPS-induced arthritis model was performed as described previously and is well-validated as a clinical model of inducing synovial arthritis. (61, 62, 63)

Mice were divided into five groups—a normal group, an LPS induced-arthritis group (a negative control), an indomethacin group (a positive group, 1 mg/kg.), and 2 groups (one group orally administered 33 mg/kg) (one group orally administered a human clinical equivalent dose of 67 mg/kg.) of MD. On day '0', mice were fed indomethacin or FlexPro MD® with two doses orally at 2-day intervals for 15 days before injecting LPS (10 ug/per mouse into the mouse intra-articular knee) one time. LPS was injected one more time 7 days after the first injection. Phosphate-buffered saline (PBS) served as the control. Indomethacin and FlexPro MD® were further administered orally at 2-day intervals for two weeks.

The Analyses of Inflammatory Markers:

At the end of the experiments, the knee joint tissues from each experimental group were collected for the analysis of inflammatory markers. The following markers were measured: interleukin 6 (IL-6), tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β), pro-inflammatory cytokines; interleukin 10 (IL-10), an anti-inflammatory cytokine; and C-Reactive protein (CRP), a marker of inflammation.

Investigation of FlexPro MD® on Cytokine Expression in the Mouse Model

To investigate the effects of FlexPro MD® on the inflammatory cytokine expression in the LPS-induced arthritis model, mice were sacrificed and the mRNAs were isolated from the knee joints. Quantitative real-time-PCR analysis was performed as described. As shown in Figure 5A, FlexPro MD® significantly reduced the mRNA levels of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β. Moreover, FlexPro MD® treatment increased IL-10 mRNA levels. (Figure 5B).
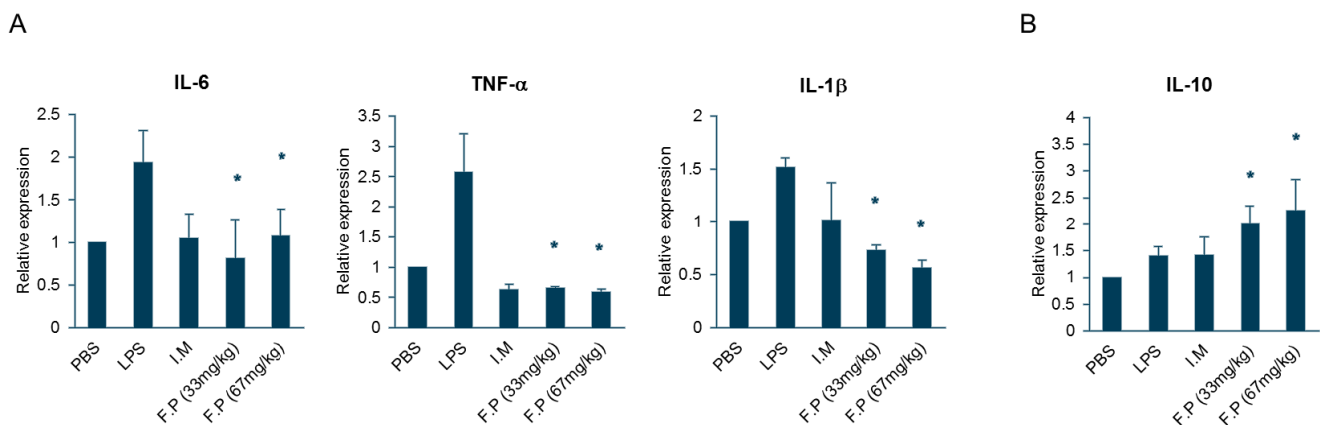

Figure 5. Flexpro MD® modulated inflammatory cytokine expression. Indomethacin (1 mg/kg) or FlexPro MD® with 2 doses (33 mg/kg and 67mg/kg) were orally administrated at 2-day intervals for 15 days before injecting LPS. At day 2 from LPS injection, indomethacin and FlexPro MD® were further administered orally at 2-day intervals for two weeks. Levels of IL-6, TNF-α, and IL-1β mRNAs were determined by real-time PCR analysis, and the values were normalized to β -actin mRNA expression. (B) As in (A), the anti-inflammatory IL-10 mRNAs were determined.

Effects of FlexPro MD® on NF-kB Dependent iNOS and COX2 Expression

To investigate the effects of FlexPro MD® on iNOS and COX-2 expression, their expression in synovia of knees was quantitatively measured by using quantitative real-time PCR. As shown in Figure 6, FlexPro MD® treatment 'significantly decreased the mRNA levels of iNOS and COX2. The data suggest that Flex-Pro MD® inhibits the NF-kB-dependent pathway in knee joints.

Figure 6.
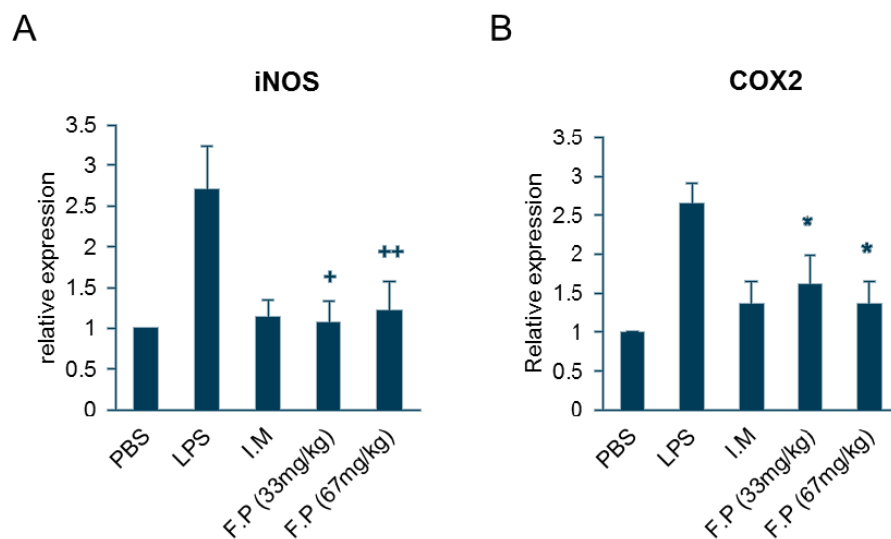
Effects of FlexPro MD® on MMP1 and MMP2 expression

