DEPARTAMENTO DE FARMACOLOGÍA
FACULTAD DE FARMACIA
UNIVERSIDAD DE SEVILLA

FUNCIONALIDAD DEL ACEITE DE OLIVA VIRGEN EXTRA EN LA ARTRITIS REUMATOIDE EXPERIMENTAL

Tesis Doctoral presentada por

MARÍA DE LOS ÁNGELES ROSILLO RAMÍREZ

Para optar al Grado de Doctora en Farmacia

Sevilla, 2015

DEPARTAMENTO DE FARMACOLOGÍA
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Las Dras. Catalina Alarcón de la Lastra Romero, Catedrática de Universidad y Marina Sánchez Hidalgo, Profesora Contratada Doctora, adscritas al Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla.

INFORMAN

Que la Tesis Doctoral titulada **"FUNCIONALIDAD DEL ACEITE DE OLIVA VIRGEN EXTRA EN LA ARRITIS REUMATOIDE EXPERIMENTAL"** presentada por la Lda. María de los Ángeles Rosillo Ramírez para optar al grado de Doctora en Farmacia con Mención Internacional ha sido llevada a cabo bajo su dirección.

Sevilla, 16 de noviembre de 2015

Dra. Catalina Alarcón de la Lastra Romero

Dra. Marina Sánchez Hidalgo

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FACULTAD DE FARMACIA
UNIVERSIDAD DE SEVILLA

Dña. Mª Dolores García Giménez, Profesora Catedrática de Farmacología y Directora del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla.

CERTIFICA:

Que la Tesis Doctoral titulada **"FUNCIONALIDAD DEL ACEITE DE OLIVA VIRGEN EXTRA EN LA ARRITIS REUMATOIDE EXPERIMENTAL"** realizada por la Lda. María de los Ángeles Rosillo Ramírez, ha sido dirigida por la Dra. Catalina Alarcón de la Lastra Romero y la Dra. Marina Sánchez Hidalgo, para aspirar al grado de Doctor en Farmacia con Mención Internacional, cumpliendo los requisitos para este tipo de trabajo.

Y para que así conste, firmo la presente.

En Sevilla, a 16 de noviembre de 2013

Dra. Mª Dolores García Giménez

Este trabajo de investigación se ha llevado a cabo gracias a la financiación de las siguientes instituciones y proyecto de investigación:

PROYECTO DE INVESTIGACIÓN

- **Valoración del Aceite de Oliva Virgen Extra en la Artritis Reumatoide Experimental: Estudio Biodirigido, Caracterización Farmacológica y Desarrollo de Ingredientes Funcionales.**
  Entidad financiadora: Consejería de Innovación, Ciencia y Empresa. Junta de Andalucía.
  Investigador principal: Catalina Alarcón de la Lastra Romero.
  Referencia: AGR-6609
  Duración: 2011- 2016

BECA PREDOCTORAL

Beca predoctoral asociada a Proyectos de Investigación de Excelencia en Equipos de Investigación Orden de 11 de Diciembre de 2007 (BOJA 4, 05/01/2008) Convocatoria 2010 (BOJA 135, 12/07/2011).

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Y gracias a las estancias realizadas en:

- Lugar: Departamento de Farmacología. Facultad de Farmacia. Universidad de Valencia, España.

  Duración: Octubre-Noviembre 2012.

  Investigador responsable: María José Alcaraz Tormo.

- Lugar: Departamento de Reumatología. Erasmus MC. Universidad Erasmus. Rotterdam, Holanda

  Duración: Septiembre-Diciembre 2014.

  Investigador responsable: Erik Lubberts.

María de los Ángeles Rosillo Ramírez, Licenciada en Farmacia por la Universidad de Sevilla en 2009. En 2010 cursó el Máster en Atención Farmacéutica y Farmacia Asistencial en la misma universidad, durante este año recibió una beca de Plan de Apoyo al Máster Oficial de la Universidad de Sevilla. Ese mismo año consiguió el Premio a la mejor Beca sobre "Tema Libre" "Actividad del zumo de la granada y sus componentes en la inflamación crónica intestinal asociada a cáncer colorrectal experimental" concedido por la Fundación Farmacéutica Avenzoar.

Desde el año 2010 forma parte del grupo de investigación Farmacología Experimental y Farmacia Clínica (CTS-259). Comenzó sus estudios de Doctorado en la Universidad de Sevilla en el año 2011. En 2012 obtuvo una beca predoctoral Adscrita a Proyectos de Investigación de Excelencia en Equipos de Investigación asociada al proyecto AGR 6609 de la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía para la realización de su Tesis Doctoral bajo la dirección de las doctoras Catalina Alarcón de la Lastra Romero y Marina Sánchez Hidalgo.

Completó su formación predoctoral en el Departamento de Farmacología de la Universidad de Valencia bajo la dirección de la doctora Mª José Alcaraz Tormo durante los meses de octubre y noviembre de 2012 y en el Departamento de Reumatología e Inmunología del Erasmus Medical Center de Rotterdam (Holanda) bajo la dirección del doctor Erik Lubberts durante los meses de septiembre a diciembre de 2014.

Ha participado en distintos proyectos, así como en diversos trabajos de investigación que han sido presentados en congresos nacionales e internacionales y que han dado lugar a distintas publicaciones en revistas internacionales.

PROYECTOS DE INVESTIGACIÓN

- Valoración del Aceite de Oliva Virgen Extra en la Artritis Reumatoide Experimental: Estudio Biodirigido, Caracterización Farmacológica y Desarrollo de Ingredientes Funcionales (AGR-6609 – Becaria predoctoral) Junta de Andalucía. Investigador principal: Dra. Catalina Alarcón de la Lastra Romero.

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ÍNDICE

ÍNDICE

INTRODUCCIÓN

1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) can be defined as a chronic inflammatory disease with systemic autoimmune component, and is mainly characterized by aggressive synovial hyperplasia, synovitis, progressive destruction of cartilage, and bone erosion with painful swelling of small joints, fatigue, prolonged stiffness, and fever caused by immune responses and specific innate inflammatory processes (Zvaifler 1965, Creemers 2004, Brooks 2006).

Global prevalence of RA has been estimated to be around 0.5-1.0% of adults in developed countries with a large variation across regions and approximately three-times more common in the female gender. The disease may begin at any age, but around 80% of all patients initiate the disease between the ages of 35 and 50 years (Rudan, Sidhu et al. 2015).

RA patients exhibit an inflammatory chronic condition, which usually affects symmetrically diarthrodial and small joints of hands and feet. RA is characterized by the inflammation of synovial joint tissues leading hyperplasia of the synovial lining cell layer and the formation of rheumatoid pannus, which is capable of destroying adjacent cartilage and bone and causing subsequent joint deformity (Verpoort, van Dongen et al. 2004). Additional characteristic features of RA synovitis include neo-angiogenesis and infiltration of immune cells such as macrophages and lymphocytes which can form aggregates. Autoimmunity, identified by the production of auto-antibodies such as rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA) precedes the clinically detectable onset of inflammatory arthritis and can last for years (Chapuy-Regaud, Nogueira et al. 2005, Wegner, Lundberg et al. 2010). The clinic outcome is extremely variable and may extend from a soft autolimitant AR to a multisystemic inflammation with a rapid outcome (Finckh 2009). In this sense, about 40% of patients, AR disease may involve an extraarticular component, affecting various organs and systems including skin, lungs, eyes, heart and blood vessels. The progressive articular or extra-articular deterioration, leads deformity, pain, functional disability and a decrease in life expectancy of

these patients. These characteristics justify its multidisciplinary approach, which includes medical treatment, physiotherapy and nutritional therapy. The different treatments are aimed primarily to suppress the inflammatory process alleviating the symptoms and signs of the disease.

The etiology of RA is unknown. A genetic predisposition suggested by a link with HLA-DR4 and related allotypes of MHC class II, the T-cell-associated protein PTPN22, STAT4 and TRAFI1/C5, among others gene polymorphisms, has been related to a worse outcoming and a higher degree of articular destruction and extra-articular manifestations (Messemaker, Huizinga et al. 2015). However, the concordance rate of RA in monozygotic twins is low but higher than that in dizygotic twins suggesting that in addition to genetic factors, environmental factors as well as their interaction can be decisive in the development and disease progression. Among environmental factors, smoking has been recognized as a major risk factor RA. In fact, smoking has been associated with an increased risk of developing seropositive RA (RF and/or ACPA) (Chang, Yang et al. 2014). Nevertheless, other environmental factors including socio-economic, hormonal, silica exposure, stimuli like viruses, bacteria and stress, and dietetic factors may contribute to the disease progression (Cutolo 2007, Gomez-Puerta, Gedmintas et al. 2013).

The pathogenesis of RA is incompletely understood but hyperplasia of the synovial membrane is a hallmark of RA pathology, which is characterized by both hyperproliferation of synovial fibroblasts and massive infiltration of inflammatory immune cells, including CD4+ T-cells and innate immune cells (Komatsu and Takayanagi 2012). Once the process starts, a progressive recruitment of inflammatory T cells and macrophages into the joints occurs through a complex series of adhesion and migratory events. In this sense, initially there is an extensive increase in the number of innate effector cells in the synovial lining layer, which becomes several layers thick. Sublining layer becomes infiltrated with inflammatory mononuclear cells, including lymphocytes and macrophages and mast cells. In fact, macrophages are central effectors of synovitis and act though release of cytokines, reactive oxygen and nitrogen intermediates,

production of prostanoids and matrix-degrading enzymes, phagocytosis and antigen presentation. In addition, neutrophils transmigrate rapidly from the intravascular compartment to synovial fluid in the joint cavity contributing to the joint swelling and synthesizing prostaglandins, proteases and reactive oxygen intermediates (Cascao, Rosario et al. 2010). All of these cells, including resident fibroblasts, produce cytokines, which together with locally produced autoantibodies and immune complexes and complement, maintain the chronic inflammation leading the membrane expansion, which forms pannus. The cell layer of fibroblast- and macrophage-like cells which compose the pannus, can produce proteinases, receptor activator of nuclear factor kappa B ligand (RANKL) and other factors causing cartilage destruction. The persistent synthesis of proinflammatory cytokines plays a remarkable role in the development of RA disease from early autoimmunity step through continuous chronic inflammation into destruction of joint tissue. In addition, the prolonged immune activation is the main responsible to erosive and systemic bone loss and an increased propensity to fall in RA patients.

