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REVIEW ARTICLE

On the Mechanisms of Action of the Low Molecular Weight Fraction of Commercial Human Serum Albumin in Osteoarthritis

David Bar-Or[1-7,*], Gregory Thomas[1-7], Leonard T. Rael[1-6], Elizabeth Frederick[1-7], Melissa Hausburg[1-6], Raphael Bar-Or[1-7] and Edward Brody[8]

[1]*Trauma Research Department, Swedish Medical Center, 501 E. Hampden Avenue, Englewood, CO 80113, USA;* [2]*Trauma Research Department, St. Anthony Hospital, 1600 W. 2nd Place, Lakewood, CO 80228, USA;* [3]*Trauma Research Department, Medical City Plano, 3901 W. 15th Street, Plano, TX 75075, USA;* [4]*Trauma Research Department, Penrose Hospital, 2222 N. Nevada Avenue, Colorado Springs, CO 80907, USA;* [5]*Trauma Research Department, Research Medical Center, 2315 E. Meyer Boulevard, Kansas City, MO 64132, USA;* [6]*Trauma Research Department, Wesley Medical Center, 550 N. Hillside Street, Witchita, KS 67214, USA;* [7]*Ampio Pharmaceuticals, Inc., 373 Inverness Parkway, #200, Englewood, CO 80112, USA ;* [8]*SomaLogic, Inc., 2945 Wilderness Place, Boulder, CO 80301, USA*

Abstract: The low molecular weight fraction of commercial human serum albumin (LMWF5A) has been shown to successfully relieve pain and inflammation in severe osteoarthritis of the knee (OAK). LMWF5A contains at least three active components that could account for these anti-inflammatory and analgesic effects.

We summarize *in vitro* experiments in bone marrow–derived mesenchymal stem cells, monocytic cell lines, chondrocytes, peripheral blood mononuclear cells, fibroblast-like synoviocytes, and endothelial cells on the biochemistry of anti-inflammatory changes induced by LMWF5A. We then look at four of the major pathways that cut across cell-type considerations to examine which biochemical reactions are affected by mTOR, COX-2, CD36, and AhR pathways. All three components show anti-inflammatory activities in at least some of the cell types.

The *in vitro* experiments show that the effects of LMWF5A in chondrocytes and bone marrow–derived stem cells in particular, coupled with recent data from previous clinical trials of single and multiple injections of LMWF5A into OAK patients demonstrated improvements in pain, function, and Patient Global Assessment (PGA), as well as high responder rates that could be attributed to the multiple mechanism of action (MOA) pathways are summarized here. *In vitro* and *in vivo* data are highly suggestive of LMWF5A being a disease-modifying drug for OAK.

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1. INTRODUCTION

Very often, trial and error, together with clinical observations, lead to the development of novel therapeutic agents. This is certainly true in traditional medicine in both Western culture and China. For example, chewing willow bark was known to reduce fever and inflammation at least as long ago as the era of Hippocrates of Cos, around 400 B.C. Today, of course, we know that this effect is due in part to the high concentration of salicins in the bark and now use purified aspirin. However, in addition to salicins, willow bark also contains polyphenols and flavonoids, which have anti-inflammatory properties. Thus, the natural product probably has a much more complex mechanism of action than aspirin alone and is potentially more effective.

*Address correspondence to this author at the Trauma Research Department, Swedish Medical Center, 501 E. Hampden Avenue, Suite 4-454, Englewood, CO 80113, USA; Tel: 303-788-4089; Fax: 303-788-4064; E-mail: dbaror@ampiopharma.com

Treating diseases with therapeutics having more than one active ingredient is both old, as described above, and modern. Combinations of therapeutic agents are increasingly tested and developed by pharmaceutical companies, especially in diseases such as cancer, where rapidly growing cancer cells with high mutation rates escape the effects of individual compounds [1]. In this review, we propose a multifaceted mode of action for a novel therapeutic agent containing at least three active ingredients and show how each component contributes to the overall anti-inflammatory and analgesic effects of the biological product, a low molecular weight fraction of commercial human serum albumin referred to as LMWF5A.

Osteoarthritis is a degenerative and inflammatory condition of the joint affecting a very large human population and is manifested by, among other symptoms, progressive loss of articular cartilage. Osteoarthritis of the Knee (OAK) specifically is characterized by the progressive breakdown of ar-

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ticular cartilage and inflammation. The resulting microenvironment and catabolic events overcome the ability of mesenchymal stem cells near articulations to differentiate into chondrocytes which repair cartilage. Recent clinical trials have explored and demonstrated beneficial effects of LMWF5A on patients with OAK [2-5], particularly those with the most severe grade OAK.

In this review, we describe the various biochemical pathways involving the known components of LMWF5A on multiple cell types and their relevance to the observed clinical effects in patients with severe OAK.

2. HUMAN SERUM ALBUMIN

Human Serum Albumin (HSA) is the most abundant protein in human plasma and serum, with a normal concentration of ~700 micromolar (μM) and a molecular weight of 66 kDa. The molecule exhibits remarkable stability: the result of 17 disulfide linkages as well as pockets of tight binding regions for an array of low molecular weight compounds for which it serves as a carrier, such as long chain fatty acids, bilirubin, and a variety of low molecular weight drugs [6]. HSA has a long clinical history dating at least as far back as 1944, when World War II stimulated research into blood fractionation with the aim of producing a blood substitute that could be available for wounded soldiers on the battlefield [7]. Although primarily administered to restore blood volume in critically ill patients, HSA has seen fluctuations in use over the years [8] and is also recommended for ameliorating outcomes in critically ill patients, especially those with septic shock, Systemic Inflammatory Response Syndrome (SIRS), burns, and severe trauma [9].

3. FORMULATION AND COMPONENTS OF LMWF5A

Clinical observations on the apparent anti-inflammatory properties of commercial HSA led to a series of scientific investigations exploring these properties, which resulted in the therapeutic agent referred to here as LMWF5A, the low molecular weight fraction of commercial HSA. To understand the active components of LMWF5A, it is important to review how the commercial HSA solution is produced. A ~96% pure fraction of HSA is obtained from pooled human plasma using a cold ethanol fractionation procedure introduced in the 1940s. Because human plasma can carry viruses, the HSA fraction is pasteurized by heating to 60 °C for 10-11 hours. Prior to pasteurization, two important additions are made to the 5% HSA fraction: 1) Sodium caprylate, also known as sodium octanoate, and 2) N-acetyl-DL-tryptophan, both to 4 mM. Sodium caprylate is known to bind strongly to HSA at multiple sites and stabilizes it from heat denaturation [10, 11]. N-acetyl-DL-tryptophan protects HSA protein from oxidative damage and is especially useful in keeping the important cysteine-34 residue in a reduced state during the 60 °C pasteurization [10]. Of note, 50-60 °C is the optimal temperature for an important protein component of commercial HSA solution, dipeptidyl peptidase 4 (DPP-4), which can cleave the two terminal amino acids, aspartate and alanine, from HSA. The resulting dipeptide can be further cyclized to form aspartyl alanyl diketopiperazine (DA-DKP), thus the pasteurization process stimulates the production of DA-DKP from HSA [12]. For reference, DA-DKP concentrations in commercial human 5% serum albumin solutions can range between 50-200 μM.

To produce LMWF5A, pasteurized 5% HSA solution is subjected to tangential flow filtration through Polyvinylidene Difluoride (PVDF) membranes with a 5000-Da molecular weight cutoff, resulting in a low molecular weight solution containing at least 20 different components visualized by mass spectrometry. Three components of LMWF5A that are known to have bioactivity and are reviewed here are DA-DKP, sodium caprylate (octanoate), and N-acetyl-DL-tryptophan.

4. EFFECT OF LMWF5A ON SPECIFIC CELL TYPES (FIG. 1)

4.1. Use of Saline as a Control Agent

All experiments summarized in this review on the effects and mechanisms of action of LMWF5A used normal physio-

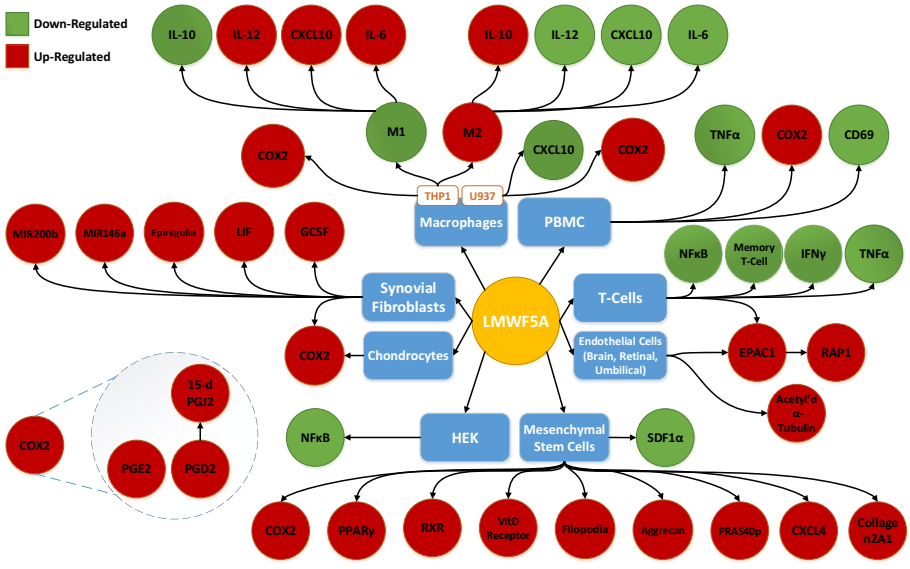

Fig. (1). Effects of LMWF5A on various cell lines. PBMC, peripheral blood monocytic cell; HEK, human embryonic kidney cell.