The irreversible destruction of the cartilage and bone that comprise synovial joints is the hallmark of both rheumatoid arthritis and osteoarthritis. Since the expression of metalloproteinases, such as MMP1 and MMP2, is elevated in arthritis and these enzymes degrade non-collagen matrix components of the joints (65), we investigated whether Flexpro MD® could inhibit MMP1 and MMP2 expression in knee joints. We found that FlexPro MD® significantly inhibited the expression of these key metalloproteinases. (Figure 7). These results suggest that FlexPro MD® can effectively protect the degradation of non-collagen matrix components.
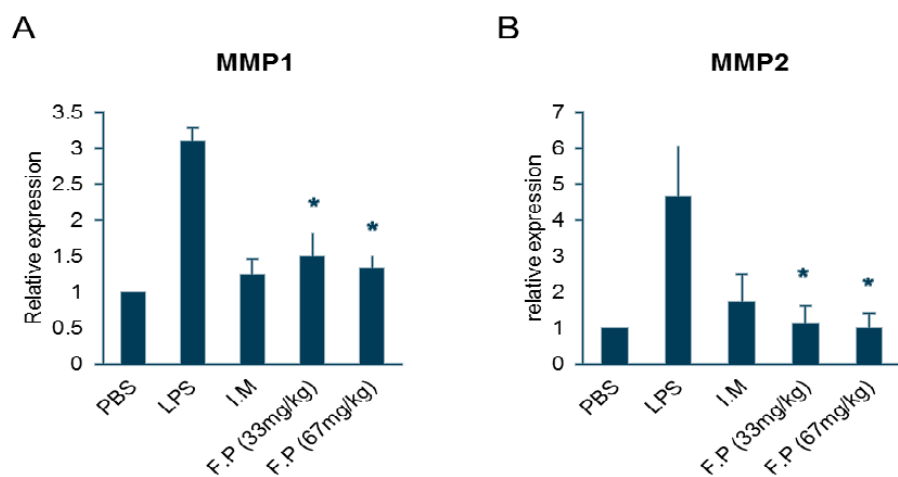
Figure 7. FlexPro MD® inhibited expression of MMP1 and MMP2. (A-B) Levels of MMP1 (A) and MMP2) (B) mRNAs were determined by real-time PCR analysis, and the values were normalized to β-actin mRNA expression.

Effects of FlexPro MD® on CRP Expression in Mouse Knee Joint Tissues

The physiological role of CRP is to activate the complement system for the disposal of dying cells, and its levels are expected to be higher at the source of an inflammatory process in the synovial fluid rather than systemically. Therefore, we measured the hs-CRP levels in the synovial fluid of the LPS-induced arthritis mouse model. (66, 67, 68)

mRNA levels of hs-CRP were quantitatively measured by using quantitative real-time PCR. The LPS-induced hs-CRP mRNA expression was significantly inhibited by FlexPro MD® treatment illustrated in Figure 8.
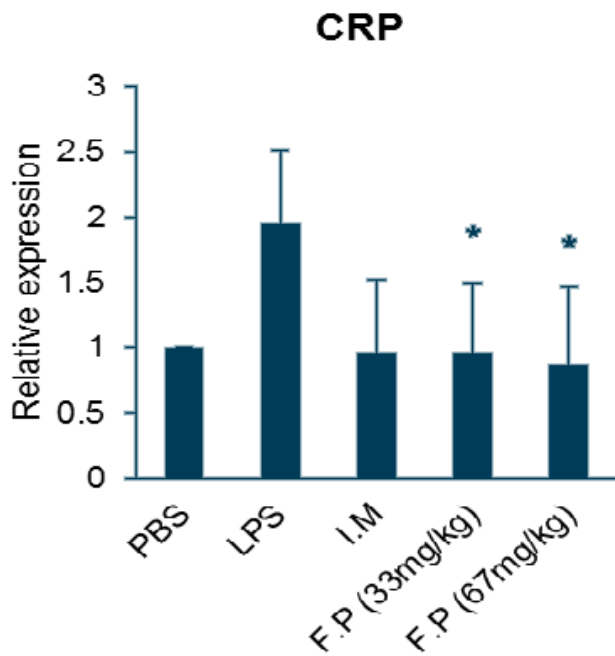

Figure 8. FlexPro MD® inhibited hs-CRP expression. Levels of hs-CRP mRNA was determined by real-time PCR analysis, and the values were normalized to B-actin mRNA expression.

Thirty days of continuous oral administration of 33 mg/kg and 67 mg/kg of FlexPro MD® in the LPS Mouse model significantly lowered the levels of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β and the key inflammatory markers indicating the up-regulation of NF-kB expression, iNOS and COX-2. FlexPro MD® also simultaneously upregulated levels of the anti-inflammatory cytokine IL-10 equal to the effects of indomethacin. The results obtained in this study show that FlexPro MD® has sufficient potential to continue its use as a novel supplement or nutra-ceutical for joint discomfort.

The macrophage-like cell line RAW264.7 is the most commonly used mouse macrophage cell line in medical research. Because of ease of cell propagation, high efficiency for DNA transfection, sensitivity to RNA interference, possession of receptors for many relevant ligands, and other properties, RAW264.7 has been chosen by the Alliance for Cellular Signaling as the primary experimental system for their large-scale study of signaling pathways. The RAW264.7 cell line has limitations for viral studies, however. (69, 70, 71)