The development of the inflammatory process in RA involves many different type of cells and a complex cytokine network. Upon activation and expansion, $CD4^+$ T cells develop into different T helper cell subsets with different cytokine profile and distinct effector functions. Activated T cells that secret IFNY, IL2, IL12, IL18, TNF are produced in the synovial fluid and expressed in the synovial membrane. Particularly, TNF and IL-1β are considered as the main proinflammatory cytokines in the pathogenesis of RA. TNF plays a fundamental role through activation of cytokine and chemokine expression, expression of endothelial-cell adhesion molecules, protection of synovial fibroblasts, promotion of angiogenesis, suppression of regulatory T cells and induction of pain (McInnes and Schett 2011). In fact, treatment with TNF-α inhibitors results in decreased inflammation and bone protection in RA patients (Scott and Kingsley 2006). IL-1 family cytokines are also abundantly expressed in RA and promote the activation of leucocytes, endothelial cells, chondrocytes and osteoclasts (Brennan and McInnes

2008). Th17 cells, via their production of IL-17, contribute crucially to joint inflammation and bone erosion in RA (Kotake, Udagawa et al. 1999, Lubberts 2010, Jung, Kim et al. 2014). IL-6 and TGF-β induce Th17 development and IL-23 promotes Th17 cell expansion. In addition, these cytokines activate macrophages to secret other proinflammatory cytokines such as TNF and IL-1β, IL-6, IL-12, RANKL expression on T cells, promote the differentiation of B cells and stimulate the release of matrix metalloproteases (MMP) provoking the degradation of the cartilage and the activation of osteoclasts leading to the bone resorption (Schett and Teitelbaum 2009, Chimenti, Triggianese et al. 2015).

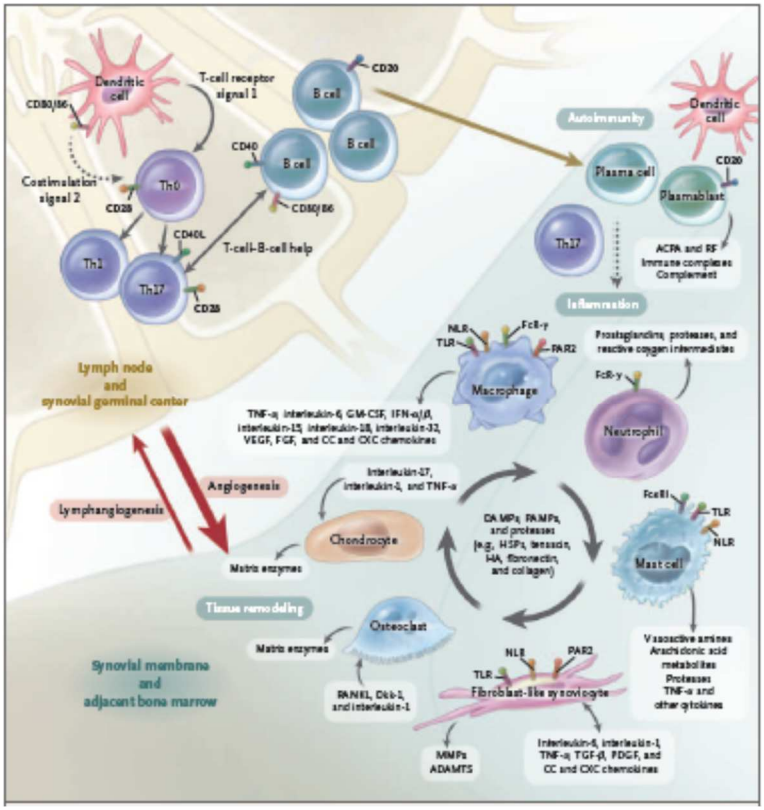

Figure 1. Adaptive and Innate Immune Processes within the Joint in Rheumatoid Arthritis (McInnes and Schett 2011)

Understanding the intracellular targets that regulate cytokines in RA can potentially lead to new therapeutic interventions. In this sense, the inhibition of key signal transduction pathways in inflammation such as mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol-3 protein kinase (PI3) pathway, janus kinase-signal transducer and activator of transcription (Jak-STAT) and nuclear factor kappa B (NFKB) would be expected to

abolish the cell activation by cytokines and production of these proinflammatory cytokines in RA (Morel and Berenbaum 2004). For example, NF-κB is activated in the synovium of patients with RA and regulates genes that contribute to inflammation including TNF, IL-6, IL8, iNOS and COX-2 (Aupperle, Bennett et al. 2001). Similarly, MAPK have attracted considerable attention as potent therapeutic targets in RA. MAPK are also key regulators of cytokines and MMP production and could also targeted in RA. All three kinases families, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), are expressed in rheumatoid synovial tissue (Schett, Tohidast-Akrad et al. 2000).

1.1. Pharmacological treatment of RA

The current pharmacological therapies for the treatment of RA are designed to fast control the disease activity and prevent the progression of damage in tissues. These include early and strict control of the activity, the use of drugs for the treatment of moderate disease, and induction of remission with use of biological drugs.

First line therapy including medications that suppress inflammation such as nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids; they act rapidly to improve pain and swelling due to RA. Disease-modifying antirheumatic drugs (DMARDs) are slower-acting compounds, not only improve symptoms but also diminish clinical and radiographic progression. In 1999, first biological-response modifier was marketed. These agents were designed to target the inflammatory mediators of tissue damage in RA (TNF-α, IL-1). The newest pharmacological target are anti-IL-6-receptor monoclonal antibodies.

NSAIDs

NSAIDs relieve the bone and joint inflammatory symptoms of RA; can be useful in the first weeks after the onset of RA symptoms while a diagnostic workup is undertaken, before a diagnosis is certain, and as bridge therapy while waiting for a slow-acting DMARD to become effective. The mechanism of action of NSAIDs is the inhibition of COX. COX is critical in the metabolism of arachidonic acid to the pro-inflammatory prostaglandins. Two isoforms of COX

have been extensively studied in humans: COX-1 and COX-2. COX-1 is expressed constitutively in the stomach, intestines, kidneys, and platelets and is involved in functions such as gastric protection from hydrochloric acid. COX-2, a predominantly inducible isoenzyme, is involved in the production of prostaglandins E2 and I2, which are upregulated in the inflammatory response (Vane and Botting 1998). NSAIDs are usually well tolerated for short periods of time; however, with chronic use they sometimes lead to gastrointestinal complications, such us ulcer formation, perforations, and bleeding (Chiba, Sato et al. 2005). Another potential adverse effect of NSAIDs is their renal toxicity.

Glucocorticoids.

Glucocorticoids have been widely used in the treatment of RA. Although high doses of glucocorticoids clearly cause unacceptable toxicity, daily doses of prednisone (or its equivalent) of ≤15 mg may diminish pain and swelling in many patients. This dosage of glucocorticoids may also have a limited disease-modifying effect in patients with RA (van Everdingen, Jacobs et al. 2002). First, they promote the expression of lipocortin-1, which inhibits the enzyme phospholipase A2 and the generation of arachidonic acid. Second, their transcription products inhibit the action of NF-κB and AP-1 protein, which act to upregulate proinflammatory molecules, like TNF and IL-1. Third, they exert an effect through membrane-associated receptors and second messengers-a nongenomic effect (De Bosscher, Schmitz et al. 1997). Glucocorticoids present a large number of adverse effects such us, diabetes, hypertension, peptic ulcer, among others. Given the slow onset of action of traditional DMARDs, low doses of glucocorticoids are often used as a bridge therapy to control symptoms until the DMARDs or biological agents become effective. Monotherapy with glucocorticoids is not recommended.

DMARDs

Methotrexate

Methotrexate low dose is used as an immunosuppressant in autoimmune and rheumatic diseases. Methotrexate treatment has demonstrated effectiveness, reliability, sustained long-

term action and high tolerability. Up to four mechanisms of action have been proposed for the drug. First, it works as an antifolate agent (Quemeneur, Gerland et al. 2003). Second, methotrexate may diminish the accumulation of toxic compounds, especially polyamines, which contribute to tissue injury in RA (Cronstein 2005). Third, methotrexate may reduce intracellular levels of glutathione (Phillips, 2003). Finally, it increases extracellular adenosine levels, an anti-inflammatory action (Montesinos, Desai et al. 2003). All of these mechanisms induce inhibition of the immune cellular proliferation and strong anti-inflammatory activity. Potential adverse effects of methotrexate include hepatitis and cirrhosis, oral ulcers, cytopenias, and interstitial pneumonitis. According these side effects, it is important monitoring patients receiving methotrexate treatment (Kremer, Alarcon et al. 1994).

Hydroxychloroquine

Hydroxychloroquine is an antimalarial that has been introduced as therapy for RA. The efficacy of hydroxychloroquine depends on the interference with antigen presentation; lysosomal membrane stabilization, and inhibition the metabolism of deoxyribonucleotides (Fox and Kang 1993, Weber and Levitz 2000). Hydroxychloroquine is rarely used as monotherapy for RA; its main clinical utility is in combination with other DMARDs.

Sulfasalazine

Sulfasalazine is cleaved in the intestine by bacterial organisms into 5-aminosalicylic acid and sulfapyridine. Sulfapyridine seems to exert the therapeutic action after intestinal absorption. The mechanism of action of sulfasalazine is based on neutrophil function inhibition, immunoglobulin levels reduction, and interference with T-cell function via suppression of NF-κB activation (Carlin, Djursater et al. 1992, Gadangi, Longaker et al. 1996). The most common toxicities are gastro intestinal-related (nausea, vomiting, diarrhea, abdominal pain) and hematologic in nature (neutropenia, thrombocytopenia), but the drug is generally considered safe and well-tolerated (Symmons, Salmon et al. 1988).