logical saline as a control. This is of great significance because most of the clinical trials that have been carried out on the therapeutic effect of LMWF5A in OAK contain a control arm of physiological saline [2]. Moreover, when analgesia in the knee after intra-articular injection is the only criterion on which the clinical results are measured, saline does have some effect. This effect is well known, and we [13] and others [14] have argued that saline is thus a poor control arm for such studies. Therefore, it is important to point out that the mechanisms described here were determined from *in vitro* studies of LMWF5A in which saline was appropriately controlled and cannot be attributable to the analgesic effects of saline observed in the clinic. Furthermore, the described biochemical reactions are elicited by unique drug components and are independent of the formulation buffer. This may help explain why the effect of LMWF5A was markedly better than that of saline when function and quality of life, as well as reduction of pain, were the trial endpoints considered in the most severe cases of OAK [2, 3].

4.2. Bone Marrow-derived Mesenchymal Stem Cells

Bone Marrow-derived Mesenchymal Stem Cells (BMMSCs) could provide a source of progenitor cells for chondrocytes and osteocytes in regenerating cartilage and bone in OA joints. As a result, studies were performed to establish if LMWF5A can affect BMMSC *in vitro* function. One of the hallmarks of LMWF5A treatment of BMMSCs is rapid cytoskeletal alterations, including but not limited to the development of filopodia, concomitant with distinct changes in cellular shape. In addition, , LMWF5A has been shown to increase the rate of BMMSC condensation in chondrogenic culture. These findings could be explained by an observed reduction in activated RhoA GTPase in BMMSCs immediately following treatment with LMWF5A. It has been well documented that F-actin organization is intimately associated with small GTPase activity and plays a pivotal role in stem cell differentiation. RhoA helps dictate cell shape and the monomeric status of cellular actin, both of which help drive chondrogenic differentiation through the transcription factor SOX9. Moreover, BMMSCs undergoing chondrogenesis change shape and exhibit decreased RhoA activity. Conversely, when RhoA is overproduced, chondrogenesis is diminished, as reflected in the loss of expression of SOX9 [15].

LMWF5A also affects a variety of mRNAs associated with chondrocyte differentiation and mobilization in the cells. For instance, during TGF-β-induced chondrogenic differentiation of BMMSCs, the mRNA levels of alpha-1 type II collagen, which is cartilage-specific, are increased ~40-fold over the levels in non-differentiating stem cell controls. Adding LMWF5A to the differentiation medium results in a further 4.3-fold increase in alpha-1 type II collagen transcription. LMWF5A also changes the ratio of CXCL12 (also known as SDF-1alpha) mRNA to CXCL12 receptor (also known as CXCR4) mRNA in BMMSCs when grown in 3D culture. Because this pair is known to be involved in attracting stem cells to arthritic lesions and inhibiting breakdown of arthritic tissue, it can be argued that LMWF5A will assist in this process in arthritic tissue as well [16]. In addition, LMWF5A stimulated the binding of 52 known transcription factors to their cognate DNA sequence binding sites at least 2-fold, five of which were stimulated more than 10-fold and AP-2 was stimulated more than 80-fold. Phosphorylation of the PRAS40 transcription factor at threonine-246 was stimulated more than 2-fold after LMWF5A treatment. Phosphorylation at this residue releases mTORC1from the inhibitory constraints of PRAS40, effectively increasing mRNA transcription through ribosomal protein S6 kinase [17]. This result is also significant because increased mTORC1 complex activity promotes stem cell survival, allowing them to grow and differentiate [18].

BMMSCs have been used as a treatment for OAK, and preliminary results have been encouraging but not conclusive [19]. Based on these findings, we hypothesized that intra-articular injection of LMWF5A could help mobilize BMMSCs into the joint, as well as increase the chondrogenic potential of resident and arriving cells.

4.3. Monocytic Cell Lines

Pathogen-Associated Molecular Patterns (PAMPs) are well-studied responses to infectious agents that trigger both the innate and adaptive immune systems. In an analogous manner, trauma, SIRS, and degenerative diseases such as OA lead to the release of Damage-Associated Molecular Patterns (DAMPs) from necrotic and dying cells. Although the DAMP response shows initial differences from the PAMP response, the responses to PAMPs and DAMPs quickly converge and equally stimulate both the innate and adaptive immune systems. Monocyte mobilization and maturation into macrophages is an early response to the signals arriving from damaged tissue (reviewed in [20]). Monocytes can give rise to two types of macrophages: pro-inflammatory and healing/anti-inflammatory [20, 21]. As a result, the polarization status of macrophages is thought to help orchestrate the course of the inflammatory response as well as the subsequent repair activities after tissue injury.

Because it is present in large concentrations (4 mM) in 5% commercial HSA solutions, an obvious active ingredient candidate in LMWF5A is NAT. This hypothesis is based on the documented biological activities of metabolites of the amino acid tryptophan. The oxidation of tryptophan is a complex metabolic pathway that results in the production of kynurenine (KYN) and associated biologically active molecules [22]. It has been suggested that tryptophan metabolites act *via* the Aryl hydrocarbon Receptor (AhR), which upon activation, increases the ratio between regulatory T cells (Treg) and T helper 17 cells (Th17), thereby modulating the immune response [23]. Interestingly, the tryptophan metabolites kynurenine and kynurenic acid are potent AhR agonists [24]. This interaction between kynurenine-like molecules and AhR is capable of generating immunosuppressive T cells [25]. Finally, various studies have suggested that AhR activation regulates immune responses via interaction with key components of nuclear factor-κB (NF-κB), an important promoter of inflammation [26, 27].

Because macrophages have a moderate to high expression level of AhR [28], we developed a cell culture model to study the anti-inflammatory effect of LMWF5A and NAT metabolic products on macrophages. Human monocytic THP-1 cells were differentiated with phorbol 12 myristate 13 acetate (PMA), which has been shown to induce all the char-

acteristics of macrophages [29], then stimulated overnight with Lipopolysaccharide (LPS) in the presence of LMWF5A. In this cell culture model, LMWF5A treatment induced large decreases in the pro-inflammatory cytokines IL-6, IL-12, and CXCL-10 and increased the production of IL-10 when LPS was included [30]. To further elucidate potential mechanisms of action, differentiated THP-1 cells were pre-treated with 2-fold dilutions of LMWF5A or a synthetic NAT metabolite (N-acetyl kynurenine, NAK) with or without an AhR antagonist (CH223191) prior toLPS stimulation. Treatment with LMWF5A caused a 50-70% decrease in IL-6 release throughout the dilution series. A dose-response inhibition of IL-6 release was observed for NAK, with maximal inhibition (50%) seen at the highest NAK concentration. Finally, an AhR antagonist partially blocked the anti-inflammatory effect of LMWF5A while completely blocking the effect of NAK. A similar inhibitory effect was observed for CXCL-10, but the AhR antagonist was not effective suggesting additional mechanisms for CXCL-10 release. These findings suggest that LMWF5A and NAK partially promote the suppression of activated macrophages via the AhR receptor.

These findings were substantiated using another monocytic cell line, U937. PMA-primed U937 cells exhibited dose-dependent reductions in LPS-induced IL-6 release (up to 80% inhibitions observed) when treated with LMWF5A. As with THP-1 cells, this response was AhR-dependent. Conversely, 30-40% increases in LPS-induced IL-10 release are observed in the presence of LMWF5A with these cells (unpublished data).

This is an important finding because LMWF5A caused a significant decrease in the release of pro-inflammatory biomarkers associated with the activation of the M1 macrophage phenotype with a concomitant significant increase in IL-10 release, a marker of M2 macrophage activation. These *in vitro* results suggest that these properties of LMWF5A are partially due to the inhibition of M1 macrophages and potential activation of an anti-inflammatory macrophage phenotype (M2) leading to pain relief, wound healing, and tissue remodeling.