The use of intra-articular injection of LPS to induce joint arthritis is a well-established experimental *in vivo* mouse model and in *in vitro* cell studies of inflammation. (60, 61, 62) The present cell study used real-time PCR to measure the levels of IL-1β, TNF-α, IL-6 and the levels of IL-10, an anti-inflammatory cytokine in LPS-treated RAW264.7 cells to delineate the effects of FP-MD treatment in this model.
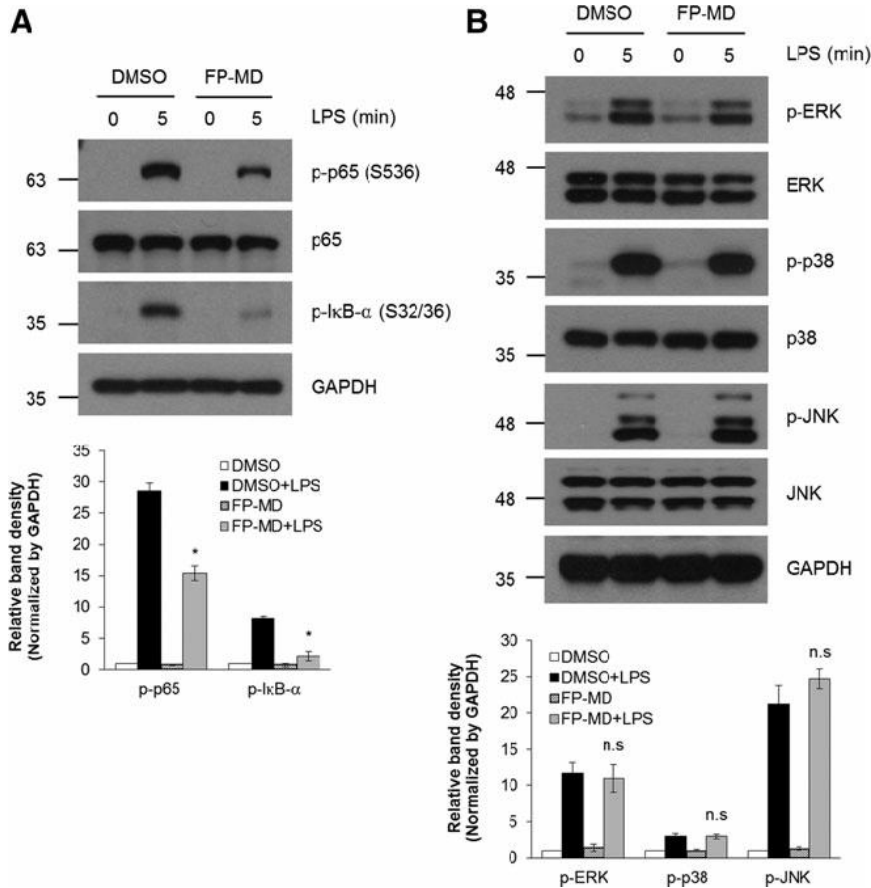
Figure 9.
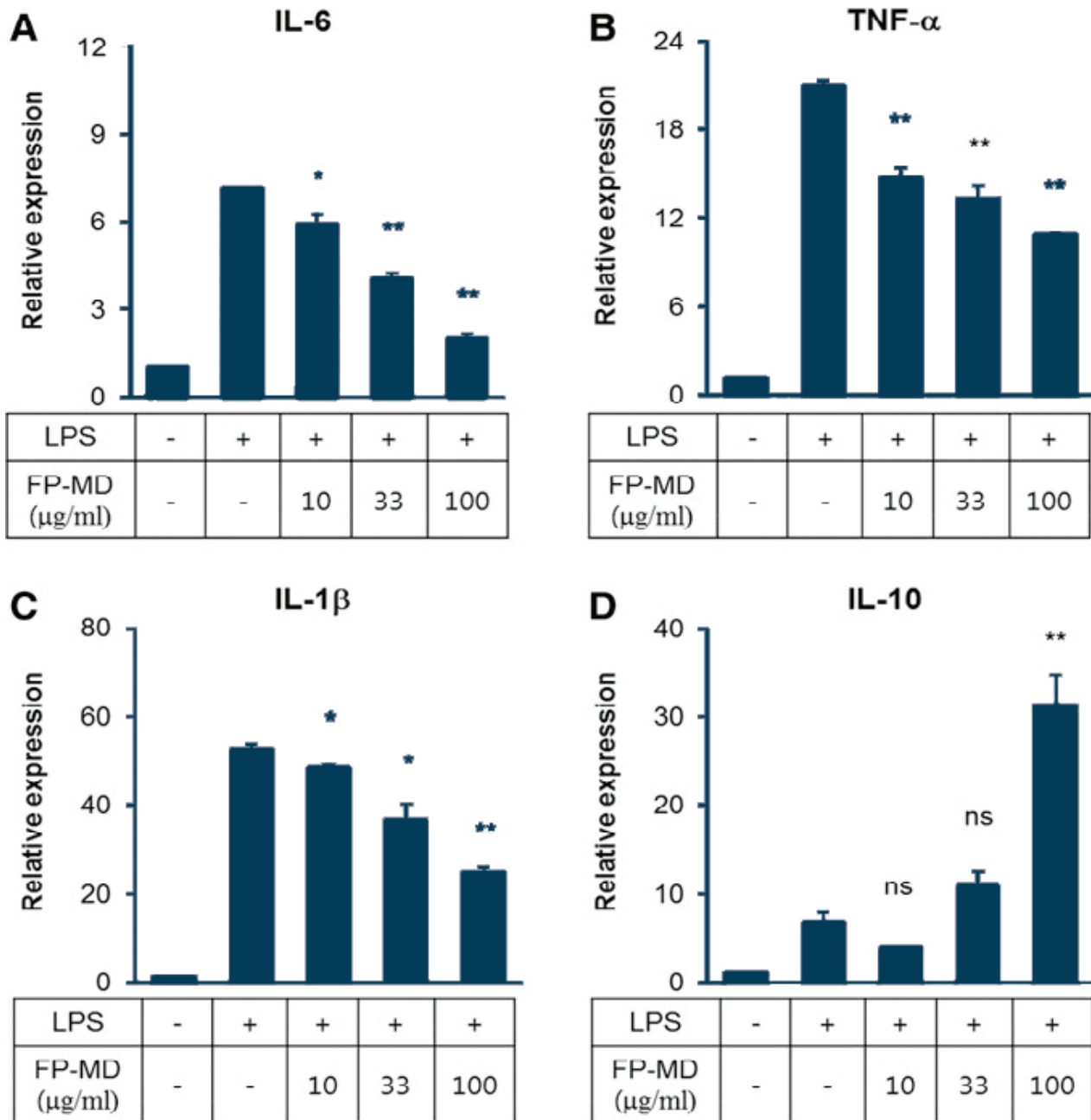
FP-MD lowered LPS-induced IL-6 levels in a linear, step-wise fashion at doses from 10 micrograms per mL, 33 micrograms per mL and 100 micrograms per mL illustrated by the bar graph in Figure 9A. FP-MD also lowered LPS-induced TNF-α levels in a step-wise, dose-dependent manner in doses from 10, 33 and 100 micrograms per mL shown in the bar graph B in Figure 9B. In Figure 1C, FP-MD lowered LPS-induced IL-1β expression levels step-wise at doses ranging from 10, 33 and 100 micrograms per mL As seen in Figure 9D, LPS alone induced the anti-inflammatory cytokine IL-10 in RAW264.7 cells at a dose level of 10 ng. per mL over a two-hour time-period. FP-MD at 10 micrograms per mL for one hour increased IL-10 expression to about half as much as LPS did. However, FP-MD elevated IL-10 expression levels higher than LPS itself at a dose of 33 micrograms per mL. At a 100 ug/mL dose, FP-MD increased IL-10 levels several times higher than LPS alone or FP-MD at the two lower doses as seen in Figure 9D.