Leflunomide

Leflunomide is an immunomodulatory drug, is a competitive inhibitor of dihydroorotate dehydrogenase, enzyme required for the de novo synthesis of pyrimidines, necessaries for the activation of lymphocytes. Therefore, blockade of the pyrimidine-synthesis pathway has antiproliferative effects. Leflunomide is generally well tolerated; hepatotoxicity and gastrointestinal intolerance are its most important adverse reaction (Olsen and Stein 2004). It is most commonly used as an alternative for methotrexate-intolerant patients.

Other antirheumatic drugs

Gold salts, their mechanism of action include the reduction of circulating B cells, immune complexes, rheumatoid factor, and immunoglobulin levels (Hirohata, Nakanishi et al. 1999). Long treatment with gold salts could present toxic reactions, including mucocutaneous reactions, proteinuria, and cytopenias.

Minocycline, from tetracycline family, in addition to their antimicrobial action, it has anti-inflammatory and immunomodulatory properties (O'Dell, Paulsen et al. 1999). Among its side effects, include autoimmune syndromes (serum sickness, polyarthritis, druginduced lupus, and vasculitis), headaches, GI intolerance, and a greying skin pigmentation.

Cyclosporine, a calcineurin inhibitor demonstrated efficacy in the treatment of RA (Sawitzke AD 2005). However, given the availability of safer DMARDs and biological agents, its role is now limited to a third-line agent after failure of first- and second-line drugs.

Biological agents

TNF- α antagonist

TNF-α, an inflammatory cytokine, that is important in the pathogenesis of RA, binds to two receptors, the type 1 TNF receptor (p55) and the type 2 TNF receptor (p75), that are expressed on many types of cells. The biologic activity of TNF-α can be attenuated by soluble TNF receptors. Patients with RA have high concentrations of TNF-α in the synovial fluid. TNF-α is localized to the junction of the inflammatory pannus and healthy cartilage, and high synovial fluid TNF-α concentrations are associated with the erosion of bone (Olsen and Stein 2004).

Infliximab

Infliximab is a chimeric molecule that joins the Fc region of human immunoglobulin G1 (IgG1) with the variable region of a mouse antibody against TNF-α (Elliot, Maini et al. 2008). It binds to soluble and membrane-bound TNF-α with high affinity, impairing the binding of TNF-α to its receptor; inducing a complement and antibody-dependent response against cells that express TNF-α. Infliximab can be used as monotherapy, but administration infliximab plus methotrexate is more effective (Lipsky, van der Heijde et al. 2000).

Etanercept

Etanercept is a soluble molecule composed of two recombinant p75 TNF-receptor proteins fused together to form a dimer (Nestorov 2005). Each of these molecules is linked to the Fc portion of human IgG1 to provide them a longer half-life. Etanercept is indicated in the treatment of refractory RA to others DMARDs included methotrexate.

Adalimumab

Adalimumab is a recombinant human IgG1 monoclonal antibody that binds to human TNF-α with high affinity, both impairing cytokine binding to its receptors and lysing cells that express TNF-a on their surface. It has a long circulating half-life (approximately 10–20 hours) and can be self-administered. Adalimumab is indicated in the treatment of refractory RA to others DMARDs included methotrexate.

IL-1 antagonist

Anakinra

IL-1 is a pro-inflammatory cytokine produced by monocytes, macrophages, and some specialized cells in the synovial lining, has inflammatory effects that include the induction of IL-6 and COX-2. In patients with rheumatoid arthritis, levels of IL-1-receptor antagonist in the damaged joint are diminished. Anakinra is a recombinant form of human interleukin- 1–receptor antagonist that targets the type I interleukin-1 receptor that is expressed in many tissues.

Anakinra in combination with methotrexate are indicated in patients who have not responded to methotrexate monotherapy (Cohen, Hurd et al. 2002).

Anti-CD20

Rituximab

Rituximab is a monoclonal antibody directed against an antigen, CD20, on the surface of all B cells other than stem cells and pre-B lymphocytes. It does not affect plasma cells since they do not possess CD20 on their cell surface. It exerts its effect by binding to CD20 on B cells and causing cell lysis by both complement-dependent and antibody-dependent cell mediated cytotoxicity. It has efficacy in RA patients who have failed conventional DMARDs and in RA patients who have failed anti- TNF agents (Edwards, Szczepanski et al. 2004, Cohen, Emery et al. 2006).

Anti-CD80/86

Abatacept

Abatacept is a fusion protein linking the extracellular domain of human cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) to the Fc portion of human IgG1. It works by competing for the binding between CD28 on the T cell and CD80/86 on the antigen-presenting cell. This is an important co-stimulatory signal that is essential for T cell activation (Fan and Leong 2007). It is effective in patients who have an inadequate response to either methotrexate or one or more TNF-inhibitors (Genovese, Becker et al. 2005).

IL-6 antagonist

Tocilizumab

Tocilizumab is a humanized anti-human IL-6 receptor antibody that specifically inhibits the biological activity of IL-6 by competitively inhibiting the binding of IL-6 to the IL-6 receptor (Paul-Pletzer 2006). Tocilizumab monotherapy has been shown to be effective in the treatment of RA and may be an option for patients that have side effects with combination biologics and DMARDs (Mima and Nishimoto 2008).

2. Nutritional therapy in RA

Clinical characteristics of RA justify its multidisciplinary approach, within which nutritional intervention is included. In the last years, it has been highlighted that consumption of some foods as well as a nutritive function, have a profound influence on health outcomes. Certain nutritional components influence the cellular metabolism and interfere in the pathological inflammatory process, so that they may act as coadjutant in the treatment of many inflammatory diseases, among which RA is included (Sales, Oliviero et al. 2009, Gonzalez Cernadas, Rodriguez-Romero et al. 2014).

Importantly, recent research has suggested that dietary patterns such as the traditional Mediterranean diet of countries that surround the Mediterranean Sea may confer protection from certain chronic diseases related to oxidative stress, inflammation and the immune system. Essentially, the traditional diet emphasizes foods from plant sources such as vegetables, fruits, nuts, and grains, fish , limited meat consumption, small amounts of wine and olive oil as the main fat source. The beneficial effects of the Mediterranean diet has been proven not only to cardiovascular diseases but also for diabetes, obesity, arthritis and cancer (Cardeno, Sanchez-Hidalgo et al. 2013). Evidence points out that Mediterranean diet decreases both pain and disease activity leading to better outcomes, and decreasing the doses of anti-inflammatory drugs, which exhibit important secondary effects (Smedslund, Byfuglien et al. 2010).

Evidence on polyunsaturated fatty acids suggests that they produce clinical improvement and inhibitory effects over the RA inflammatory response. Fatty acids can influence inflammation through a variety of mechanisms, including acting via cell surface and intracellular receptors/sensors that control inflammatory cell signaling and gene expression patterns. Some effects of fatty acids on inflammatory cells appear to be mediated by, or at least are associated with, changes in fatty acid composition of cell membranes. Changes in these compositions can modify membrane fluidity, lipid raft formation, cell signaling leading to altered gene expression, and the pattern of lipid and peptide mediator production. During inflammatory diseases,

eicosanoids are produced from polyunsaturated fatty acids present in cellular membranes. Inflammatory activity of these molecules depends on the nature of their precursors (Calder 2011).

Cells involved in the inflammatory response are typically rich in the n-6 fatty acid arachidonic acid, so when arachidonic acid (n-6) is present, pro-inflammatory molecules are released. Whereas eicosapentaenoic acid (EPA) (n-3)-derived eicosanoids are weakly inflammatory but the contents of arachidonic acid and of the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be altered through oral administration of EPA and DHA.

In this way, fish oils, rich in n-3 polyunsaturated fatty acids, increase the content of eicosapentaenoic-eicosanoids and decrease arachidonic acid in immune and endothelial cells leading to a lower inflammatory activity. EPA and DHA give rise to resolvins, which are anti-inflammatory, and inflammation resolving. Mechanisms underlying the anti-inflammatory actions of marine n-3 fatty acids also include disruption of lipid rafts, inhibition of activation of the pro-inflammatory transcription factor nuclear factor kappa B so reducing expression of inflammatory genes, activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor γ and binding to the G protein coupled receptor GPR120 (Calder 2015). Thus, fatty acid exposure and the fatty acid composition of human inflammatory cells influences their function. As a result of their anti-inflammatory actions marine n-3 fatty acids have therapeutic efficacy in rheumatoid arthritis, although benefits in other inflammatory diseases and conditions have not been unequivocally demonstrated (Calder 2012).

Fish oil has been shown to slow the development of arthritis in animal models and to reduce disease severity. A number of randomized controlled trials of marine n-3 PUFAs have been performed in patients with RA. Evidence is seen for a fairly consistent, but modest, benefit of marine n-3 PUFAs on joint swelling and pain, duration of morning stiffness, global assessments of pain and disease activity, and use of non-steroidal anti-inflammatory drugs The therapeutic dose of n-3 fatty acids is not clear (Miles and Calder 2012).

Recently a randomized controlled trial (RCT) of high-dose v. low-dose fish oil in recent-onset RA demonstrated that the group allocated to high-dose fish oil had increased remission and decreased failure of DMARD therapy. In addition, fish oil was associated with benefits additional to those achieved by combination 'treat-to-target' DMARDs with similar methotrexate use. These included reduced triple DMARD failure and a higher rate of the American College of Rheumatology remission (Smedslund, Byfuglien et al. 2010).

As regards with olive oil , and specifically the beneficial effect of extra virgin olive oil (EVOO) consumption has been ascribed to non-polar lipids or its high monounsaturated fatty acid (MUFA) content present in the major fraction of EVOO (98–99%) (Berbert, Kondo et al. 2005, Bermudez, Lopez et al. 2011). However, EVOO also contains multiple minor components with important biological properties. Nowadays, it is clear that many of the beneficial effects of ingesting EVOO are due to its minor polyphenol compounds such as flavonoids, lignans (acetoxypinoresinol), secoiridoids (oleuropein-aglycone and ligstroside aglycone) and their hydrolysis products hydroxytyrosol (HT) and tyrosol (Ty), respectively, among others.