4.4. Chondrocytes

OAK is characterized by the progressive breakdown of articular cartilage, and chondrocytes are the major cellular component of cartilage [31]. Residing within OAK cartilage, two dysfunctional chondrocyte phenotypes observed are 1) chondrocytes undergoing apoptosis, which contributes to the hypocellularity of OA cartilage and its inability to regenerate, and 2) fibroblast-like chondrocytes (OA-FLCs), which no longer express structural proteins required for cartilage maintenance and function [32-34]. One of the structural proteins required for cartilage function is collagen type II, the expression of which is progressively lost, and the cells dedifferentiate into OA-FLCs displaying fibroblast characteristics within OA cartilage [32, 34]. Interestingly, primary human chondrocytes when grown in monolayer culture begin to lose collagen type II expression, becoming more fibrotic, similar to the OA-FLCs observed in OA cartilage. Remarkably, *in vitro* derived OA-FLCs can revert to their normal phenotype when grown in solid media such as 0.5% low melting temperature agarose gels [35]. For our experiments, we derived *in vitro* OA-FLCs by passaging primary human OA chondrocytes 9-13 times, during which they lost expression of chondrogenic genes, thus becoming OA-FLC cells. As mentioned above, we have previously shown that in human mesenchymal stem cells grown in chondrogenic differentiation conditions, LMWF5A enhances expression of the collagen type II α 1 chain (*Col2a1*) gene [16], which encodes collagen type II [36]. The promoter of *Col2a1* is directly regulated by the transcription factor SOX9 (SRY-box 9). Along with other functions, SOX9 is required for chondrogenesis during embryonic development, is maintained in adult articular chondrocytes, and promotes chondrocyte cell survival [37]. We have shown that LMWF5A upregulates SOX9 expression, re-differentiating OA-FLCs into functional chondrocytes, resulting in increased collagen type II expression and resistance to apoptosis [38].

4.5. Peripheral Blood Mononuclear Cells

Some of the earliest experiments on the mechanisms of action of LMWF5A and its known active components were performed on Peripheral Blood Mononuclear Cells (PBMCs). PBMCs are a quickly purified fraction of whole blood containing primarily T-cell lymphocytes ($CD3^+$), B-cell lymphocytes ($CD20^+$), monocytes ($CD14^+$), and natural killer (NK) cells ($CD56^+$). PBMCs, as well as T-cell lines growing in culture, can be stimulated through the T-cell complex to secrete the inflammatory cytokines interferon-gamma and TNF-alpha. LMWF5A significantly inhibits the secretion of these two cytokines from PBMCs, and this inhibitory property has been shown to partially reside in the DA-DKP component of LMWF5A [39]. In a further study of this phenomenon, DA-DKP was demonstrated to increase the levels of the GTPase Rap1 in these cells, which in turn decreases the phosphorylation of the transcription factors ATF-2 and c-Jun and the subsequent production of interferon-gamma and TNF-alpha [40]. More recently, these experiments were extended to show that LMWF5A containing a similar concentration of DA-DKP to that used above increases the production of the anti-inflammatory cytokine IL-10 while inhibiting pro-inflammatory cytokines. Similar results were observed when a cloned T-cell line was used instead of fresh PBMCs.

Further recent probing of this system led to another exciting discovery regarding the anti-inflammatory properties of LMWF5A in PBMCs. One of the earliest responses to stimulation of the innate immune system is the activation of phospholipase A2 (PLA2), which cleaves cellular membranes and liberates arachidonic acid. PLA2 also stimulates synthesis of cyclooxygenases-1 and -2 (COX-1 and COX-2). COX-2 catalyzes the transformation of arachidonic acid into prostaglandin H_2. Prostaglandin H_2 is the precursor to prostaglandin E_2 and, in a complex series of steps, the anti-inflammatory prostaglandins J_2, D2, and 15d-PGJ2 (Fig. **2**).

During induced inflammatory responses, COX-2–dependent synthesis of prostaglandin E_2 takes place immediately and then diminishes by 48 hours after the initial insult. A switch then occurs as the activity of the enzyme catalyzing PGE_2 synthesis, microsomal prostaglandin E synthase (mPGES-1), is replaced by the activity of PGD synthase,

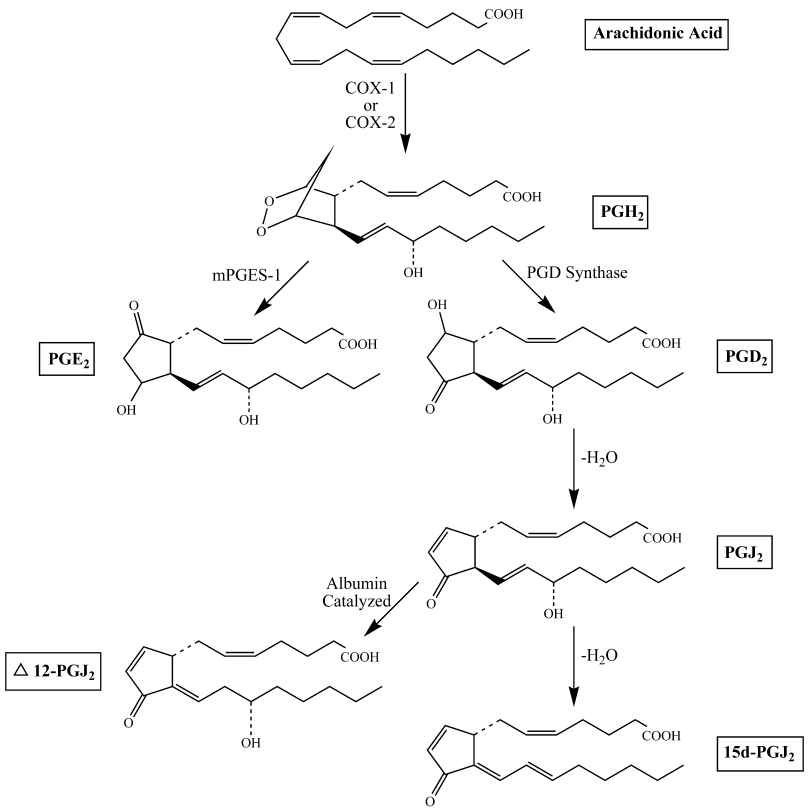

Fig. (2). Prostaglandin synthesis pathways. Following the activation of PLA2 by pro-inflammatory cytokines, arachidonic acid is liberated from cellular membranes. COX-1 and COX-2 convert arachidonic acid to the unstable prostaglandin PGH_2. PGH_2 can be converted to the pro-inflammatory prostaglandin PGE_2 by mPGES-1 or converted to PGD_2, a prostaglandin with well-known anti-inflammatory effects. These effects are mostly due to further non-enzymatic conversions of PGD_2 to PGJ_2 and then 15d-PGJ_2. Intriguingly, PGJ_2 can also convert to Δ12-PGJ_2 in the presence of albumin.

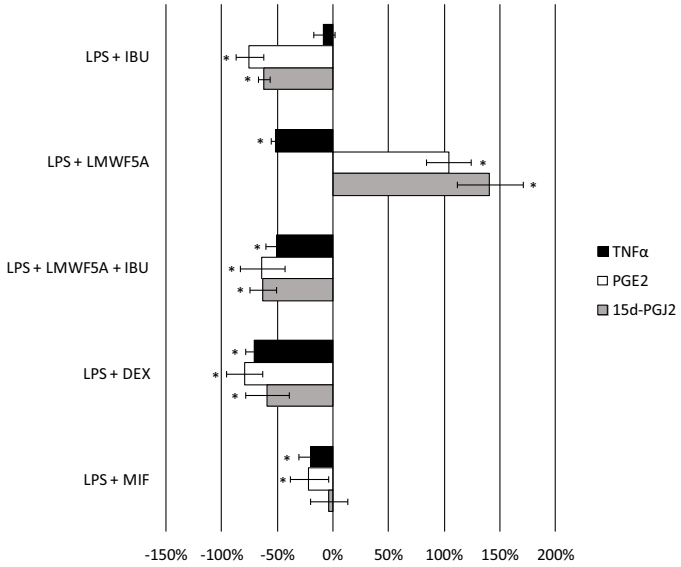

Fig. (3). Combined release data for TNFα, PGE_2, and 15d-PGJ_2 are presented as mean ± SD after repeating the experiment on 3 different occasions. * = $p < 0.05$ versus saline control. LPS, lipopolysaccharide; IBU, ibuprofen; DEX, dexamethasone; MIF, mifepristone.

which (still in a COX-2–dependent fashion) catalyzes the synthesis of PGD_2, which in turn is catalyzed into the anti-inflammatory prostaglandin 15d-PGJ_2 and others (for a review, see [41]). This same pathway has been shown to exist in chondrocytes and synoviocytes from OA patients [42-44]. LMWF5A was demonstrated to act differently on the COX2 and prostaglandin pathway than Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) like ibuprofen and the steroid dexamethasone in LPS-treated PBMCs. Each of these anti-inflammatory agents showed a distinct pattern of inhibitory and stimulatory behavior with respect to TNF-alpha, COX-2, and the tested prostaglandins, but the most striking result was that only LMWF5A stimulated the release of the anti-inflammatory prostaglandin 15d-PGJ_2 [45] (Fig. **3**).

4.6. Fibroblast-Like Synoviocytes

Further validation of this mechanism of action comes from experiments performed on fibroblast-like synoviocytes cultured from OA patients. The synovial membrane and synovial fluid participate in the inflammatory process during both early and advanced OAK (and presumably other joints). Synovial fluid contains inflammatory cytokines as well as inflammatory cells, and fibroblast-like synoviocytes isolated from OA patients are targets of the DAMP system to secrete such cytokines [46, 47]. As in PBMCs, LMWF5A stimulates the production of both COX-2 and prostaglandin E_2 when fibroblast-like synoviocytes are challenged with IL1-beta and, especially, TNF-alpha; more significantly, LMWF5A increases release of the anti-inflammatory prostaglandin PGD_2, especially in response to TNF-alpha stimulation [48].