Since one function of IL-10 is to downregulate the levels of other pro-inflammatory cytokines, since IL-10 can block NF-kB, AP1 and p38 and is involved in the regulation of the JAK-STAT pathway. IL-10 suppresses CD4 T-cell activation and LPS-induced TNF-α, IL1b, IL-12 and IFN-y from the TLR (Toll Like Receptor) triggered myeloid lineage cells. (72, 73, 74, 75, 76, 77, 78, 79)

NF-kB and MAPK (mitogen-activated protein kinases) are necessary signaling pathways for the up-regulation of cytokine expression in LPS-induced inflammation. To determine whether FP-MD regulates NF-kB and MAPK activation, phosphorylation levels of NF-kB, JNK, p65 and IkB were compared to a DMSO control.

Figure 10.

Seen in Figure 10A, FP-MD strongly reduced LPS phosphorylation of NF-kB p65 and IkB-a versus a DMSO placebo.

In Figure 10B, FP-MD had no effect in phosphorylation levels of ERK, p38 or JNK. This demonstrates that FP-MD specifically inhibits only the NF-kB signaling pathway. ERK, p38 and JNK are MAPK's that are not directly affected by FlexPro MD® in this LPS-induced inflammation model in the RAW264.7 cell line.

The *in vitro* results using RAW264.7 cells further demonstrate the FlexPro MD® can modulate inflammation by lowering the pro-inflammatory cytokines IL-6, TNF-α and IL-1β found to be present at high levels in arthritic joints. These results also demonstrate that FlexPro MD® lowers these pro-inflammatory cytokines through the NF-kB pathway by inhibiting phosphorylation of p65 and IkB-a without affecting the phosphorylation of ERK, p38 and JNK, which are all MAPK's, another major alternative pathway to inflammation. The other *in vitro* benefit of FlexPro MD® is that adding it to the RAW264.7 cells increased IL-10 levels at 33 ug/mL but dramatically elevated IL-10 levels at 100 ug/mL IL-10 is an anti-inflammatory cytokine that inhibits TNF-α, IL-1β and IL-6. (89)

The unique combination of LMW-HA and AST in FlexPro MD® provides several benefits—only LMW-HA restores I kappa B alpha protein levels. The predominant form of NF-kB is a heterodimer composed of p50 and p65 subunits. In unstimulated cells, the NF kappa B heterodimer is kept as an inactive cytoplasmic complex by inhibitory proteins, such as I kappa B alpha and I kappa B beta. I kappa B alpha levels are elevated by LMW-HA, but not by HMW-HA. The induction of I kappa B expression was not observed for other glycosaminoglycans (GAGs) tested. (81, 82)

Lower molecular weight hyaluronic acid has excellent oral bioavailability—LMW-HA fragments bind specifically to connective tissue which maximizes interaction with target synovial fluid-producing cells. LMW-HA fragments may promote site repair by stimulation of the innate immune system, bringing the joint back to normal homeostasis. In skin studies of keratinocytes, LMW-HA increases the self-defense of skin epithelial cells, the keratinocytes, which then produce B-Defensin-2 through TLR activation and this is not accompanied by an inflammatory response—no production of IL-1β, TNF-α, IL-6 or IL-8 was observed. (96)

LMW-HA enhances osteoclast formation and helps restore osteoclast function in RAW264.7 cells. LMW-HA activates the differentiation of RAW264.7 cells into osteoclasts via RANKL in a dose-dependent manner, whereas HMW-HA has almost no effect. RANKL is a member of the tumor necrosis family and triggers osteoclastogenesis by forming a complex with RANK. Bone resorption is a multi-step, dynamic turn-over process that slows in the arthritic joint. (97) Stimulating osteoclast formation and restoring osteo-clast function in the arthritic synovial joint by LMW-HA holds promise that arthritic joint function can be restored. It was earlier reported that HA activates CD44 in bone marrow stromal cells by stimulating RANKL expression. Bone marrow stromal cells have the same lineage as alveolar macrophages. (98) In the study under discussion here, the researchers found that the expression of CD44 function-blocking monoclonal antibody remarkably inhibited the effect of LMW-HA on the signal introduction of c-Src and RANK in RAW264.7 cells. The expression of CD44 in osteoclasts "leads to the localized degradation of HA in the joint cavity, which, in turn, leads to induction of osteoclast formation and activation mediated by LMW-HA in the surrounding tissues." (96)

The AST in FlexPro MD®is a superoxide dismutase (S.O.D.) mimetic. A major hallmark of arthritic synovial fluid is low levels of S.O.D., the enzymatic function of which is to dismutate two superoxide radicals into the far less reactive hydrogen peroxide. AST completely traverses the lipophilic and hydrophilic multi-layer components of cell and organelles membranes to use its biphasic quenching of free radicals regardless of whether radicals are generated or passing through hydrophilic or lipophilic layers. AST enters the mitochondria of chondrocytes. In chondrocytes, increased IL-1β levels stimulates them to shift their production from collagen type I and II to produce and increase MMP-13 via upregulation of NF-kB, while increased TNF-α levels inhibit the P13K/AKT pathway which suppresses proteoglycan/ collagen Extra Cellular Matrix (ECM) products. AST, unlike RA drugs that are immuno-suppressive, lowers TNF-α levels without suppressing the immune system. Increased mitochondrial dysfunction induces JNK signaling pathways which eventually leads to chondrocyte apoptosis. (83, 84, 85, 86, 87, 88)

Discussion of the LPS Mouse Data and the *in vitro* Raw Cell Data vis-à-vis the Human Clinical Trial Data:

These results using oral FlexPro MD® in an LPS-mouse model of joint inflammation help delineate the effects discovered in a 107-subject clinical trial of FlexPro MD®, which demonstrated surprisingly robust and rapid onset observed as early as day 7 in the active 'Group B'. On day 56, the percentage of subjects that were 'pain free' determined by WOMAC and VAS were 63% and 68%, respectively, whereas less than 30% of the subjects in Group A and C were 'pain free'. Group A in this study was treated with a standard glucosamine/ chondroitin product and Group C was given a palm oil placebo.

All the subjects in the FlexPro MD® group had lower anti-inflammatory markers, although the changes were not statistically significant. There was a trend toward a reduction in expression of the pro-inflam-matory genes with none of the genes analyzed showing an increase in expression. 83% (31/38) of subjects

had at least one gene that exhibited a 25% or greater decrease in expression on Day 29 relative to Day 0. 79% of subjects had at least one gene that exhibited a 25% or greater decrease in expression on Day 57 relative to Day 0 levels.)