Recently we have evaluated the effects of dietary EVOO on type II collagen-induced arthritis (CIA) in mice. EVOO diet significantly reduced joint edema and cartilage destruction, preventing the arthritis development (Rosillo, Sanchez-Hidalgo et al. 2015). Besides, we also have studied the effects of the oral administration of the polyphenol extract (PE) from EVOO in the arthritis model of CIA in mice. We demonstrated that PE decreased joint edema, cell migration, cartilage degradation and bone erosion by reducing the levels of proinflammatory cytokines and prostaglandin E2 in the joint as well as the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 (Rosillo, Alcaraz et al. 2014). We could suggest that the responsibility for such beneficial properties could to be assigned to both an adequate fatty acid profile of EVOO and the high proportion of phenolic compounds in according with our recent data (Sanchez-Fidalgo, Cardeno et al. 2013, Rosillo, Alcaraz et al. 2014, Aparicio-Soto, Sanchez-Hidalgo et al. 2015). Also the presence of other valuable minor components present in the

unsaponifiable fraction of the oil such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, phytosterols with important antiinflammatory properties (Cardeno, Magnusson et al. 2014, Sanchez-Fidalgo, Villegas et al. 2015) could play a pivotal role. Moreover, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it is not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action.

Antioxidant supplements and diets have long been advocated for the treatment of RA, osteoarthritis (OA) and other inflammatory arthritis. In RA, reactive oxygen species and other free radicals are associated with the inflammation process via numerous pathways (Mahajan 2004). These include the role of nitrous oxide in regulating vascular tone, superoxide in fibroblast proliferation and hydrogen peroxide in the transcription of cytokines IL-2 and TNF-α. During inflammation, oxidation modifies low-density lipoproteins, inactivates α-1-protease inhibitor, damages DNA and causes lipid peroxidation. Reactive oxygen species also damage cartilage and the extracellular matrix and inhibit collagen and proteoglycan synthesis. Evidence that increased oxidative stress or deficient antioxidant status are important in the pathogenesis of RA comes from several studies .Epidemiological studies have shown that low intake of dietary antioxidants is associated with the incidence of RA. Furthermore, animal studies have demonstrated an anti-inflammatory role for some antioxidants including superoxide dismutase (SOD) and vitamin E in experimentally induced arthritis (Canter, Wider et al. 2007). Clinical trials testing the efficacy of vitamin E in the treatment inflammatory arthritis have been methodologically weak and have produced contradictory findings.

In addition, in a randomized clinical trial in which 64 women with RA who fulfilled the eligibility criteria were randomly allocated to an intervention or a control group. Vitamin K1 or placebo was administered to the participants for 8 weeks. Vitamin K1 supplementation did not alter joint destruction and immune status in the patients with RA compared with the controls (Shishavan, Gargari et al. 2015). Thus, there is presently no convincing evidence that selenium,

vitamin A, vitamin C, Vitamin K1 or the combination product selenium ACE is effective in the treatment of any type of arthritis.

Furthermore, to its well-documented involvement in mineral homeostasis, vitamin D seems to have broad effects on human health that go beyond the skeletal system. Prominent among these so-called non-classical effects of vitamin D are its immunomodulatory properties. The association of vitamin D deficiency with RA severity supports the hypothesis of a role for vitamin D in the initiation or progression of the disease, or possibly both. However, whether 25(OH)D status is a cause or consequence of RA is still incompletely understood and requires further analysis in prospective vitamin D supplementation trials (Jeffery, Raza et al. 2015).

Suboptimal vitamin D status was recently acknowledged as an independent predictor of cardiovascular diseases and all-cause mortality in several clinical settings, and its serum levels are commonly reduced in Rheumatoid Arthritis (RA). Patients affected by RA present accelerated atherosclerosis and increased cardiovascular morbidity and mortality with respect to the general population. In a recent study, RA patients with moderate disease activity presented with low vitamin D levels, low CD34+ cell count, increased PWV and cIMT; we found that vitamin D deficiency is associated to CD34+ cell reduction in peripheral blood, and with fibrinogen levels. This suggests that vitamin D might contribute to endothelial homeostasis in patients with RA (Lo Gullo, Mandraffino et al. 2015).

3. Dietary polyphenols in the control of RA

Polyphenols are the biggest group of phytochemicals, and many of them have been found in plant-based foods. Dietary polyphenols have receive much attention among nutritionist, food scientist and consumer due their roles in human health (Tsao 2010). More than 8000 phenolic structures are currently known, and among them over 4,000 flavonoids have been identified (Pandey and Rizvi 2009).

It has been reported that dietary polyphenols have pronounced anti-inflammatory, anti-cancerous and immunomodulatory effects to reduce the onset of disease progression (Liu 2003).

Their beneficial effects on rheumatoid arthritis derive from their ability to the regulation of B and T cell responses, the inhibition of relevant signalling pathways such as JAK-STAT, MAPKs and NF-κB thus controlling the production of inflammatory mediators such as cytokines, chemokines, proinflammatory enzymes (Fouda and Berika 2009, Hou, Wu et al. 2009).

Most of the phytochemicals are represented by polyphenols, which are divided into several classes according to their chemical structures (Bruneton 2001).

- **Phenolic acids** that are present in artichoke, green coffee, tea, rosemary and other fruits. They can be further divided into two main types, benzoic acid (C_6-C_1) and cinnamic acid (C_6-C_3).
- **Lignans** that are present in sesame and line seeds. It has been described their antimicrobial, antifungal and antitussive proprieties.
- **Diarylheptanoids and arylalkanones** are derived from phenylpropane acid, including curcuminoids and gingerols.
- **Stilbenes** present in the human diet in low quantities. Resveratrol is the main studied compound of this group.
- **Flavonoids** including six groups: Flavones, Flavonols, Flavanones, Flavanols, Anthocyanins and Isoflavones.

A wide range of evidences have demonstrated the anti-inflammatory and antiarthritic effects of several dietary polyphenols *in vitro* (Table 1) and *in vivo* (Table 2).

3.1. Phenolic acids.

Phenolic acids can be further divided into two main types, benzoic acid (C_6-C_1) and cinnamic acid (C_6-C_3). While fruits and vegetables contain many free phenolic acids, in grains and seeds (particularly in the bran or hull) phenolic acids are often in the bound form. There are some evidences *in vitro* and *in vivo* studies where demonstrates that some of these phenolic acids could show antiarthritic effects.

3.1.1. Benzoic acid derived

Yoon et al. have shown anti-inflammatory effects of gallic acid using fibroblast-like synoviocytes (RA-FLS) cells isolated from the synovial tissues of patients with RA. They demonstrated that gallic acid could induce apoptosis in RA-FSL through regulation of apoptosis related protein expressions such as caspase-3, Bcl-2, Bax, p53 and pAkt. As well, gallic acid was able to reduce pro-inflammatory cytokines (IL-1β, IL-6), chemokines (CCL-2/MCP-1, CCL-7/MCP-3), COX-2, and MMP-9 gene expression (Yoon, Chung et al. 2013). On the other hand, treatment with protocatechuic acid significantly reduced the paw swelling in adjuvant-induced arthritis (AIA) in rats. Additionally, these treatment could decease lipid peroxides (LPO) expression and reactive free radicals nitric oxide (NO) levels and increase antioxidant enzyme expression, such as, superoxide dismutase (SOD), catalase and glutathione (GSH) in liver. The antiarthritic activity of protocatechuic acid was found to be equivalent to that of diclofenac sodium (Lende, Kshirsagar et al. 2011).

3.1.2. Cinnamic acid derived

It is well know that T cells are involved in initialling disease process and, even more, emerging evidence suggests that these cells also perpetuate RA pathogenesis at the late stage by secreting various mediators, such as receptor activator of NK-kB ligand (RANKL), IL-17 and osteopontin, which influence osteoclasts and FLS (Cho, Yoon et al. 2004, Xu, Nie et al. 2005). Rosmarinic acid has shown has apoptotic activity toward T cells from RA patients and further verified target T cell subsets. CD3+CD25+ activated T-cell subsets from most of the RA patients displayed significantly higher apoptosis rates than did the PBMCs and total CD3+ T cells. Furthermore, activated and effector CD4+ T cells, including CD4+CD25+ and CD4+CD45RO+ T cells, had a tendency of being more susceptible to rosmarinic acid-induced apoptosis than that of resting and naïve T-cell subsets. Rosmarinic acid was able to induce the release of cytochrome *c* from mitochondria and the blockage of mitochondrial depolarization inhibited apoptosis (Hur, Suh et al. 2007). On the other hand, Hsu et al., have reported that Rosmarinic acid decreased

the number of multinucleated osteoclasts in a dose-dependent manner using osteoclast-precursor cell line RAW264.7 stained with tartrate-resistant acid phosphatase (TRAP). The effects of this phenolic acid on osteclatogenesis were further confirmed by using primary murine bone marrow-derived macrophages (BMM) stimulated with RANKL. Rosmarinic acid was able to significantly reduced the *c-fos,* nuclear factor of activated T cells c1*(NFATc1)*, *TRAP, OSCAR, DC-STAMP, and ß3 integrin* mRNA expression through inhibition of phosphorylation of p38 and ERK MAPKs and blocking the IkB degradation and supressing the subsequent nuclear translocation of NF-kB p65 (Hsu, Cheng et al. 2011). Similar activity showed chlorogenic acid who inhibited RANKL- mediated osteoclast differentiation by down-regulation of phosphorylation of p38, JNK and ERK MAPKs and IkB degradation. Chlorogenic acid, also supressed the mRNA expression of *NFATc1, TRAP* and *OSCAR* in RANKL-treated BMMs (Kwak, Lee et al. 2013). According to the *in vivo* studies, rosmarinic acid has been verified that ameliorated CIA, as manifested by reduction of synovitis and depletion of COX-2 positive cells in affected joints (Youn, Lee et al. 2003). The immunomodulatory effect of chlorogenic acid, was described by Chauhan et al. in AIA in rats. In this model, chlorogenic acid was able to control the total (CD3) and differentiated (CD4 and CD8) T cells count and supressed CD80/86 co-stimulatory molecule. Also this phenolic acid had the ability of diminish Th1 cytokines (IL-2, IFN-γ and IL-12) and elevate Th2 cytokines (IL-4 and IL-10) in $CD4^+$ cells (Chauhan, Satti et al. 2012). *p*-cumaric acid also showed immunomodulatory and anti-inflammatory in AIA in rats. The cinnamic acid derived improved the arthritic disease through reduced the macrophage phagocytic index, circulating immune complex in sera and synovial TNF-α expression (Pragasam, Venkatesan et al. 2013).