In addition, LMWF5A has been shown in synoviocytes and chondrocytes to increase the expression of miR146a and miR200b (unpublished data), two microRNAs that are known to downregulate proteins involved in the inflammatory response [49, 50]. The increased expression of these microRNAs may contribute to the overall anti-inflammatory action of LMWF5A.

4.7. Human Endothelial Cells

The vascular endothelium provides an essential barrier function and regulation is a critical event in the inflammatory response [51]. To determine if LMWF5A and/or its components can alter endothelial function, *in vitro* permeability models were employed. Our findings suggest that endothelial cells treated with LMWF5A exhibited reduced macromolecular permeability and increased trans-endothelial resistance [52].

Vascular permeability is intimately associated with changes in the endothelial cell cytoskeleton [53]. Treatment of endothelial cells in culture with LMWF5A induces a rapid, calcium-dependent acetylation of alpha-tubulin and a concomitant decrease in macromolecular permeability [54]. Endothelial permeability depends on the polymerization state of microtubules, which are composed of tubulin. Permeability can be affected by other microtubule-associated proteins, which in turn affect the polymerization state of tubulin [52]. Destabilization of microtubules with nocodazole and vinblastine increases permeability through Rho GTPase activation [55]. Conversely, stabilization of the microtubule with paclitaxel increases the acetylation of tubulin and decreases permeability [52].

In addition to microtubule changes, the LMWF5A component DA-DKP exhibits a capacity as well to alter the actin cytoskeleton. When exposed to 100 μM final concentrations of DA-DKP for one-hour, endothelial cells displayed increased cortical actin rearrangements as detected by fluorescently conjugated phalloidin (unpublished findings). Furthermore, increased trans-endothelial cell resistance was also observed under similar culture conditions.

Based on our finding that DA-DKP upregulates intracellular levels of active GTP-bound RAP-1 in T-cells, we hypothesized that DA-DKP functions, in part, through exchange protein directly activated by cAMP or EPAC. This protein is one of the primary guanine nucleotide exchange factors for RAP-1 and has been found to regulate endothelial cell function through VE-cadherin and actin remodeling events [56]. To establish this, siRNA EPAC1 knockdown was performed on these cells and reduction in protein translation was confirmed by western blot analysis of total cellular protein extracts. Preliminary findings indicate that loss of EPAC attenuates DA-DKP-induced cortical actin rearrangements and DA-DKP-induced increases in trans-endothelial resistance. Interestingly, studies suggest that EPAC1 colocalizes with microtubules and its activation plays a role in endothelial microtubule dynamics [57]. Furthermore, EPAC/RAP activation can enhance endothelial barrier function by increasing cortical actin and down regulating Rho GTPase, implying that cross-talk exists between these pathways.

5. LMWF5A'S MECHANISMS OF ACTION INTEGRATE ALONG MULTIPLE PATHWAYS (FIG. 4)

Many of the mechanisms of action in the specific cell types noted above have been demonstrated in more than one cell type. Thus, we now examine the global effect of LMWF5A on various pathways to understand how it contributes to pain relief and healing in OAK, as well as to consider the potential of this therapeutic for the more acute problems of trauma, SIRS, and sepsis. All *in vitro* effects of LMWF5A found to date can be interpreted as causes of the known clinical findings measured after LMWF5A injection into OA knee joints, including pain relief, increased mobility, and improved global self-assessment [2, 4, 5]. In essence, the downstream effects of the pathways described below can be summarized as 1) Modification of the inflammatory response and 2) Promotion of healing of inflamed tissue. The question of whether LMWF5A causes reversal of the cartilage destruction that characterizes severe OAK has not been definitively answered, but we also discuss results relevant to this issue below.

5.1. mTOR Pathway

BMMSCs differentiate into chondrocytes after activation of the mTOR pathway [58]. This is, in part, a consequence of Akt phosphorylation, which leads to the phosphorylation of PRAS40, an inhibitor of mTORC1, resulting in the dissociation of PRAS40 from the mTORC1 complex, allowing mTOR to stimulate stem cell differentiation. Chondrocyte formation, as opposed to the formation of other differentiated cell types, depends on other factors [59]. LMWF5A promotes PRAS40 phosphorylation and a subsequent increase in the levels of many mRNAs, most significantly collagen type II [16]. Therefore, LMWF5A stimulates what appears to be the beginning of chondrocyte differentiation.

Stimulation of mTOR activity *via* PRAS40 siRNA knockdown in human amniotic fluid stem cells leads to apoptosis of the outer cells in a three-dimensional embryonic bud but leaves the inner cells intact to differentiate [18]. This result is not seen when these stem cells are grown in two-dimensional monolayers. An important difference between this siRNA knockdown experiment and the increased phosphorylation of PRAS40 activity caused by LMWF5A is that in the latter, phosphorylated PRAS40, now dissociated from the mTORC1 complex, can bind to any of seven human 14-3-3 proteins and may play an additional role in chondrogene-

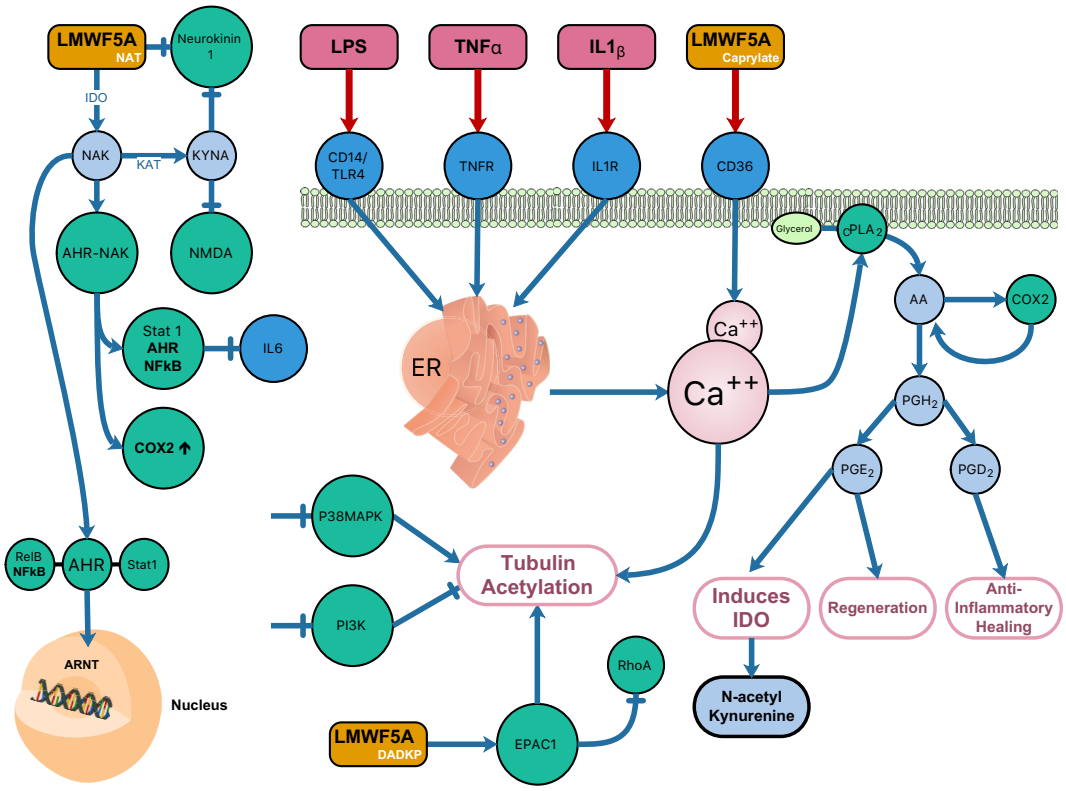

Fig. (4). Effects of various stimuli and LMWF5A on inflammation. Endoplasmic Reticulum (ER) stress causes the release of intracellular calcium, which in turn localizes and activates cytoplasmic phospholipase A2 (cPLA2) to the plasma membrane, liberating arachidonic acid (AA) which is a substrate for COX-2 (cyclooxygenase-2) to form the precursor prostaglandin PGH_2 and, through enzymatic steps, PGE_2 and PGD_2 and their metabolites. PGE_2 induces indoleamine 2,3-dioxygenase (IDO), which is involved in the kynurenine pathway, to form N-Acetyl Kyneurenine (NAK) from N-acetyl Tryptophan (NAT), transforming into kynurenic acid through the action of Kynurenine Amino Transferase (KAT). AhR, aryl hydrocarbon receptor; NMDA, N methyl-D- aspartate.

sis [17]. It has been suggested in the literature that the changes in actin polymerization seen during the differentiation of BMMSCs (and other cell types) are a result of the activation of this same Akt pathway [60].

5.2. COX-2 Pathway

The production of both prostaglandin E_2 and prostaglandin J_2 depend on COX-2 pathway activation [61, 62]. Osteoarthritic chondrocyte death induced by NO depends on the COX-2–dependent production of PGE_2 [63]. Under pro-inflammatory conditions in fibroblast-like synoviocytes from OA patients, LMWF5A increases COX-2 mRNA production. However, the production of prostaglandin E_2 was significantly increased in TNF-alpha–stimulated cells but not IL1-beta–stimulated cells. Nonetheless, the release of the anti-inflammatory prostaglandin D_2 was increased after stimulation by both inducers of inflammation [48]. These results suggest that LMWF5A would also induce anti-inflammatory prostaglandins in closely related cells, such as stem cells in the knee joint [64].