Table B. Taqman® Assay

GENE	DAY 29	DAY 57
IL-1β	26%	15%
IL-6	34%	23%
NFK-B1	18%	15%
NOS-2a	31%	33%
PTG-S2	24%	26%
TGF-B3	26%	28%
TNF	24%	13%

77% of the subjects who had lower NF-kB whole blood gene expression levels in the FlexPro MD® treated group also had lower whole blood levels of PTGS2 (COX-2). This confirms that the downregulation of NF-kB measured in whole blood is reflected by the lower levels of COX-2, one of the two NF-kB primary biomarkers in a sub-group of the subjects. However, most of the 38 subjects who had lower pain scores or who were 'pain-free' did not show that specific inflammatory biomarker relationship in whole blood, and their pain reductions may be due to other effects, perhaps related to changes in nocioception caused by FlexPro MD®. Furthermore, a modest reduction in two or more pro-inflammatory genes may have an additive or synergistic effect on joint inflammation, whether through nocioception or other mechanisms.

The inhibitory efficacy of FlexPro-MD® in reducing inflammatory cytokines, MMPs and CRP, while raising the anti-inflammatory IL-10 levels in an LPS-induced mouse arthritic joint model further details the mechanisms responsible for FlexPro MD®'s effectiveness for the attenuation of inflammation in arthritis. The LPS-induced inflammatory cytokine inhibition in this mouse model provides more specific details explaining the extraordinary pain relief results reported in the above unpublished double-blinded placebo controlled human clinical trial of FlexPro MD® conducted in subjects suffering from mild to moderate knee pain.

The further elucidation of FlexPro MD®'s mechanisms of action was demonstrated in an *in vitro* study using RAW264.7 mouse macrophage cells, where the exact mechanism of inhibition of iNOS, TNF-α and IL-1β were due to strongly decreasing phosphorylation of NF-kB p65 and IkBx versus a DMSO placebo, while having no effect on phosphorylation levels of ERK, p38 or JNK, all MAPK kinases. This demonstrates *in vitro* that FP-MD specifically inhibits only the NF-kB signaling pathway and not through the MAPK pathway.

Nocioception

Pain and the immune system influence each other in complex ways, making it difficult to determine which is cause and effect. However, lowering pro-inflammatory cytokines clearly lowers severe pain. (90) IL-1β and TNF-α, the first cytokines formed after tissue damage, act directly on receptors on sensorial neurons, leading to a cascade of other cytokines, prostanoids, nitric oxide, chemokines, kinins and ATP. TNF-α and IL-1β also contribute to activating the complement pathway. The complement pathway causes glial cell proliferation and hypertrophy in the Central Nervous System (CNS), causing release of TNF-α, IL-1β and IL-6, resulting in a network of independent activation. (91, 92, 93, 94, 95)

FlexPro MD® has nocioception properties by lowering IL-1β and TNF-α in arthritic synovial joints, while elevating levels of IL-10, which also is known to lower IL-1β and TNF-α as a part of its anti-inflammatory function. The LPS-induced arthritic joint synovial clinical study in mice, described above, demonstrates FlexPro MD®'s ability to lower TNF-α and IL-1β, while elevating the anti-inflammatory cytokine IL-10 in a dose-dependent manner.

The rapid onset of relief (7 days) reported in the human trial described above demonstrates that FlexPro MD® lowers generalized discomfort of the surrounding supportive tissues of the knee and/or hip." By day 56 of that study, the active group treated with FlexPro MD® reported a 55% reduction of discomfort from their baseline levels. On day 56, the percentage of subjects that were 'pain free' determined by WOMAC and VAS were 63% and 68%, respectively, whereas less than 30% of the subjects in Group A treated with a standard dose glucosamine/chondroitin supplement, and Group C, a placebo group, reported pain relief.

A further *in vitro* study delineated the precise mechanism of FlexPro MD®'s modulation of inflammation by lowering the pro-inflammatory cytokines Il-6, TNF-α and IL-1β found to be present at high levels in arthritic joints. These results demonstrate that FlexPro MD® lowers these pro-inflammatory cytokines through the NF-kB pathway by inhibiting phosphorylation of p65 and IkB-a.

Whether FlexPro MD® acts directly on sensorial neurons remains to be determined in future studies.

For more information about FlexPro MD® or Flexuron® Plus, Valensa's proprietary lower molecular weight hyaluronic acid combined with Zanthin® Astaxanthin, please contact moreinfo@valensa.com or call (877) 876-8872.

References:

1. Felson, DT, Lawrence, RC, Dieppe, PA, Hirsch, R, Helmick, CG, Jordan, JM, et al. Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med*. 2000 Oct 17;133(8):635-46.

2. Martin, JA, Buckwalter, J. Aging, articular cartilage chondrocyte senescence and osteoarthritis. *Biogerontology*. 2002;3(5):257–64.

3. Emery P, Williamson D.J., MacKay, I. R. Role of cytokines in rheumatological inflammation. *Concepts of Immunopathology* 1987; 4: 171-9.

4. Gowen M, Wood D. D., Ihrie E. J. McGuire M K B, Russell R G. An interleukin-l like factor stimulates bone resorption in vitro. *Nature* 1983; 306: 378-80.

5. Thomson B M, Mundy G R, Chambers T.J. Tumor necrosis factor a and B induce osteoblastic cells to stimulate osteoclast bone resorption. *J Immunol* 1987; 138: 775-9.

6. Crofford, L. Use of NSAIDs in Treating Patients with Arthritis. *Arthritis Res Ther*. 2013: 15 (Suppl 3): S2.

7. Godwin, M. Intra-articular Steroid Injections for Painful Knees – Systematic Review with Meta-Analysis. *Can Fam Physician* 2004; 50:241-248.

8. Tashiro T, Seino S, Sato T, Matsuoka R, Masuda Y, Fukui N. Oral administration of polymer hyaluronic acid alleviates symptoms of knee osteoarthritis: a double-blind, placebo-controlled study over a 12-month period. *Scientific World Journal*. 2012; 2012:167928.

9. Suzuki, Y, Fukushima, M, Sakuraba, K, Sawaki, K, Sekigawa, K. Krill oil Improves Mild Knee Joint Pain: A Randomized Control Trial. *PLoS One*. 2016; 11(10).

10. Kidd, P.. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Altern Med Rev*. 2011 Dec;16(4):355-64

11. Edouard, P, Rannou, F, Coudeyre, E. Animal evidence for hyaluronic acid efficacy in knee trauma injuries. Review of animal-model studies. *Phys Ther Sport*. 2013 May; 14 (2): 116-23.