3.2. Flavonoids

3.2.1. Flavones

Apigenin and luteolin are flavones abundantly present in common fruits and vegetables such as chamomile tea and celery among others food. It has been demonstrated that apigenin has potential as antiarthritic agent using *in vitro* model (Shin, Kim et al. 2009). This dietary- plant

flavonoid was able to inhibit the proliferation of RA-FSLs by inducing apoptosis through activation of the effectors caspase-3 and caspase-7. Apigenin treatment resulted in activation of ERK MAPK, and when the cells were treated with the ERK inhibitor, apigenin-induced apoptosis was reduced.

On the other hand, the authors demonstrated that the induction of the apoptosis and the activation of ERK by apigenin, it was due to the apigenin-induced generation of ROS (Shin, Kim et al. 2009). Similar results showed Sun et al., apigenin treatment was able to induce apoptosis of RA-FSLs, which was coupled with increased caspase-3 expression and activity and decreased Bcl-2/Bax ratio. These results were likely associated with downregulation of PI3-K/Akt activity (Sun, Jiang et al. 2012). Hou et al., reported that luteolin inhibited the proliferation of collagen-induced arthritis (CIA) rat synovial fibroblasts, which was accompanied by a decrease in MMP-1 and MMP-3 secretion. Besides, cytokine production (IL-6, IL-8, IL-15 and TGF-β) were found to be lower in CIA rat synovial fibroblasts treated with luteolin. The authors also demonstrated that luteolin treatment caused a delay of cells in the G2/M phase and this polyphenol inhibited the MAPK/ERKs and PI3K-Akt pathways (Hou, Wu et al. 2009). Other *in vitro* study on SW982 cell line, demonstrated that luteolin treatment resulted in inhibition of IL-1β-induced MMP-1, MMP-3, TNF-α and IL-6 production. Moreover, IL-1b-induced activator protein-1 (AP-1) and NF-κB activation were inhibited by luteolin (Choi and Lee 2010). Nobiletin is a citrus flavone present especially in tangerines. Nobiletin shown anti-inflammatory effects since it was able to downregulate the production of MMP-1, MMP-3 and MMP-9 and PGE_2 in IL-1 stimulated rabbit synovial fibroblast, moreover this citrus polyphenol, inhibited the proliferation of these cells (Ishiwa, Sato et al. 2000). This citrus flavone ameliorated CIA in mice. The reduced arthritis index and severity were accompanied by a suppression of osteoclastogenesis and TRAP activity in RAKNL-stimulated RAW264.7 cells. Moreover, nobiletin treatment resulted in inhibition of activation of MAPKs as well as AP-1/NF-kB pathways (Murakami, Song et al. 2007). On the other hand, in another CIA study, nobiletin was able to

improve the severity of damage and the authors, also demonstrated that this flavone reduced aggrecanase-1/a disintegrin and MMP with thrombospondin-like motifs (ADAMTS)-4 and ADAMTS-5 mRNA expression in CIA mice (Imada, Lin et al. 2008).

3.2.2. Flavonols

Quercetin, kaempferol and myricetin are found in several foods, including onions, berries, grapes and red wine and exhibit a wide range of antiarhritic effects. Sato et al. demonstrated in 1997 that quercetin supresses IL-8 and MPC-1 mRNA in human synovial fibroblats stimulate with TNF-α. H_2O_2 mediated induction of IL-8 and MCP-1 genes was also inhibited by quercetin in synovial fibroblats. Besides quercetin inhibited the induction of NF-kB by TNF-α (Sato, Miyazaki et al. 1997). Other study in RA-FLS treated with quercetin reported that quercetin inhibited the IL-1β-induced proliferation. In addition, quercetin downregulated the expression of MMP-1, MMP-3, COX-2 and production of PGE_2 in RA-SFs via inhibition of IL-1β-induced activation of NF-kB and phosphorylation of p38, JNK and ERK MAPKs pathway (Sung, Lee et al. 2012). The apoptotic pathway of quercetin in RA-SFs was described by Xiao et al., DNA fragmentation assay showed that quercetin elevated the apoptosis of RA-SFs, accompanying with enhanced caspase-3 and caspase-9 cleavages. Moreover, quercetin caused loss of mitochondrial membrane potential and cytochrome C release to cytosol and also decreased Bcl-2/Bax ratio. Additionally, quercetin elevated phosphorylation at ser15. Indicating that quercetin-induced apoptosis of RA-SFs was through mitochondrial pathway, in which p53 played an important role (Xiao, Hao et al. 2013). In vitro antioxidants studies have revealed the scavenging activity of ABTS and DPPH radicals, inhibition of nitric oxide and the superoxide radical scavenging capacity of quercetin (using thioglycolic acid-capped cadmium telluride quantum dots (QDs) as nanocarrier) (Jeyadevi, Sivasudha et al. 2013). Administration of QDs-quercetin complex showed a reduction in inflammation and improvement in cartilage regeneration in AIA in rats. Histology of hind limb tissue confirmed the complete cartilage regeneration in AIA rats treated with QDs-quercetin complex. This treatment reduced the expressions lipid peroxidation

and showed increase in activities of antioxidant enzymes such as SOD, GSH, glutathione peroxidase (GPx), catalase (CAT) levels in paw tissue. C-reactive protein, rheumatoid factor, red blood cells and white blood cells count and erythrocyte sedimentation rate of experimental animals were also brought back to normal levels (Jeyadevi, Sivasudha et al. 2013). Oral administration of quercetin in rat model of AIA ameliorated all markers of inflammation such as MCP-1, IL-1β and C-reactive protein and markers of oxidative stress such as 13/15 LOX restoring plasma antioxidant capacity. A protective effect of quercetin was verified being that HO-1 protein levels were upregulated in the animals treated. In this study, the authors deepened signalling pathways possibly involved, in this way, they demonstrated that quercetin was able to inhibit phosphorylation of ERK MAPKs and activation of NF-kB pathway (Gardi, Bauerova et al. 2015). On the other hand, kaempferol has also shown exert anti-inflammatory and anti-arthritic proprieties. As describe Yoon et al., kaempferol inhibits IL-1β- induced proliferation of RA-SFs by apoptotic way. Likewise, kaempferol treatment downregulated the expression of MMP-1, MMP-3, COX-2 and the production of PGE_2 in IL-1β-stimulated RA-SFs. According to the involved pathways, this flavonol inhibited the phosphorylation of p38, JNK and ERK MAPKs and the activation of NF-kB signalling pathways (Yoon, Lee et al. 2013).

A recent study testing a possible immunomodulatory activity of kaempferol in arthritis showed that kaempferol could strengthen the suppression function of Treg cells and prevent the pathological symptom of CIA in a rat model. Mechanistically, kaempferol treatment could stabilize FOXP3 protein level in vivo and in vitro. Furthermore, this polyphenol could inhibit PIM1-mediated FOXP3 phosphorylation at S422 to promote FOXP3 transcriptional activity (Lin, Luo et al. 2015). Finally, Lee et al., demonstrated that myricetin was able to decreased IL-1β-induced production of IL-6 and MMP-1 in SW982 syovial cells. Moreover, myricetin diminished the phosphorylation of p38 and JNK MAPKs (Lee and Choi 2010).

3.2.3. Flavanones

Flavanones are polyphenolic compounds highly and almost exclusively present in citrus. Experimental observation have shown that these compounds, including hesperidin and naringin present anti-inflammatory and antiarthritic proprieties. Orally administration of hesperidin to CIA mice, result in improvement of clinical scores. Furthermore, hesperidin treatment was able to improve histological features such as reduced the damage of interchondral joints and supressed the increases of infiltration of inflammatory cells and pannus formation. This citrus flavonoid also, downregulated TNF-α mRNA expression in lesions (Kawaguchi, Maruyama et al. 2006). Similarly results, have been demonstrated by Li et al., using a model of AIA in rats. Hesperidin supressed secondary paw swelling and reduced the polyarthritis index of AIA rats. These macroscopic results were companied by amelioration of joint destruction and inhibition of bone destruction, synovial hyperplasia and inflammatory cells infiltration. In addition, hesperidin enhanced concanavalin-A (ConA)-induced T-lymphocyte proliferation and recovered ConA-induced IL-2 production by solenocytes in comparison with AIA control group. Production of inflammatory cytokines such as IL-1, IL-6 and TNF-α were also determinate in peritoneal macrophages from treated rats and stimulated with LPS. In this way, hesperidin was able to inhibit the production of these pro-inflammatory cytokines (Li, Li et al. 2008). Other study of hesperidin in CIA model in rats reveals the anti-inflammatory and antioxidant effects of this molecule. Hesperidin treatment supressed the evolution of CIA rats and ameliorated the changes at histological level and was able to restore damage alteration to a great extent. Administration of hesperidin showed a significant reduction in neutrophil activation and infiltration in the joints, also decreased TBARS concentration by inhibiting lipid peroxidation in the cartilage tissue. The antioxidant enzymes GSH and SOD were upregulated by hesperidin, and cartilage catalase activity and nitrite content were lowered (Umar, Kumar et al. 2013).

Finally, the anti-inflammatory and antiarthritic effect of naringin were shown using an AIA rat model. In this study, administration of naringin supressed the paw swelling in AIA rats

and decreased histopathological changes of joint tissues. Moreover, naringin suppressed the production of TNF-α, IL-1β and IL-6 in serum of arthritic rats. TUNEL assay demonstrated that naringin induced apoptosis of AIA synovial cells via regulation of the protein expression of Bcl-2 and Bax (Zhu, Wang et al. 2015).