Recent advances have shed new light on the role of prostaglandins, and in particular PGE_2, in inflammation and healing. While traditionally viewed as a pro-inflammatory molecule, PGE_2 appears to exhibit a broad range of contextual effects on the progression and resolution of the inflammatory response. Studies suggest that PGE_2 exerts a phase of post-inflammation immune suppression and tolerance that could reduce auto-immune tissue damage [65]. In addition, M2 or anti-inflammatory macrophage polarization can be induced by PGE_2 though cAMP-dependent pathways [66]. Of note, hybrid M1/2 subsets of macrophages have been identified, deemed resolution phase macrophages, that express high levels of COX2 that may signal post resolution lymphocyte repopulation [67]. PGE_2 can also promote the migration and homing of mesenchymal stem cells, potentially through the upregulation of CXCR4 [68, 69]. Furthermore, in mouse models, PGE_2 deficiency exacerbates collagen-induced arthritis [70]. Interestingly, both mesenchymal stem cells and myeloid-derived suppressor cells mediate immune suppression by COX2/PGE_2 as well as tryptophan metabolism [71]. Taken together, PGE_2 and other eicosanoid species provide immune direction response across all phases of the response.

5.3. CD36 Pathway

More recently, it has become clear that LMWF5A may exert some of its actions through the CD36 pathway. CD36 is a membrane-bound protein, also commonly known as Fatty Acid Translocase (FAT), thrombospondin receptor for thrombospondin-1, platelet glycoprotein 4, and scavenger receptor class B member 3 (SCARB3). CD36 is present on many cell types and binds many ligands, of which oxidized LDL and octanoate anion are relevant here. CD36 signaling is involved in a variety of diseases as well as in different cellular processes, such as angiogenesis and apoptosis. The

foci for the mode of action of LMWF5A with respect to this pathway are primarily (1) CD36 signaling in macrophages (and their precursor monocytes) and chondrocytes and (2) the effect of octanoate on CD36 receptor activity and downstream signaling (Fig. **1**).

CD36, as the fatty acid translocase, binds octanoate and transmits stress to the endoplasmic reticulum system [72, 73]. Oxidized LDL, a product of reactive oxygenated species (which are known to be associated with OAK), also reacts with CD36 in its role as a scavenger receptor. The outcomes of these reactions are similar, if not identical. These signals lead to the liberation of intracellular stores of calcium ions and a change in calcium flux into cells. This, in turn, leads to the liberation of arachidonic acid by the action of phospholipase A2, which feeds into the COX-2 pathway, and the release of prostaglandins, particularly prostaglandin E_2 as an immediate response [74].

Research of cardiovascular and metabolic diseases has unveiled large differences between males and females in CD36 mRNA (both in rats and humans) and CD36 protein (in rats). For example, females show more than double the levels of human CD36 mRNA than males [75]. In our experiments on fibroblast-like synoviocytes from OA patients, we confirmed this finding and showed that LMWF5A inhibits the amount of CD36 mRNA produced after inducing inflammation with IL1-beta Decreasing CD36 expression after inflammation is expected to be anti-inflammatory, as CD36 increases lead to, among other things, the inhibition of macrophages' ability to phagocytose cellular debris. Thus, LMWF5A should help macrophages fight inflammatory responses [76].

We emphasize here that the mTOR pathway, COX-2 pathway, and CD36 pathway (probably through its action on COX-2 stimulation) all contribute to the important anti-apoptotic effects of LMWF5A that we observed on mesenchymal stem cells and chondrocytes. This overlapping of effects is seen even more strongly when we examine the effect of each of the three known components of LMFW5A on the inflammatory response and healing action in OA and other inflammatory diseases.

5.4. Effects of LMWF5A's Components on Inflammatory Responses and Healing

Not every effect of LMWF5A on every cell type can be definitively attributed to one of its three major components, but some experimental outcomes do seem to be primarily attributable to one specific component. For example, the action of DA-DKP seems to be sufficient to suppress TNF-alpha and IFN-gamma in LPS-stimulated PBMCs and T cells, and tryptophan derivatives were explicitly tested and found to have no capacity to inhibit these two inflammatory cytokines [39]. Moreover, DA-DKP and tryptophan metabolites seem to be the component that stimulates the production of the cytokines IL-8 and migration inhibitory factor (MIF) in the human macrophage cell line THP-1. The effect of sodium caprylate (octanoate) in macrophages, monocytes, chondrocytes, and synoviocytes is also clear: it binds to and stimulates CD36, which leads to the release of first pro-inflammatory and then anti-inflammatory prostaglandins. Other ligands (such as oxidized LDL) may contribute to CD36 engagement, and clearly other components of LMWF5A also contribute to the synthesis of anti-inflammatory cytokines and prostaglandins, but the partial role of sodium caprylate in this pathway is undisputed. We show the known and supposed roles of the three known components of LMWF5A determined by our experimental findings to date (Fig. **1**).

5.5. LMWF5A as a Disease-modifying Therapeutic

One important measure of a therapeutic agent is whether treatment is disease-modifying, that is whether the therapeutic agent simply relieves symptoms of a disease or actually changes the course of the disease to make it less harmful to the patient. Classic examples of disease-modifying drugs have been especially well characterized for rheumatoid arthritis and are termed DMARDs [77]. Analogous drugs for OAK are under investigation, and their potential use is being contemplated [78]. In presenting the effects and mechanisms of action of LMWF5A above, we emphasized those results that provide strong suggestive evidence for LMWF5A as a disease-modifying drug. These results reflect anti-inflammatory and cellular effects that have implications for the use of LMWF5A, not only in OAK, but also potentially in critical care diseases, such as sepsis and SIRS.

The concept of a disease-modifying drug for OAK is regulatory rather than medical in nature. Because of the success of DMARDs for rheumatoid arthritis, the same idea has been extended to therapeutics for a variety of chronic, debilitating diseases. Both the FDA in the US and the EMA in Europe have written draft guidelines for the properties required to attain disease-modifying drug status for OA therapeutics [79, 80]. Such classification requires evidence of reversing or dramatically slowing the disease process rather than simply showing symptomatic relief [81]. Although there is no definitive proof that LMWF5A is a disease-modifying OA drug (DMOAD), there are a variety of findings suggesting this possibility.

First, because LMWF5A has at least three active components that combat the inflammatory pathways seen in OA, the *a priori* possibility of it being a disease-modifying drug is higher than if it were a single-component therapeutic. This is somewhat analogous to the example given in the introduction, where we suggested that the multiple-component willow bark is likely a better anti-inflammatory agent than the single-component aspirin. In support of the potential DMOAD status of LMWF5A are results from three different fields: cell biology experiments, radiological examination, and clinical outcomes.

Osteoarthritis results from the destruction of cartilage, overcoming the ability of mesenchymal stem cells near articulations to differentiate into chondrocytes and synoviocytes, which make normal cartilage and synovium. As described above, LMWF5A stimulates BMMSCs to differentiate into apparent chondrocytes that show many of the hallmarks of normal cartilaginous cells [16]. Although this does not definitively show that the kinetics of such regeneration *in vivo* would be sufficient to overcome the destructive process, it is evidence to support the likelihood that LMWF5A is a DMOAD.

The STRUT study was a phase II investigation of the efficacy and safety of three intra-articular injections of LMWF5A or saline in patients with OAK. Pain relief was reported in the two groups (N=40) at 20 weeks after the first injection [82]. Although it was not reported with the 20-week results, MRI data were also taken at baseline and 52 weeks after the first injection. Because OAK is frequently a local disease [83, 84], the MRI measurements were taken from patients with a medial lesion (n=10), a lateral lesion (n=10), or no area of denuded cartilage or symmetrical (medial and lateral) lesions. Changes in cartilage thickness were measured across six anatomically defined sub-regions, with patients with medial disease and lateral disease examined in six distinct sub-regions. Among patients with medial disease, those treated with LMWF5A had less cartilage-thickness loss than those treated with saline in all six medial sub-regions. The mean cartilage loss for the medial lesions was 3 μm for those treated with LMWF5A and 34 μm for those treated with saline. Among patients with lateral lesions, those treated with LMWF5A had less cartilage loss than those treated with saline in five of the six measured sub-regions. Only patients treated with LMWF5A had increases in cartilage thickness in two of the six lateral sub-regions. These findings support the possibility that LMWF5A is a DMOAD by slowing or preventing further cartilage destruction.

Finally, an IRB-approved follow up to the STRUT (AP007) study was carried out ~3.5 years after the completion of the initial STRUT study. Using a telephone questionnaire, patients who had been given three biweekly intra-articular injections of either LMWF5A or saline 3.5 years previously as part of STRUT were asked whether they had undergone a Total Knee Replacement (TKR) since that time. Delaying TKR would be considered positive evidence for LMWF5A acting as a DMOAD. Fifteen of the 39 respondents had undergone TKR. Among the 16 patients with the most severe OAK (Kellgren-Lawrence (KL) score of 4), 40% of those treated with LMWF5A had undergone TKR, whereas 83% of those treated with saline had undergone TKR [4, 5, 85]. This type of study should be repeated with a larger sample size, but the existing data are highly suggestive of LMWF5A being a DMOAD. Clinical trial results were published separately and are supported by the *in vitro* MOAs described in this review.