12. Krill oil. Monograph. *Altern Med Rev*. 2010 Apr; 15 (1): 84-6.

13. Huang, L, Chen, WP. Astaxanthin ameliorates cartilage damage in experimental osteoarthritis. *Mod Rheumatol*. 2015 Sep; 25 (5): 768-71.

14. Gomez – Aristtizabal, A, Kim, KP, Viswanathan, S. A Systematic Study of the Effect of Different Molecular Weights of Hyaluronic Acid on Mesenchymal Stromal Cell-Mediated Immunomodulation. *PLoS One.* 2016 Jan 28; 11 (1): e0147868.

15. Buddhachat, K, Siendee, p, Chomdei, S, et al. Effects of different omega-3 sources, fish oil, krill oil, and green-lipped mussel against cytokine-mediated canine cartilage degradation. In vitro *Cell Dev Biol Anim*. 2017 May; 53 (5): 448- 457.

16. Schuchardt, JP, Schneider, I. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations comparative bioavailability of fish vs. krill oil. *Lipids Health Dis.* 2011 Aug 22; 10: 1 45.

17. Park DR, Ko, R, Kwon SH, Min, B, Yun, SH, Kim, MH Minatelli J, Hill S, Lee SY. FlexPro MD, a Mixture of Krill Oil, Astaxan-thin, and Hyaluronic Acid Suppresses Lipopolysaccharide-Induced Inflammatory Cytokine Production Through Inhibition of NF-kB. *J Med Food*. 2016 Dec;19(12):1196-1203.

18. Shi WJ, Tjoumakaris FP, Lendner M, Freedman KB. Biologic injections for osteoarthritis and articular cartilage damage: can we modify disease?

19. Cohen, S, van den Bekerom, MP, Mulier, M, Haverkamp, D. Hyaluronic acid in the treatment of knee osteoarthri-tis: a systematic review and meta-analysis with emphasis on the efficacy of different products. *Biodrugs*. 2012 Aug 1;26(4):257-68.

20. Bengtson Nash, SM, Schlabach, M, Nichols, PD. A nutritional-toxicological assessment of Antarctic krill oil versus fish oil dietary supplements. *Nutrients*. 2014 Aug 28;6(9):3382-402.

21. Jergensen, K, Skibsted, LH. Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. *Z Lebensm Unters Forsch*. 1993 May; 196(5): 423-9.

22. Iglseka-Kalwat, J, Gossciriska, J, Nowak, J. Carotenoids as natural antioxidants. *Postepy Hig Med Dosw* (Online) 2015 Apr 7; 69: 418-28.

23. Bagchi, D. Oxygen Free Radical Scavenging Abilities of Vitamin C, E, beta-Carotene, Grape Seed Proanthocyanidins Ex-tract and Astaxanthins In Vitro. Pharmacy Sciences Creighton University School of Health Sciences, June 2001.

24. U.S. Patent 8,481,072. Composition and Method to Alleviate Pain.

25. Parks, W, Wilson, CL, Lopez-Boado, YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature Reviews Immunology* 4, 617-629 (August 2004).

26. Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). American College of Rheumatology.

27. Bellamy N. Outcome measurement in osteoarthritis clinical trials. *J Rheumatol* 1995; 22 Suppl 43: 49–51.

28. Haefell, M, Eifering, A. Pain Assessment. *Eur Spine J.* 2006 Jan; 15: (Suppl 1): S17–S24.

29. Lequesne M., Mery, C. et al. Indexes of severity for osteoarthritis of the hip and knee. Scand. *J Rheu- matology*.1987; Supplement 65: 85-89.

30. Lequesne M.G. The algo-functional indices for hip and knee osteoarthritis. *J Rheumatol*. 1997; 24: 779-781.

31. Viala-Danten, M, Martin, S, Guillemin, I, Hays, R. Evaluation of the reliability and validity of the Medical Outcomes Study sleep scale in patients with painful diabetic peripheral neuropathy during an international clinical trial. *Health Qual Life Outcomes*. 2008; 6: 113.

32. Wolfe F, Michaud K, Li T. Sleep disturbance in patients with rheumatoid arthritis: evaluation by medical outcomes study and visual analog sleep scales. *J Rheumatol*. 2006 Oct; 33 (10): 1942-51.

33. Rejas, J, Ribera, MV, Ruiz, M, Masrramon, X. Psychometric properties of the MOS (Medical Outcomes Study) Sleep Scale in patients with neuropathic pain. *Eur J Pain*. 2007 Apr; 11 (3): 329-40.

34. Sacco, E, Tienforti, D, D'Addressi, A, et al. Social, economic, and health utility considerations in the treatment of overac-tive bladder. Open Access *J Urol*. 2010; 2: 11–24.

35. Garcia-Borreguero, D, Patrick, J, Dubrava, S, Becker, PM, Lankford, A, et al. Pregabalin Versus Pramipexole: Effects on

Sleep Disturbance in Restless Legs Syndrome. *Sleep*. 2014 Apr 1; 37 (4): 635–643.

36. Danten, M, Martin, S, Guillemin, J, Hays, R. Evaluation of the Medical Outcomes Study Sleep Scale in Patients with Painful Diabetic Peripheral Neuropathy. *Health Qual. Life Outcomes*. 2008; 6: 11.

37. A Double-Blind Placebo Controlled, Human Clinical Study to Evaluate the Safety and Efficacy of a Proprietary "Joint Health" Dietary Supplement in Subjects with Non-Arthritic Knee Joint and Connective Tissue Pain and Function.

38. Brosseau L, Balmer S, Tousignant M, O'Sullivan JP, Goudreault C, Goudreault M, et al. Intra- and intertester reliability and criterion validity of the parallelogram and universal goniometers for measuring maximum active knee flexion and extension of patients with knee restrictions. *Arch Phys Med Rehabil* 2001; 82: 396-402.

39. Watkins MA, Riddle DL, Lamb RL, Personius WJ: Reliability of goniometric measurements and visual estimates of knee range of motion obtained in a clinical setting. *Physical Therapy* 1991, 71: 90-6.

40. Rothstein J, Miller P, Roettger R. Goniometric reliability in a clinical setting. Elbow and knee measurements. *Phys Ther* 1983; 63: 1611-5.

41. Johnson, KN, Potter, AJ, Phillips, A. Measurement Properties of Short Lower Extremity Functional Exercise Tests in People with Chronic Obstructive Pulmonary Disease: Systematic Review. *Phys. Ther*. 2017 Jun 12.