3.2.4. Isoflavones

Soy bean isoflavones have been identified as phytochemical therapeutic flavonoids for the treatment of RA. Genistein and daidzein possess structural similarities with selective estrogen receptor modulators such as tamoxifen and synthetic isoflavones (Mohammad-Shahi, Haidari et al. 2011). *In vitro* studies have revealed the anti-inflammatory and antiarthritic proprieties of genistein in human synovial fibroblasts. Genistein inhibited anchorage-dependent proliferation of synovial cells induced by IL-1β, TNF-α, or EGF, virtually inhibited S-phase entry, caused cell cycle arrest in G1 restriction point and inhibited colony growth of rheumatoid synovial cells induced by EGF. Genistein also downregulated MMP-2 and MMP-9 expression induced by IL-1β or TNF-α (Zhang, Dong et al. 2012). Genistein treatment decreased the secretion of IL-1β, IL-6, and IL-8 from TNF-α-stimulated MH7A cells. Furthermore, genistein prevented TNF-α-induced NF-κB translocation as well as phosphorylation of IκB kinase-α/β and IκBα, and also suppressed TNF-α-induced AMPK inhibition (Li, Li et al. 2014).

In vivo studies have also shown antiarthritic effect of isoflavones. One study reported that genistein orally treatment in CIA rats, decreased primary paw inflammation. Dietary genistein exhibited a different pattern of immunomodulation, augmenting lymphocyte activation with increased IL-4 secretion, but decreased IFN-γ by cultured spleen lymphocytes subjected to CIA inflammation (Wang, Zhang et al. 2008). Treatment with genistein and daidzein in CIA rats resulted in not only a reduction in disease symptoms but also a delay in the onset of symptoms. The ear thickness in treated rats was lowered. Prevention of the tissue damage and joint inflammation was also observed following treatment with two soy isoflavones. Genistein and daidzein were able to reduce TNF-α, IL-6, adiponectin and leptin serum concentrations

(Mohammad-Shahi, Haidari et al. 2011). Mohammad-Shahi demonstrated that genistein and daidzein also played an important role on the extra-articular complications in CIA model in rats. In this model, both isoflavones were able to restore paraoxonase and arylesterase enzyme activity, which was decreased in serum from CIA rats. Paraoxonase low concentration is associated with a lower HDL- cholesterol and a higher level of malondialdehyde and C-reactive protein. Genistein and daidzein could also reverse the increased levels of malondialdehyde resulting from CIA (Mohammadshahi, Haidari et al. 2013).

3.2.5. Antocyanins

Anthocyanin belongs to a subgroup of bioactive flavonoids responsible for the blue, purple, and red colour of many fruits, flowers, and vegetables such as grapes, pommegranate and berries. It has been shown to have protective effects in *in vitro* and *in vivo* arthritis models. Malvidin, major grape anthocyanin, was able to improve AIA clinical score; both therapeutic and preventive oral protocols of malvidin were efficient in reducing arthritic signs. Finally, the level of nitrites was decreased following both therapeutic and preventive treatment with malvidin from peritoneal macrophages. Thus confirming the reduction of macrophages inflammatory state *in vivo* in the presence of malvidin (Decendit, Mamani-Matsuda et al. 2013). Delphinidin, presents in cranberries and Concord grapes, was found to be a specific inhibitor of histone acetyltransferase. Delphinidin treatment inhibited NF-kB function by inhibiting acetylation-dependent nuclear translocation of p65 in TNF-α-stimulated human rheumatoid arthritis synovial cell line (MH7A). Furthermore, this antocyanin efficiently suppressed TNF-α-induced expression of IL-6, COX-2, and IL-1β in MH7A cells (Seong, Yoo et al. 2011). Cyanidin-3-glucoside inhibited receptor activator of RANKL-mediated osteoclast and formation and downregulated the expression of osteoclast differentiation marker genes (*Acp5, CtsK, Oscar, Tm7sf4, Atp6v0d2,* and *Nfatc1)* Pretreatment differentiation with cyanidin-3-glucoside considerably reduced the induction of ERK, JNK, and p38 MAPKs by RANKL in osteoclast precursor cells. Furthermore, cyanidin-3-glucoside dramatically inhibited the expression of c-Fos and Nfatc1, which are

important transcription factors for osteoclast differentiation and activation. The formation of osteoclasts in coculture of bone marrow cells and calvaria-derived osteoblasts was also inhibited by cyanidin-3-glucoside treatment (Park, Gu et al. 2015).

3.2.6. Flavanols

Tea polyphenols have received considerable public attention due to the positive association between tea consumption and beneficial health effects. The putative health benefits attributed to green tea (*Camellia sinensis, Theaceace*) is due to high concentrations of polyphenolic compounds known as catechins, including epigallocatechin-gallate (EGCG), epigallocatechin (EGC), epicatechin-gallate (ECG), and epicatechin (EC).

One study showed that EGCG pretreatment inhibited both the constitutive and IL-1β-induced chemokine MCP-1/CCL2 production, regulated upon activation, normal T-cell expressed and secreted (RANTES/CCL5) production, growth-regulated oncogene (Gro-α/CXCL1) production, and epithelial neutrophil-activating peptide 78 (ENA-78/CXCL5) production, and MMP-2 activation by RA-SFs (Ahmed, Pakozdi et al. 2006). This was achieved by EGCG via selective inhibition of the IL-1β-induced protein kinase Cδ and NF-κB pathways (Ahmed, Pakozdi et al. 2006). It was also shown, that EGCG was effective in inhibiting IL-1β-induced MMP-1, MMP-3, and MMP-13 in human tendon fibroblasts (Corps, Curry et al. 2004). Synovial fibroblast IL-6 production has been shown to inhibit bone formation and to concomitantly stimulate bone resorption and pannus formation (Cronstein 2007). Other study showed that EGCG pretreatment inhibits IL-1β-induced IL-6 and vascular endothelial growth factor synthesis in RA-SFs (Ahmed, Marotte et al. 2008). Yun et al., showed that EGCG treatment resulted in inhibition of TNFα-induced production of MMP-1 and MMP-3 at the protein and mRNA levels in RA-SFs by inhibiting AP-1 DNA binding activity (Yun, Yoo et al. 2008). Ahmed et al., demonstrated that caspase-3 activation by EGCG suppressed RA-SFs growth, and this effect was mimicked by Akt and NF-κB inhibitors. Mcl-1 degradation by EGCG sensitized RA-SFs to TNFα- induced cleavage of poly ADP-ribose polymerase protein and apoptosis (Ahmed, Silverman et al. 2009).

EGCG-containing green tea extract in drinking water ameliorated CIA in mice. The reduced CIA incidence and severity was reflected in a marked inhibition of the inflammatory mediators COX-2, IFN-γ, and TNF-α in arthritic joints of green tea-fed mice. Additionally, total IgG and type II collagen-specific IgG levels were found to be lower in serum and arthritic joints of green tea-fed mice (Haqqi, Anthony et al. 1999). Morinobu et al., showed that EGCG treatment reduced bone resorption as determined by tartrate-resistant acid phosphatase positive multinucleated cells, bone resorption activity, and osteoblast-specific gene expression of the transcription factor NFATc1. They also studied the in vivo effect of EGCG in CIA mice, where administration of EGCG inhibited inflammation (Morinobu, Biao et al. 2008). Another study showed that EGCG ameliorated arthritis and macrophage infiltration, and caused a reduction in the amount of MCP-1/CCL2-synthesizing osteoblasts in rat CIA model (Lin, Chang et al. 2008). EGCG orally treatment ameliorated clinical symptoms, reduced histological scores in arthritic mice and also lowered serum type-II collagen IgG2a antibodies. EGCG significantly suppressed T cell proliferation and relative frequencies of CD4 T cells, CD8 T cells and B cell subsets including marginal zone B cells, T1 and T2 transitional B cells, while increasing the frequency of CD4+ Foxp3+ regulatory T cells (Tregs) and indoleamine-2,3-dioxygenase (IDO) expression by CD11b+ dendritic cells (DC). Joint homogenates from EGCG-fed mice exhibited increased levels of Nrf-2 and HO-1 compared with control CIA mice (Min, Yan et al. 2015). Intra-articular injection of EGCG in CIA mice, showed a significant reduction in cartilage degradation in prophylactic- and therapeutic-groups, by histomorphological scoring of the articular cartilage (Natarajan, Madhan et al. 2015). Administration of EGCG to rat adjuvant-induced arthritis resulted in an inhibition of IL-6 levels in the serum and joints of EGCG-treated animals. This study also showed that EGCG enhances the synthesis of soluble gp130 protein, an endogenous inhibitor of IL-6 signalling and trans-signalling. The inhibition of arthritis in EGCG-treated rats correlated to the reduction in MMP-2 activity in the joints compared with the activity level in arthritic rats (Ahmed, Marotte et al. 2008). Green tea extract administration in drinking water ameliorated rat adjuvant-

induced arthritis via the inhibition of serum IL-17 levels, with a concomitant upregulation of serum IL-10 levels (Kim, Rajaiah et al. 2008).

3.3. Diarylheptanoids and arylalkanones

3.3.1. Gingerols

Ginger (*Zingiber officinale, Roscoe*) is one of the most widely used spices and is a common condiment for a variety of foods and beverages. It contains a large number of phytochemical constituents.

Ginger extract treatment was able to inhibit TNF-α-induced IL-8 and IL-6 of human synovial cells, showing similar anti-inflammatory effect than betamethasone (Ribel-Madsen, Bartels et al. 2012). Ginger extract intraperitoneally could ameliorate the clinical scores, disease incidence, joint temperature, swelling and cartilage destruction, accompanying with reduction of serum levels of IL-1β, IL-2, IL-6, TNF-α and anti-CII IgG in CIA rats (Fouda and Berika 2009). The arthritic score was decreased after adjuvant arthritis induction in rats that received ginger and turmeric rhizomes powder. Histological examination of the ankle joints from arthritic rats treated with ginger and turmeric rhizomes powder revealed that, the treatment resulted in improvement of histopathological changes characteristic from arthritic joints. On the other hand, the mixture powder administration was able to suppress changes in haematological parameters of arthritic rats, such as leucocytosis, thrombocytosis, iron deficiency anaemia, serum hypoalbuminemia or globulinemia; and also improves biochemical parameters in sera, like lipid profile, protein content, kidney functions and oxidative stress. Suggesting, that ginger and turmeric rhizomes powder has beneficial effects against not only articular complications but also against many extra-articular complications of experimentally induced RA (Ramadan and El-Menshawy 2013).