CONCLUSION

Upregulation of beneficial prostaglandins in healing and resolution of inflammation, activation of the AhR receptor through the kynurenine pathway, partial activation of CD36, activation of mTORC1, upregulation of transcription factors involved in chondrogenesis, and cytoskeleton changes associated with an increased endothelial cell barrier and with stem cell migration and differentiation are demonstrated by the effects of LMWF5A on various cell lines represented in the human joint. LMWF5A appears to have biological activities through numerous molecular pathways and is a promising drug for the treatment of osteoarthritis.

LIST OF ABBREVIATIONS

AhR	=	Aryl hydrocarbon Receptor
BMMSC	=	Bone Marrow-derived Mesenchymal Stem Cell
DA-DKP	=	Aspartyl Alanyl Diketopiperazine
DAMP	=	Damage-Associated Molecular Patterns
DMARD	=	Disease-Modifying Drug in Rheumatoid Arthritis
DMOAD	=	Disease-Modifying Osteoarthritis Drug
DPP-4	=	Dipeptidyl Peptidase 4
FAT	=	Fatty Acid Translocase
HSA	=	Human Serum Albumin
KL score	=	Kellgren-lawrence score
LMWF5A	=	Low Molecular Weight Fraction of Commercial Human Serum Albumin
LPS	=	Lipopolycaccharide
MIF	=	Migratory Inhibitory Factor
MOA	=	Mechanism of Action
NAK	=	N-acetyl Kynurenine
NF-κB	=	Nuclear Factor-κB
NK	=	Natural Killer Cells
NSAID	=	Non-Steroidal Anti-inflammatory Drug
OA-FLC	=	Osteoarthritis Fibroblast-like Chondrocytes
OAK	=	Osteoarthritis of the Knee
PAMP	=	Pathogen-associated Molecular Patterns
PBMC	=	Peripheral Blood Mononuclear Cells
PGA	=	Patient Global Assessment
PMA	=	Phorbol 12 Myristate 13 Acetate
PVDF	=	Polyvinylidene Difluoride
SCARB3	=	Scavenger Receptor Class B Member 3
SIRS	=	Systemic Inflammatory Response Syndrome
Th17	=	T helper 17 Cells
TKR	=	Total Knee Replacement
Treg	=	Regulatory T Cells

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

D.B.-O. is a director, Scientific Advisory Board (SAB) member, shareholder, owns stock options, and uncompensated intellectual property rights at Ampio Pharmaceuticals. G.W., E.F., and R.B.-O. are compensated employees, shareholders, and stock option owners at Ampio Pharmaceuticals. L.T.R. and M.H. are shareholders and stock option owners at Ampio Pharmaceuticals. E.B. is a SAB member and owns stock options at Ampio Pharmaceuticals.

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REFERENCES

[1] Zimmermann GR, Lehár J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 2007; 12(1-2): 34-42. [http://dx.doi.org/10.1016/j.drudis.2006.11.008] [PMID: 17198971]

[2] Bar-Or D, Salottolo KM, Loose H, *et al.* A randomized clinical trial to evaluate two doses of an intra-articular injection of LMWF-5A in adults with pain due to osteoarthritis of the knee. PLoS One 2014; 9(2)e87910 [http://dx.doi.org/10.1371/journal.pone.0087910] [PMID: 24498399]

[3] Cole B, McGrath B, Salottolo K, Bar-Or D. LMWF-5A for the treatment of severe osteoarthritis of the knee: Integrated analysis of safety and efficacy. Orthopedics 2018; 41(1): e77-83. [http://dx.doi.org/10.3928/01477447-20171114-05] [PMID: 29156068]

[4] Salottolo K, Cole B, Bar-Or D. Intra-articular injection of the anti-inflammatory compound LMWF-5A in adults with severe osteoarthritis: a double-blind prospective randomized controlled multi-center safety and efficacy trial. Patient Saf Surg 2018; 12: 11. [http://dx.doi.org/10.1186/s13037-018-0158-0] [PMID: 29910837]

[5] Schwappach J, Schultz J, Salottolo K, Bar-Or D. Incidence of total knee replacement subsequent to intra-articular injection of the anti-inflammatory compound LMWF-5A versus saline: a long-term follow-up study to a randomized controlled trial. Patient Saf Surg 2018; 12: 14. [http://dx.doi.org/10.1186/s13037-018-0162-4] [PMID: 29881459]

[6] Yang F, Zhang Y, Liang H. Interactive association of drugs binding to human serum albumin. Int J Mol Sci 2014; 15(3): 3580-95. [http://dx.doi.org/10.3390/ijms15033580] [PMID: 24583848]

[7] Janeway CA. Clinical use of products of human plasma fractionation. JAMA 1944; 126(11): 674-80. [http://dx.doi.org/10.1001/jama.1944.02850460004002] [PMID: 16695125]

[8] Human albumin administration in critically ill patients: systematic review of randomised controlled trials. BMJ 1998; 317(7153): 235-40. [http://dx.doi.org/10.1136/bmj.317.7153.235] [PMID: 9677209]

[9] Vincent J-L, Russell JA, Jacob M, *et al.* Albumin administration in the acutely ill: what is new and where next? Crit Care 2014; 18(4): 231. [http://dx.doi.org/10.1186/cc13991] [PMID: 25042164]

[10] Anraku M, Tsurusaki Y, Watanabe H, Maruyama T, Kragh-Hansen U, Otagiri M. Stabilizing mechanisms in commercial albumin preparations: octanoate and N-acetyl-L-tryptophanate protect human serum albumin against heat and oxidative stress. Biochim Biophys Acta 2004; 1702(1): 9-17. [http://dx.doi.org/10.1016/j.bbapap.2004.07.002] [PMID: 15450846]

[11] Faroongsarng D, Kongprasertkit J. The role of caprylate ligand ion on the stabilization of human serum albumin. AAPS PharmSciTech 2014; 15(2): 465-71. [http://dx.doi.org/10.1208/s12249-014-0076-0] [PMID: 24470225]

[12] Bar-Or D, Slone DS, Mains CW, Rael LT. Dipeptidyl peptidase IV activity in commercial solutions of human serum albumin. Anal Biochem 2013; 441(1): 13-7. [http://dx.doi.org/10.1016/j.ab.2013.06.002] [PMID: 23770236]

[13] Bar-Or D, Rael LT, Brody EN. Use of saline as a placebo in intra-articular injections in osteoarthritis: potential contributions to nociceptive pain relief. Open Rheumatol J 2017; 11(1): 16-22. [http://dx.doi.org/10.2174/1874312901711010016] [PMID: 28400868]

[14] Altman RD, Devji T, Bhandari M, Fierlinger A, Niazi F, Christensen R. Clinical benefit of intra-articular saline as a comparator in clinical trials of knee osteoarthritis treatments: A systematic review and meta-analysis of randomized trials. Semin Arthritis Rheum 2016; 46(2): 151-9. [http://dx.doi.org/10.1016/j.semarthrit.2016.04.003] [PMID: 27238876]

[15] Mathieu PS, Loboa EG. Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. Tissue Eng Part B Rev 2012; 18(6): 436-44. [http://dx.doi.org/10.1089/ten.teb.2012.0014] [PMID: 22741572]

[16] Bar-Or D, Thomas GW, Rael LT, Gersch ED, Rubinstein P, Brody E. Low molecular weight fraction of commercial human serum albumin induces morphologic and transcriptional changes of bone marrow-derived mesenchymal stem cells. Stem Cells Transl Med 2015; 4(8): 945-55. [http://dx.doi.org/10.5966/sctm.2014-0293] [PMID: 26041739]

[17] Wiza C, Nascimento EBM, Ouwens DM. Role of PRAS40 in Akt and mTOR signaling in health and disease. Am J Physiol Endocrinol Metab 2012; 302(12): E1453-60. [http://dx.doi.org/10.1152/ajpendo.00660.2011] [PMID: 22354785]

[18] Fuchs C, Rosner M, Dolznig H, Mikula M, Kramer N, Hengstschläger M. Tuberin and PRAS40 are anti-apoptotic gatekeepers during early human amniotic fluid stem-cell differentiation. Hum Mol Genet 2012; 21(5): 1049-61. [http://dx.doi.org/10.1093/hmg/ddr535] [PMID: 22090422]

[19] Cui G-H, Wang YY, Li C-J, Shi C-H, Wang W-S. Efficacy of mesenchymal stem cells in treating patients with osteoarthritis of the knee: a meta-analysis. Exp Ther Med 2016; 12(5): 3390-400. [http://dx.doi.org/10.3892/etm.2016.3791] [PMID: 27882169]

[20] Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol 2010; 10(6): 427-39. [http://dx.doi.org/10.1038/nri2779] [PMID: 20498669]

[21] Wammers M, Schupp A-K, Bode JG, *et al.* Reprogramming of pro-inflammatory human macrophages to an anti-inflammatory phenotype by bile acids. Sci Rep 2018; 8(1): 255. [http://dx.doi.org/10.1038/s41598-017-18305-x] [PMID: 29321478]