42. Dobson, F, Hinman, RS, Hall, M, Marshall, CJ, Sayer, T. Reliability, and measurement error of the Osteoarthritis Research Society International (OARSI) recommended performance-based tests of physical function in people with hip and knee osteoarthritis. *Osteoarthritis Cartilage*. 2017 Jun 22. S1063-4584 (17) 31053-1.

43. Van der Giessen LJ, Liekens D, Rutgers KJM, Hartman A, Mulder PGH, Oranje AP. Validation of Beighton score and prevalence of connective tissue signs in 773 Dutch-Caucasian Children. *J Rheumatol* 2001; 28: 2726-30.

44. Beighton P, Solomon L, Soskolne CL. Articular mobility in an African population. *Ann rheum dis*. 1973; 13 (5): 413–418.

45. Beighton P, Horan F. Orthopaedic aspects of the Ehlers-Danlos syndrome. *J bone joint surg*. 1969; 13 (3): 444–453.

46. Fleiss J. The design and analysis of clinical experiments. New York: John Wiley and Sons; 1986. Reli- ability of measurement; pp. 1–32.

47. Remvig, L, Jensen, DV, Ward, RC. Are diagnostic criteria for general joint hypermobility and benign joint hypermobility syndrome based on reproducible and valid tests? A review of the literature. *J Rheumatol.* 2007 Apr; 34 (4): 798-803.

48. Salaffi, F, Lardini, G, Canesi, B, Mannoni, A. et al. Reliability and validity of the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index in Italian patients with osteoarthritis of the knee. *Osteoarthritis and Cartilage*, vol 11, Issue 8, August 2003: 551-560.

49. McConnell S, Kolopack P, Davis AM. The Western Ontario and Mc-Master Universities Osteoarthritis Index (WOMAC): a review of its utility and measurement properties. *Arthritis Rheum*. 2001; 45: 453–61.

50. Boonstran AM, Schiphorst-Preuper, HR, Reneman, MF, et al. Reliability and validity of the visual analogue scale for disability in patients with chronic musculoskeletal pain. *Int J Rehabil* Res. 2008 Jun; 31 (2): 165-9.

51. McCormack HM, Horne DJ, Sheather S. Clinical applications of visual analogue scales: a critical re- view. *Psychol. Med*. 1988; 18: 1007–19.

52. Bellamy, N, Campbell, J, Stevens, J, Pilch, L. Validation study of a computerized version of the Western Ontario and Mc-Master Universities VA3.0 Osteoarthritis Index. *J Rheumatol*. 1997 Dec; 24 (12): 2413-5.

53. Joyce CR, Zutshi DW, Hrubes VF, Mason RM. Comparison of fixed interval and visual analogue scales for rating chronic pain. *Eur J Clin Pharmacol*. 1975; 8: 415–20.

54. Cicero AF, Rosticci M, Morbini M, Cagnati M, Grandi E, Parini A, Borghi C. Lipid-lowering and an- ti-inflammatory effects of omega 3 ethyl esters and krill oil: a randomized, cross-over, clinical trial. *Arch Med Sci*. 2016 Jun 1; 12 (3): 507-12.

55. Berge RK, Ramsvik MS, Bohov P, Svardal A, Nordrehaug JE, Rostrup E, Bruheim I, Bjørndal B. Krill oil reduces plasma triacylglycerol level and improves related lipoprotein particle concentration, fatty acid composition and redox status in healthy young adults - a pilot study. *Lipids Health Dis*. 2015 Dec 15; 14: 163.

56. Shaheen, Z, Corbett, J. Macrophage Expression of Inflammatory Genes in Response to EMCV Infection. *Biomolecules* 2015, 5, 1938-1954.

57. Serbina, N, Salazar-Mather, TP, Biron, CA, Kuziel, WA. TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. 2015, Vol 1; 1; 59-70.

58. Sokolove, J, Lepus, C. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis*. 2013 Apr; 5 (2): 77–94.

59. Abramson, S. Nitric oxide in inflammation and pain associated with osteoarthritis. *Arthritis Res Ther*. 2008; 10 (Suppl 2): S2.

60. Fan, Y., Wang, Y., Han, C., Pan, Y., Huang, H., Ye, Y., Luo, L., Yin, Z. Dipyrithione inhibits lipopolysaccharide-induced iNOS and COX-2 up-regulation in macrophages and protects against endotoxic shock in mice. *FEBS Lett*. 2008, 582: 1643-1650.

61. Abu-Ghefreh, AA, Masocha, W. Enhancement of anti-nociception by co-administration of minocycline and a non-steroidal anti-inflammatory drug indomethacin in naïve mice and murine models of LPS-induced thermal hyperalgesia and monoarthritis. BMC *Musculoskelet Disord*. 2010 Dec 1; 11: 276.

62. Nakayama, M, Niki, Y, Kawasaki, T, Takeda, Y, Horiuchi, K, Sasaki, A. Enhanced susceptibility to lipo- polysaccharide-induced arthritis and endotoxin shock in interleukin-32 alpha transgenic mice through induction of tumor necrosis factor alpha. *Arthritis Res Ther*. 2012 May 21; 14 (3): R120.

63. Chen, WT, Mahmood, U, Weisleder, R, Tung, CH. Arthritis imaging using a near-infrared fluorescence folate-targeted probe. *Arthritis Res Ther*. 2005; 7 (2): R310-7.

64. Venkatesha, S.H. et al. Cytokine-Modulating Strategies and Newer Cytokine Targets for Arthritis Therapy. *Int J Mol Sci*. 2014 Dec 31; 16 (1): 887-906.

65. Moita, E, Gil-Izquierdo, A, Sousa, C, et al. Integrated Analysis of COX-2 and iNOS Derived Inflammatory Mediators in LPS-Stimulated RAW Macrophages Pre-Exposed to *Echium plantagineum* L. Bee Pollen Extract. *PloS One.* 2013; 8 (3): e59131.

66. Zamani B, Jamali R, Ehteram H. Synovial fluid adenosine deaminase and high-sensitivity C-reactive protein activity in differentiating monoarthritis. *Rheumatol Int*. 2012 Jan; 32 (1): 183-8.

67. Parvizi, J., Jacovides, C., Adeli, B., Jung, K.A., Hozack, W.J. Mark B. Coventry Award: synovial C-reactive protein: a prospective evaluation of a molecular marker for periprosthetic knee joint infection. *Clin Orthop Relat Res*. 2012 470: 54-

68. Zamani B, Jamali R, Ehteram H. Synovial fluid adenosine deaminase and high-sensitivity C-reactive protein activity in differentiating monoarthritis. *Rheumatol Int*. 2012 Jan; 32 (1): 183-8.