3.3.2. Curcumin

Curcumin (diferuloylmethane) is a yellow pigment found in the rhizome of turmeric (*Curcuma longa L., Zingiberaceae*) which has a wide range of pharmacological and biological

activities. Curcumin has been shown protective effect against IL-1β-stimulated human chondrocytes. Treatment with curcumin relieved suppression of collagen type II (cartilage specific matrix marker) and β1-integrin synthesis and inhibited caspase-3 activation by IL-1β in human chondrocytes. Besides, chondrocytes treated with curcumin showed less severe cellular degeneration, caused by IL-1β stimulation, at the ultrastructural level; the chondrocytes regained a flattened shape and numerous microvilli-like cytoplasmic processes (Shakibaei, Schulze-Tanzil et al. 2005). Low concentration of curcumin was sufficient to inhibit IL-1β induced activation of NF-κB, caspase-3 and cyclooxygenase-2 in canine mesenchymal stem cells (MSCs), enabling growth factor induced chondrogenesis in MSCs. In IL-1β stimulated co-cultures of MSCs and primary chondrocytes, treatment with curcumin, enhanced the production of collagen type II, cartilage specific proteoglycans (CSPGs), β1-integrin, as well as activating MAPKs signalling and suppressing caspase-3 and cyclooxygenase-2, enabling co-culture induced chondrogenesis in MSCs (Buhrmann, Mobasheri et al. 2010). Studies of curcumin on RA SFs have been demonstrated that curcumin was able to inhibit proliferation of RA SFs through apoptotic way including inhibition of Blc-2 protein and X-linked inhibitors of the apoptosis protein (X-IAP) expression, proteolytic activation of caspase-3 and caspase-9, and the concomitant degradation of poly (ADP-ribose) polymerase protein (PARP). Furthermore, curcumin decreased the expression levels of COX-2 mRNA and protein, which was correlated with the inhibition of PGE_2 synthesis (Park, Moon et al. 2007). Other study has been shown that curcumin could inhibit IL-1β and PMA-induced IL-6 of RA SFs and MH7A cells. Curcumin treatment also blocked PMA-induced VEGF grow factor expression in RA SFs. Deepening signalling pathway, curcumin could inhibit IL-1β-induced phosphorylation of NF-kBp65 at SER536 and degradation of IκB-α also inhibit phosphorylation of ERK MAPK in MH7A cells. Lastly, curcumin induced cell death through apoptosis (Kloesch, Becker et al. 2013). Curcumin also inhibited inflammatory processes through suppression of collagenase and stromelysin expression in HIG-82 synoviocytes (Jackson, Higo et al. 2006).

Regarding *in vivo* studies, administration of curcumin intraperitoneally, caused attenuation of the arthritic index in CIA mice. It was accompanied by a reduction of B-cell-activating factor belonging to the TNF family (BAFF), IFN-γ and IL-6 sera levels after curcumin treatment (Huang, Xu et al. 2013). Other study shows that curcumin treatment ameliorated symptoms of arthritis in AIA rats and preserved radiological alterations in joints of arthritic rats. The blood of the treated rats presented less number of leukocytes in comparison with the control rats. Sera levels of TNF-α, MDA, nitrites, citrullinated protein antibodies and C-reactive protein were downregulated after curcumin administration. However, curcumin increase GSH, SOD and catalase activity (Arora, Kuhad et al. 2015). Zheng et al., confirmed the protective effect of curcumin in an AIA model in rats, curcumin treatment showed a therapeutic effect on AIA rats similar to methotrexate. The polyphenol was able to improve the histopathological changes, including less inflammatory cell infiltration, less synovial thickening and less proliferation of synovial tissue and fibrous tissue. TNF-α and IL-1β levels were decreased in synovial fluid and blood and NF-kB expression was lowered in the synovial tissue after curcumin administration (Zheng, Sun et al. 2015).

3.4. Stilbenes

3.4.1. Resveratrol

Resveratrol (trans-3,40,5-trihydroxystilbene) is a polyphenol found in various fruits and vegetables and is abundant in grape skins and red wines.

A number of studies have shown the apoptotic effect of resveratrol in RA-SFs (Byun, Song et al. 2008, Nakayama, Yaguchi et al. 2012). Byun et al., demonstrated for the first time that exposure to resveratrol caused apoptosis in RA-SFs through activation of caspase-9, caspase-3 and caspase-8 and PARP cleavage and mitochondrial cytochrome c release. Moreover, resveratrol induced apoptosis inducing the translocation of apoptosis-inducing factor (AIF) to the nucleus (Byun, Song et al. 2008). On the other hand, Nakayama et al., showed that resveratrol induces MH7A cell apoptosis by activating caspase-9 and the effector caspase-3 along mitochondrial

disruption as a result of reduced Bcl-XL expression, allowing cytochrome c release from the mitochondria into the cytosol, in a sirtuin 1-dependent manner (Nakayama, Yaguchi et al. 2012). Resveratrol also activated caspase-3/7, which has a pro-apoptotic effect and decreased the expression of genes related to cell proliferation and cell cycle in cultured IL-1β-stimulated RA-FLS (Glehr, Fritsch-Breisach et al. 2013). Resveratrol supressed TNF-α induced PI3kinase/Akt pathway activation by decreasing p-Akt phosphorylation correlated with downregulation of IL-1β and MMP-3 in RA-FLS (Tian, Chen et al. 2013). Additionally, a decreased of MMP-1, MMP-3 and MMP-9 production in the cell culture supernatant and a reduction on the RANKL and osteoprotegrin gene expression were obtained when resveratrol was analysed in IL-1β-stimulated RA-FLS (Glehr, Breisach et al. 2013). The anti-osteoclastogenic effect of resveratrol was prove by Shakibaei et al., they demonstrated that treatment with resveratrol inhibited the RANKL-induced formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and inhibited NF-kB activation and suppressed the activation of IκBα kinase and IκBα phosphorylation and degradation. In addition, activation of Sirt-1 (a histone deacetylase) by resveratrol induced Sirt-1-p300 association in bone-derived and preosteoblastic cells, leading to deacetylation of RANKL-induced NF-κB, inhibition of NF-κB transcriptional activation, and osteoclastogenesis (Shakibaei, Buhrmann et al. 2011). Resveratrol also, has been tested on *in vivo* arthritis models. Intraarticular injection of resveratrol in lipopolysaccharide-induced arthritis in rabbits was able to reduce histopathological damage in arthritis rabbits including decreased cartilage destruction, reduced loss of matrix proteoglycan content in the cartilage and diminished synovial inflammation (Elmali, Baysal et al. 2007). Other study shows how resveratrol could reduce disease incidence, number of involved paws, footpad thickness and clinical index of CIA mice. Resveratrol also markedly reduced mononuclear and polymorphonuclear cell infiltration into the joint, synovial hyperplasia and adjacent cartilage and bone erosion at histological level in treated CIA mice. Consistent with the attenuated clinical parameters, resveratrol prevented the development of serum collagen-specific IgG2a and IgG1.

Among the serum cytokine levels, IFN-γ, TNF-α, IL-17, IL-6, IL-1 and IL-4 levels were reduced. Moreover, isolated draining popliteal lymph nodes (DLN) CD4 T cells from resveratrol-protected mice showed reduced expression of IL-17 and IFN-γ; resveratrol also decreased the numbers of total cells, CD4 IL-17+ Th17 cells and or CD4 IFN-γ + Th1 cells in DLN of CIA mice (Xuzhu, Komai-Koma et al. 2012). Chen et al., demonstrated that resveratrol was able to reduce paw swelling and decrease the arthritis scores of AIA rats. Resveratrol also reduced the proliferation of concanavalin A-stimulated spleen cells. Articular cartilage degeneration with synovial hyperplasia and inflammatory cell infiltration was suppressed and the production of COX-2 and PGE2 in AIA rats was reduced by treatment with resveratrol (Chen, Lu et al. 2014).

3.5. Other dietary polyphenols

Besides dietary polyphenols described above, there are several non-flavonoid polyphenol found in foods that are considered important to human health. Among these, ellagitanins, hydrolysable tannins, and its derivates are found in berry fruits; on the other hand hydroxytyrosol, a phenolic alcohol and the secoiridoids oleuropein and oleocanthal are present in extra virgin olive oil (EVOO).

The pomegranate-derived polyphenols, punicalagin and ellagic acid (ellagitanins), inhibited MMP- 13-mediated degradation of CII *in vitro*. Multiple binding interactions of punicalagin and ellagic acid with CII were demonstrated by surface plasmon resonance studies and molecular docking simulations. Punicalagin inhibited the degradation of proteoglycan and CII release on IL-1β-induced degradation bovine cartilage. Anti-inflammatory effects of punicalagin were tested in an AIA rat model. Punicalagin was able to diminish the disease development and paw volume of AIA rats (Jean-Gilles, Li et al. 2013). Topical treatment with ellagic acid resulted in reducing joint swelling and inflammation-induced morphological changes in histological analysis of AIA in rats. Consistent with the attenuated paw swelling and histopathological changes, ellagic acid-topical was able to supress the levels of the pro-inflammatory cytokines TNF-α and IL1-β of arthritis rats (Mo, Panichayupakaranant et al. 2013).

Polyphenolics compounds of EVOO have also been shown antiarthritic effect *in vitro* and *in vivo.* In this way, oleocanthal decreased lipopolysaccharide-induced $iNOS_2$ synthesis in ATDC-5 murine chondrocytes without significantly affecting cell viability. Besides, oleocanthal pre-treatment was able to inhibit LPS-induced iNOS protein (Iacono, Gomez et al. 2010).