[22] Stone TW, Forrest CM, Darlington LG. Kynurenine pathway inhibition as a therapeutic strategy for neuroprotection. FEBS J 2012; 279(8): 1386-97. [http://dx.doi.org/10.1111/j.1742-4658.2012.08487.x] [PMID: 22248239]

[23] Stevens EA, Mezrich JD, Bradfield CA. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. Immunology 2009; 127(3): 299-311. [http://dx.doi.org/10.1111/j.1365-2567.2009.03054.x] [PMID: 19538249]

[24] Nguyen NT, Nakahama T, Le DH, Van Son L, Chu HH, Kishimoto T. Aryl hydrocarbon receptor and kynurenine: recent advances in autoimmune disease research. Front Immunol 2014; 5: 551. [http://dx.doi.org/10.3389/fimmu.2014.00551] [PMID: 25400638]

[25] Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol 2010; 185(6): 3190-8. [http://dx.doi.org/10.4049/jimmunol.0903670] [PMID: 20720200]

[26] Kimura A, Naka T, Nakahama T, *et al.* Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. J Exp Med 2009; 206(9): 2027-35. [http://dx.doi.org/10.1084/jem.20090560] [PMID: 19703987]

[27] Vogel CFA, Khan EM, Leung PSC, *et al.* Cross-talk between aryl hydrocarbon receptor and the inflammatory response: a role for nuclear factor-κB. J Biol Chem 2014; 289(3): 1866-75. [http://dx.doi.org/10.1074/jbc.M113.505578] [PMID: 24302727]

[28] Kreitinger JM, Beamer CA, Shepherd DM. Environmental immunology: lessons learned from exposure to a select panel of immunotoxicants. J Immunol 2016; 196(8): 3217-25. [http://dx.doi.org/10.4049/jimmunol.1502149] [PMID: 27044635]

[29] van Helden SFG, van Leeuwen FN, Figdor CG. Human and murine model cell lines for dendritic cell biology evaluated. Immunol Lett 2008; 117(2): 191-7. [http://dx.doi.org/10.1016/j.imlet.2008.02.003] [PMID: 18384885]

[30] Rael LT, Bar-Or R, Banton KL, *et al.* The anti-inflammatory effect of LMWF5A and N-acetyl kynurenine on macrophages: Involvement of aryl hydrocarbon receptor in mechanism of action. Biochem Biophys Rep 2018; 15: 61-7. [http://dx.doi.org/10.1016/j.bbrep.2018.06.006] [PMID: 30073204]

[31] Michael JWP, Schlüter-Brust KU, Eysel P. The epidemiology, etiology, diagnosis, and treatment of osteoarthritis of the knee. Dtsch Arztebl Int 2010; 107(9): 152-62.

[http://dx.doi.org/10.3238/arztebl.2010.0152] [PMID: 20305774]

[32] Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 2001; 3(2): 107-13. [http://dx.doi.org/10.1186/ar148] [PMID: 11178118]

[33] Tesche F, Miosge N. New aspects of the pathogenesis of osteoarthritis: the role of fibroblast-like chondrocytes in late stages of the disease. Histol Histopathol 2005; 20(1): 329-37. [PMID: 15578449]

[34] Zhong L, Huang X, Karperien M, Post JN. Correlation between gene expression and osteoarthritis progression in human. Int J Mol Sci 2016; 17(7): 1126. [http://dx.doi.org/10.3390/ijms17071126] [PMID: 27428952]

[35] Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982; 30(1): 215-24. [http://dx.doi.org/10.1016/0092-8674(82)90027-7] [PMID: 7127471]

[36] Nishimura R, Hata K, Takahata Y, Murakami T, Nakamura E, Yagi H. Regulation of cartilage development and diseases by transcription factors. J Bone Metab 2017; 24(3): 147-53. [http://dx.doi.org/10.11005/jbm.2017.24.3.147] [PMID: 28955690]

[37] Lefebvre V, Dvir-Ginzberg M. SOX9 and the many facets of its regulation in the chondrocyte lineage. Connect Tissue Res 2017; 58(1): 2-14. [http://dx.doi.org/10.1080/03008207.2016.1183667] [PMID: 27128146]

[38] Hausburg MA, Frederick ED, McNair P, *et al.* Clinically relevant redifferentiation of fibroblast-like chondrocytes into functional chondrocytes by the low molecular weight fraction of human serum albumin. Clin Exp Rheumatol 2018; 36(5): 891-5. [PMID: 30272545]

[39] Bar-Or D, Thomas GW, Bar-Or R, *et al.* Commercial human albumin preparations for clinical use are immunosuppressive in vitro. Crit Care Med 2006; 34(6): 1707-12. [http://dx.doi.org/10.1097/01.CCM.0000217923.53680.4C] [PMID: 16625113]

[40] Shimonkevitz R, Thomas G, Slone DS, Craun M, Mains C, Bar-Or D. A diketopiperazine fragment of human serum albumin modulates T-lymphocyte cytokine production through rap1. J Trauma 2008; 64(1): 35-41. [http://dx.doi.org/10.1097/TA.0b013e3181589ff9] [PMID: 18188096]

[41] Bar-Or D, Rael LT, Thomas GW, Brody EN. Inflammatory pathways in knee osteoarthritis: potential targets for treatment. Curr Rheumatol Rev 2015; 11(1): 50-8. [http://dx.doi.org/10.2174/1573397111666150522094131] [PMID: 26002457]

[42] Fahmi H, Pelletier JP, Mineau F, Martel-Pelletier J. 15d-PGJ(2) is acting as a 'dual agent' on the regulation of COX-2 expression in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2002; 10(11): 845-8. [http://dx.doi.org/10.1053/joca.2002.0835] [PMID: 12435328]

[43] Kawahito Y, Kondo M, Tsubouchi Y, *et al.* 15-deoxy-delta(12,14)-PGJ(2) induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. J Clin Invest 2000; 106(2): 189-97. [http://dx.doi.org/10.1172/JCI9652] [PMID: 10903334]

[44] Silva Quinteiro M, Henrique Napimoga M, Gomes Macedo C, *et al.* 15-deoxy-Δ12,14-prostaglandin J2 reduces albumin-induced arthritis in temporomandibular joint of rats. Eur J Pharmacol 2014; 740: 58-65. [http://dx.doi.org/10.1016/j.ejphar.2014.07.002] [PMID: 25016088]

[45] Thomas GW, Rael LT, Hausburg M, *et al.* The low molecular weight fraction of human serum albumin upregulates production of 15d-PGJ2 in peripheral blood mononuclear cells. Biochem Biophys Res Commun 2016; 473(4): 1328-33. [http://dx.doi.org/10.1016/j.bbrc.2016.04.072] [PMID: 27095392]

[46] Nair A, Kanda V, Bush-Joseph C, *et al.* Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands *via* soluble CD14. Arthritis Rheum 2012; 64(7): 2268-77. [http://dx.doi.org/10.1002/art.34495] [PMID: 22492243]

[47] Wang CT, Lin YT, Chiang BL, Lin YH, Hou SM. High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. Osteoarthritis Cartilage 2006; 14(12): 1237-47. [http://dx.doi.org/10.1016/j.joca.2006.05.009] [PMID: 16806998]

[48] Frederick ED, Hausburg MA, Thomas GW, Rael LT, Brody E, Bar-Or D. The low molecular weight fraction of human serum albumin upregulates COX2, prostaglandin E2, and prostaglandin D2 under inflammatory conditions in osteoarthritic knee synovial fibroblasts. Biochem Biophys Rep 2016; 8: 68-74. [http://dx.doi.org/10.1016/j.bbrep.2016.08.015] [PMID: 28955943]

[49] Rusca N, Monticelli S. MiR-146a in immunity and disease. Mol Biol Int 2011; 2011437301 [http://dx.doi.org/10.4061/2011/437301] [PMID: 22091404]

[50] Wendlandt EB, Graff JW, Gioannini TL, McCaffrey AP, Wilson ME. The role of microRNAs miR-200b and miR-200c in TLR4 signaling and NF-κB activation. Innate Immun 2012; 18(6): 846-55. [http://dx.doi.org/10.1177/1753425912443903] [PMID: 22522429]

[51] Kumar P, Shen Q, Pivetti CD, Lee ES, Wu MH, Yuan SY. Molecular mechanisms of endothelial hyperpermeability: implications in inflammation. Expert Rev Mol Med 2009; 11e19. [http://dx.doi.org/10.1017/S1462399409001112] [PMID: 19563700]

[52] Petrache I, Birukova A, Ramirez SI, Garcia JGN, Verin AD. The role of the microtubules in tumor necrosis factor-α-induced endothelial cell permeability. Am J Respir Cell Mol Biol 2003; 28(5): 574-81. [http://dx.doi.org/10.1165/rcmb.2002-0075OC] [PMID: 12707013]

[53] Vandenbroucke E, Mehta D, Minshall R, Malik AB. Regulation of endothelial junctional permeability. Ann N Y Acad Sci 2008; 1123(1): 134-45. [http://dx.doi.org/10.1196/annals.1420.016] [PMID: 18375586]