69. [http://www.signaling-gateway.org/aboutus/CellPrepLab.html]

70. Ward JM, Erexson CR, Faucette LJ, Foley JF, Diikstra C, Cattoretti G: Immunohistochemical markers for the rodent immune system. *Toxicol Pathol*. 2006, 34: 616-630.

71. Hartley, J, Evans, L, Green, K Naghasfar, Z. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology* 2008; 5:1.

72. Saraiva M, O'Garra A, et al. The regulation of IL-10 production by immune cells". Nature Reviews. *Immunology*. Mar 2010 10 (3): 170–81.

73. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. *The Journal of Experimental Medicine.* Nov 1991: 174 (5): 1209–20.

74. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. *J. Experimental Medicine*. Oct 1991:174 (4): 915–24.

75. Akdis CA, Joss A, Akdis M, Faith A, Blaser K. A molecular basis for T cell suppression by IL-10: CD28-associated IL-10 receptor inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding". *FASEB Journal*. 2000: 14 (12): 1666–8.

76. Joss A, Akdis M, Faith A, Blaser K, Akdis CA. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway". *European Journal of Immunology*. Jun 2000: 30 (6): 1683–90.

77. Varma TK, Toliver-Kinsky TE, Lin CY, Koutrouvelis AP, Nichols JE, Sherwood ER. Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice. *Infection and Immunity*. Sep 2001: 69 (9): 5249–63.

78. Opp, MR, Smith EM, Hughes TK. Interleukin-10 (cytokine synthesis inhibitory factor) acts in the central nervous system of rats to reduce sleep. *Journal of Neuroimmunology*. July 1995: 60 (1–2): 165–8.

79. Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *Journal of Immunology*. 1998: 160 (12): 5936–44.

80. Clark, AG, Jordan, J.M., Vilim, V., Renner, J.B., Dragomir, A.D., Luta, G. & Kraus, V.B. (1999) Serum cartilage oligomeric protein reflects osteoarthritis presence and severity. *Arthritis and Rheumatism* Vol. 42, No. 11 (November 1999), pp. 2356-2364.

81. Noble, PW, McKee, CM, Cowman, M. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *J Exper Med*. vol. 183: (5): 2373-2378, 1996.

82. Nathan, C., C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. Wright. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/ CD18 integrins. *J. Cell Biol*. 109: 1341-1349.

83. Ambati, R.R. et al., Astaxanthin: Sources, Extraction, Stability, Biological Activities, and Its Commercial Applications. A Review. *Marine Drugs* 12:128-152, 2014.

84. Chen, W, Xiong, Y, Shi, PF, Bao, JP, Wu, LD. Astaxanthin reduces matrix metalloproteinase expression in human chondrocytes. *Internat Immunopharmacology*. Mar 2014, 19: (1): 174-177.

85. Pei, LP, Dong, FH, Hui, B. Effects of Astaxanthin on the damage of osteoblast induced by H2O2. Zhongguo, Gu Shang. 2008 Mar;21(3):187-9.

86. Huang LJ, Chen WP. Astaxanthin ameliorates cartilage damage in experimental osteoarthritis. *Mod Rheumatol*. 2015 Sep;25(5):768-71.

87. Bikadi, Z, Hazai, E, Zsila, F, Lockwood, SF. Molecular modeling of non-covalent binding of homochiral (3S,3'S)-astaxanthin to matrix metalloproteinase-13 (MMP-13). *Bioorg Med Chem*. 2006 Aug 15;14(16):5451-8.

88. Lee, DH, Kim, CS, Lee, YJ. Astaxanthin protects against MPTP/MPP+-induced mitochondrial dysfunction and ROS production in vivo and in vitro. *Food Chem Toxicol*. 2011 Jan;49(1):271-80.

89. de Oliveria, C, Sakata, R, Issy, AM, Gerola, LR, et al. Cytokines and Pain. *Rev Bras Anestesiol*. 2011; 61: 2: 255-265.

90. Watkins, LR, Maier, SF. Beyond Neurons: Evidence that Immune and Glial Cells Contribute to Pathological Pain States. *Pysiol. Rev*. 2002; 82: 981-1011.

91. Zhang, JM, An, J. Cytokines, Inflammation, and Pain. *Int Anesthesiol Clin*. 200; 45: 27-37.

92. Obata, H, Eisenach, JC, Hussain, H, et al. Spinal Glial Activation Contributes to Postoperative Mechanical Sensitivity in the Rat. *J Pain*. 2006; 7: 816-832.

93. Miller, RJ, Jung, H, Bhangoo, SK, White, FA. Cytokine and chemokine regulation of sensory neuron function. *Handb Exp Pharmacol*. 2009; (194): 417-49.

94. Sommer, C, White, F. Cytokines, Chemokines, and Pain. 1st Ed. Seattle, *IASP Press*, 2010; 279-302.

95. Hudmon, A, Choi, JS, Tyrrell, L. et al. Phosphorylation of Sodium Channel Nav1.8 by p38 Mitogen Activated Protein Kinase Increases Current Density in Dorsal Root Ganglion Neurons. *J Neurosci*. March 19, 2008; 28 (12): 3190 –3201.

96. Gotoh et.al. Effects of molecular weight of hyaluronic acid and its action mechanisms on experimental joint pain in rats. *Annuals of Rheumatic Diseases* 1993; 52: 817-822.

97. Maharjan et.al. High and lower molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation *PloS ONE* 6:(1-10) doi: 10.1371/journal.pone.0026078.

98. Gariboldi, S, Palazzo, M, Zanobbio, L, et al. Lower Molecular Weight Hyaluronic Acid Increases the Self-defense of Skin Epithelium by Induction of B-Defensin 2 via TLR2 and TLR4. *J. Immunol*. 2008, 181: 2103-2110.

99. Ariyoshi W, Takahashi T, Kanno T, Ichimiya H, Takano H, Koseki T, Nishihara T. Mechanisms involved in enhancement of osteoclast formation and function by lower molecular Weight hyaluronic acid. *J Biol Chem*. 2005 May 13;280(19):18967-72.

100. Cao, JJ, Singleton, PA, Majundar, S, Boudignon, B, et al. Hyaluronan increases RANKL expression in bone marrow stromal cells through CD44. 2005. *J Bone Miner Res*; 20: 30-46.