[54] Thomas GW, Rael LT, Hausburg M, Frederick ED, Brody E, Bar-Or D. The low molecular weight fraction of commercial human serum albumin induces acetylation of α-tubulin and reduces transcytosis in retinal endothelial cells. Biochem Biophys Res Commun 2016; 478(4): 1780-5. [http://dx.doi.org/10.1016/j.bbrc.2016.09.026] [PMID: 27613088]

[55] Verin AD, Birukova A, Wang P, *et al.* Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation. Am J Physiol Lung Cell Mol Physiol 2001; 281(3): L565-74. [http://dx.doi.org/10.1152/ajplung.2001.281.3.L565] [PMID: 11504682]

[56] Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 2005; 105(5): 1950-5. [http://dx.doi.org/10.1182/blood-2004-05-1987] [PMID: 15374886]

[57] Sehrawat S, Cullere X, Patel S, Italiano J Jr, Mayadas TN. Role of Epac1, an exchange factor for Rap GTPases, in endothelial microtubule dynamics and barrier function. Mol Biol Cell 2008; 19(3): 1261-70. [http://dx.doi.org/10.1091/mbc.e06-10-0972] [PMID: 18172027]

[58] Phornphutkul C, Wu K-Y, Auyeung V, Chen Q, Gruppuso PA. mTOR signaling contributes to chondrocyte differentiation. Dev Dyn 2008; 237(3): 702-12. [http://dx.doi.org/10.1002/dvdy.21464] [PMID: 18265001]

[59] Xiang X, Zhao J, Xu G, Li Y, Zhang W. mTOR and the differentiation of mesenchymal stem cells. Acta Biochim Biophys Sin (Shanghai) 2011; 43(7): 501-10. [http://dx.doi.org/10.1093/abbs/gmr041] [PMID: 21642276]

[60] Xue G, Hemmings BA. PKB/Akt-dependent regulation of cell motility. J Natl Cancer Inst 2013; 105(6): 393-404. [http://dx.doi.org/10.1093/jnci/djs648] [PMID: 23355761]

[61] Gilroy DW, Colville-Nash PR, McMaster S, Sawatzky DA, Willoughby DA, Lawrence T. Inducible cyclooxygenase-derived 15-deoxy(Delta)12-14PGJ2 brings about acute inflammatory resolution in rat pleurisy by inducing neutrophil and macrophage apoptosis. FASEB J 2003; 17(15): 2269-71. [http://dx.doi.org/10.1096/fj.02-1162fje] [PMID: 14563690]

[62] Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clark MJ, Willoughby DA. Inducible cyclooxygenase may have anti-inflammatory properties. Nat Med 1999; 5(6): 698-701. [http://dx.doi.org/10.1038/9550] [PMID: 10371510]

[63] Notoya K, Jovanovic DV, Reboul P, Martel-Pelletier J, Mineau F, Pelletier JP. The induction of cell death in human osteoarthritis chondrocytes by nitric oxide is related to the production of prostaglandin E2 *via* the induction of cyclooxygenase-2. J Immunol 2000; 165(6): 3402-10. [http://dx.doi.org/10.4049/jimmunol.165.6.3402] [PMID: 10975859]

[64] Jones EA, English A, Henshaw K, *et al.* Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. Arthritis Rheum 2004; 50(3): 817-27. [http://dx.doi.org/10.1002/art.20203] [PMID: 15022324]

[65] Newson J, Motwani MP, Kendall AC, *et al.* Inflammatory resolution triggers a prolonged phase of immune suppression through COX-1/mPGES-1-derived prostaglandin E 2. Cell Rep 2017; 20(13): 3162-75. [http://dx.doi.org/10.1016/j.celrep.2017.08.098] [PMID: 28954232]

[66] Luan B, Yoon Y-S, Le Lay J, Kaestner KH, Hedrick S, Montminy M. CREB pathway links PGE2 signaling with macrophage polarization. Proc Natl Acad Sci USA 2015; 112(51): 15642-7. [http://dx.doi.org/10.1073/pnas.1519644112] [PMID: 26644581]

[67] Bystrom J, Evans I, Newson J, *et al.* Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. Blood 2008; 112(10): 4117-27. [http://dx.doi.org/10.1182/blood-2007-12-129767] [PMID: 18779392]

[68] Lu X, Han J, Xu X, *et al.* PGE2 promotes the migration of mesenchymal stem cells through the activation of FAK and ERK1/2 pathway. Stem Cells Int 2017; 20178178643 [http://dx.doi.org/10.1155/2017/8178643] [PMID: 28740516]

[69] Wang Y, Lai S, Tang J, *et al.* Prostaglandin E2 promotes human CD34+ cells homing through EP2 and EP4 *in vitro*. Mol Med Rep 2017; 16(1): 639-46. [http://dx.doi.org/10.3892/mmr.2017.6649] [PMID: 28560401]

[70] Frolov A, Yang L, Dong H, Hammock BD, Crofford LJ. Anti-inflammatory properties of prostaglandin E2: deletion of microsomal prostaglandin E synthase-1 exacerbates non-immune inflammatory arthritis in mice. Prostaglandins Leukot Essent Fatty Acids 2013; 89(5): 351-8. [http://dx.doi.org/10.1016/j.plefa.2013.08.003] [PMID: 24055573]

[71] Vladimirovna IL, Sosunova E, Nikolaev A, Nenasheva T. Mesenchymal stem cells and myeloid derived suppressor cells: common traits in immune regulation. J Immunol Res 2016; 20167121580 [http://dx.doi.org/10.1155/2016/7121580] [PMID: 27529074]

[72] Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010; 140(6): 900-17. [http://dx.doi.org/10.1016/j.cell.2010.02.034] [PMID: 20303879]

[73] Tabas I. The role of endoplasmic reticulum stress in the progression of atherosclerosis. Circ Res 2010; 107(7): 839-50. [http://dx.doi.org/10.1161/CIRCRESAHA.110.224766] [PMID: 20884885]

[74] Kuda O, Jenkins CM, Skinner JR, *et al.* CD36 protein is involved in store-operated calcium flux, phospholipase A2 activation, and production of prostaglandin E2. J Biol Chem 2011; 286(20): 17785-95. [http://dx.doi.org/10.1074/jbc.M111.232975] [PMID: 21454644]

[75] Ståhlberg N, Rico-Bautista E, Fisher RM, *et al.* Female-predominant expression of fatty acid translocase/CD36 in rat and human liver. Endocrinology 2004; 145(4): 1972-9. [http://dx.doi.org/10.1210/en.2003-0874] [PMID: 14684613]

[76] Chuang P-C, Lin Y-J, Wu M-H, Wing L-YC, Shoji Y, Tsai S-J. Inhibition of CD36-dependent phagocytosis by prostaglandin E2 contributes to the development of endometriosis. Am J Pathol 2010; 176(2): 850-60. [http://dx.doi.org/10.2353/ajpath.2010.090551] [PMID: 20035060]

[77] Ruderman EM. Overview of safety of non-biologic and biologic DMARDs. Rheumatol 2012; 51(Suppl 6): vi37-43. [http://dx.doi.org/10.1093/rheumatology/kes283]

[78] Losina E, Daigle ME, Suter LG, *et al.* Disease-modifying drugs for knee osteoarthritis: can they be cost-effective? Osteoarthritis Cartilage 2013; 21(5): 655-67. [http://dx.doi.org/10.1016/j.joca.2013.01.016] [PMID: 23380251]

[79] European Medicines A. Guideline on clinical investigation of medicinal products used in the treatment of osteoarthritis. 1999.

[80] FDA US. Guidance for Industry Clinical Development Programs for Drugs, Devices, and Biological Products Intended for the Treatment of Osteoarthritis (OA). In: 1999; pp. 800-99.

[81] Barr AJ, Conaghan PG. Disease-modifying osteoarthritis drugs (DMOADs): what are they and what can we expect from them? - MedicographiaMedicographia. Medicographia 2013; 35: 189-96.

[82] Schwappach J, Dryden SM, Salottolo KM, Bar-Or D. Preliminary trial of intra-articular LMWF-5A for osteoarthritis of the knee. Orthopedics 2017; 40(1): e49-53. [http://dx.doi.org/10.3928/01477447-20160926-02] [PMID: 27684085]

[83] Ukachukwu V, Duncan R, Belcher J, *et al.* Clinical significance of medial versus lateral compartment patellofemoral osteoarthritis: cross-sectional analyses in an adult population with knee pain. Arthritis Care Res (Hoboken) 2017; 69(7): 943-51. [http://dx.doi.org/10.1002/acr.23110] [PMID: 27696767]

[84] Weidow J, Mars I, Kärrholm J. Medial and lateral osteoarthritis of the knee is related to variations of hip and pelvic anatomy. Osteoarthritis Cartilage 2005; 13(6): 471-7. [http://dx.doi.org/10.1016/j.joca.2005.01.009] [PMID: 15922181]

[85] Salottolo K, Stahl E. Minimal clinically important improvement response in patients with severe osteoarthritis of the knee: Short report from a survey of clinicians. J Orthop 2018; 15(2): 424-5. [http://dx.doi.org/10.1016/j.jor.2018.03.034] [PMID: 29881169]