Other similar study, also showed that oleocanthal pre-treatment suppresses the expression of inflammatory mediators including IL-6 and MIP-1α at the protein and the mRNA level in LPS-stimulated ATDC-5 murine chondrocytes (Scotece, Gomez et al. 2012). Impellizzeri et al., confirmed the beneficial effects of oleuropein aglycone on an experimental model of CIA. Treatment with oleuropein aglycone ameliorated the clinical signs and improved histological status in the joint and paw of CIA mice. The degree of oxidative and nitrosative damage was also reduced in oleuropein aglycone-treated mice. Plasma levels of the proinflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokine (MIP-1α and MIP-2) expression and neutrophil infiltration (MPO) were also reduced by oleuropein aglycone in arthritic mice (Impellizzeri, Esposito et al. 2011). The protective effect of hydroxytyrosol in a CIA model in rats was carried out by Silva et al., in this study, they showed that hydroxytyrosol treatment decreased paw edema, histological damage, COX-2 and iNOS expression, and markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation, improving articular function in treated animals (Silva, Sepodes et al. 2015).

Tabla 1. *In vitro* studies of dietary polyphenols in RA.

Polyphenol	Source	Experimental System	Mechanism/s of Action	Efficacious []	Ref
Phenolic acids					
Benzoic acid derived					
Gallic Acid	Tea	RA-FSL	Gallic acid induces apoptosis of RA FLS through regulation of apoptosis related protein expressions (caspase-3, Bcl-2, Bax, p53 and pAkt) and reduces the expression of pro-inflammatory genes (IL-1β, IL-6), chemokines (CCL-2/MCP-1, CCL-7/MCP-3), COX-2, and MMP-9.	0.1 and 1 μM	(Yoon, Chung et al. 2013)
Cinnamic acid derived					
Rosmarinic Acid	Basil, rosemary, thyme and peppermint	RA PBMCs	Rosmarinic Acid induces the preferential apoptotic activity of activated T cells and effector T cells via mitochondrial pathway.	50 μM	(Hur, Suh et al. 2007)
		Murine bone marrow-derived macrophages	Rosmarinic acid inhibits RANKL-mediated osteoclast differentiation by down-regulation MAPKs and NF-kB pathway and suppressing *NFATc1*, *TRAP* and *OSCAR* mRNA expression	60 and 120 μM	(Hsu, Cheng et al. 2011)
Chlorogenic acid	Peach, prunes and green cofee	Murine bone marrow-derived macrophages	Chlorogenic acid inhibits RANKL-mediated osteoclast differentiation by down-regulation MAPKs and NF-kB pathway and suppressing *NFATc1*, *TRAP* and *OSCAR* mRNA expression	28, 70 and 141 μM	(Kwak, Lee et al. 2013)
Flavonoids					
Flavones					
Apigenin	Parsley, celery and chamomile tea	MH7A cells	Apigenin induces apoptosis through activation of the effectors caspase-3 and caspase-7 and activation of ERK MAPK.	100 μM	(Shin, Kim et al. 2009)
		RA-FSL	Apigenin induces apoptosis of RA-FSL through increase caspase-3 expression and activity and decrease Bcl2/Bax ratio, mediated by downregulation of PI3-K/Akt activity	20 μM	(Sun, Jiang et al. 2012)
Luteolin	Celery, broccoli, green pepper, parsley and thyme.	Rat synovial fibroblasts	Luteolin inhibits the proliferation of synovial fibroblasts in CIA rats. Decreases the secretion of MMP-1 and -3 and the expression of IL-6, IL-8, IL-15, and TGF-β, through inhibition of MAPK/ERKs and PI3K-Akt pathways.	20 μM	(Hou, Wu et al. 2009)
		SW982	Treatment with luteolin significantly inhibited IL-1β-induced MMPs (MMP-1 and -3) and cytokines (TNF-α and IL-6) production. Luteolin also inhibited JNK and p38 MAPKs activation and the transcription factors AP-1 and NF-κB.	1 and 10 μM	(Choi and Lee 2010)

Nobiletin	Tangerine	Rabbit synovial fibroblasts and articular chondrocytes.	Nobiletin downregulates the production of MMP-1, MMP-3 and MMP-9 and PGE_2 and COX-2 of IL--stimulated rabbit synovial cells and inhibits proliferation of rabbit synovial fibroblasts in the growth phase.	4, 8, 16, 32 µM	(Ishiwa, Sato et al. 2000)
		RAW264.7	Nobiletin suppresses RANKL-induced osteoclastogenesis in macrophages through inhibition of MAPKs and the resultant regulation of transcription factors (AP-1 and NFκB)	4, 20 and 50 µM	(Murakami, Song et al. 2007)
Flavonols					
Quercetin	Onion	RA-FSL	Quercetin suppresses TNF-α induced IL-8 and MCP-1 mRNA. H_2O_2 mediated induction of IL-8 and MCP-1 genes are inhibited by quercetin. Quercetin inhibites the activation of NF-kappa B by TNF-α.	50, 100 and 200 µM	(Sato, Miyazaki et al. 1997)
		RA-FSL	Quercetin inhibits IL-1β- induced proliferation of RA-FSL and downregulates MMP-1, MMP-3, COX-2 expression and PGE2 production. Through inhibition of ERK, p38, JNK phosphorylation and activation of NF-kB by IL-β	100 µM	(Sung, Lee et al. 2012)
		RA-FSL	Quercetin induces the apoptosis of RA-FSL, through enhances caspase-3 and caspase-9 cleavages and through mitochondrial pathway.	100, 200 and 300 µM	(Xiao, Hao et al. 2013)
Kaempferol	Apples, grapes, tomatoes, green tea, potatoes, onions, broccoli, Brussels sprouts, squash, cucumbers, lettuce, green beans, peaches, blackberries, raspberries, and spinach	RA-FSL	Kaempferol inhibites the proliferation of IL-1β-stimulated RA-FSL, as well as the mRNA and protein expression of MMP-1, MMP-3, COX-2 and PGE_2 induced by IL-1β. Through inhibition of ERK, p38, JNK phosphorylation and activation of NF-kB by IL-β.	100 µM	(Yoon, Lee et al. 2013)
Myricetin	Vegetables, fruits, nuts, berries, tea and red wine.	SW982	Myricetin decreases IL-1β-induced production of IL-6 and MMP-1 in synovial cells. Moreover, myricetin diminishes the phosphorylation of JNK and p38 MAPKs.	10 µM	(Lee and Choi 2010)
Isoflavones					
Genistein	Lupin, fava beans, soybeans, kudzu, and psoralen	RA-FSL	Genistein inhibits proliferation of RA-FSL induced by IL-1β, TNF-α and EGF; also inhibits MMP-2 and MMP-9 expression induced by IL-1β or TNF-α .	37 µM	(Zhang, Dong et al. 2012)
		MH7A cells	Genistein decreases the secretion of IL-1β, IL-6, and IL-8 from TNF-α-stimulated MH7A cells. Genistein prevented TNF-α-induced NF-κB translocation as well as phosphorylation of IκB kinase-α/β and IκBα, and suppressed TNF-α-induced AMPK inhibition.	5, 10 and 20 µM	(Li, Li et al. 2014)
Antocyanins					
Delphinidin	Grapes	MH7A cells	Delphinidin inhibits TNF-α-induced NF-κB function and suppresses expression of IL-6, COX-2 and IL-1β.	10 and 30 µM	(Seong, Yoo et al. 2011)
Cyanidin	Grapes, bilberry, blackberry,	Osteoclast precursor cells	Cyanidin inhibits osteoclasts formation and downregulates expression of osteoclast	100 µM	(Park, Gu et al. 2015)

	blueberry, cherry, cranberry		differentiation marker genes, inhibiting phosphorylation of MAPKs		
Flavanols					
EGCG	Green tea	RA-FSL	EGCG inhibits IL-1β-induced MCP-1/CCL2, RANTES/CCL5, Gro-α/CXCL1, ENA-78/CXCL5 production ans MMP-2 activation, via inhibition of protein kinase Cδ and NF-κB pathway.	10 and 20 μM	(Ahmed, Pakozdi et al. 2006)
		Human tendon fibroblast	EGCG inhibits IL-1β-induced MMP-1, MMP-3 and MMP-13	2.5 and 25 μM	(Corps, Curry et al. 2004)
		RA-FSL	EGCG inhibits IL-1β induced IL-6 and VEGF.	10 and 200 μM	(Ahmed, Marotte et al. 2008)
		RA-FSL	EGCG inhibits TNF-α-induced MMP-1 and MMP-3 expression by inhibiting AP-1 DNA binding activity.	0.12, 0.25 and 0.5 μM	(Yun, Yoo et al. 2008)
		RA-FSL	EGCG inhibits TNF-α induced Mcl-1 protein expression. EGCG actives caspase 3 activity, mediated via down-regulation of the TNFα-induced Akt and NF-κB pathways	50 μM	(Ahmed, Silverman et al. 2009)
Diarylheptanoids and arylalkanones					
Gingerol	Ginger (*Zingiber officinale*)	RA-FSL	Ginger extract inhibits TNF-α-induced IL-8 and IL-6		(Ribel-Madsen, Bartels et al. 2012)
Curcumin	*Curcuma longa*	Human chondrocytes	Curcumin relieves suppression of collagen type II, β1-integrin and caspase-3 activation.	50 μM	(Shakibaei, Schulze-Tanzil et al. 2005)
		Co-culture canine mesenchymal stem cells and primary chondrocytes	Curcumin enhances the production of collagen type II, CSPGs, β1-integrin and activating MAPKs signaling and suppressing caspase-3 and COX-2.	5 μM	(Buhrmann, Mobasheri et al. 2010)
		RA-FSL	Curcumin induces apoptosis in RA-FSL by inhibition of Blc-2 and X-IAP expression, activation of caspase-3 and caspase-9 and degradation of PARP. Also, inhibits COX-2 expression and PGE_2 production.	50, 75 and 100 μM	(Park, Moon et al. 2007)
		RA-FSL, MH7A	Curcumin blocks IL-1β and PMA-induced IL-6 expression, blocks PMA-induced VEGF expression, blocks activation of NF-κB and ERK1/2 and induces cell death through apoptosis.	12.5, 25 and 50 μM	(Kloesch, Becker et al. 2013)
		HIG-82 rabbit synoviocytes	Curcumin inhibits inflammatory processes through suppression of collagenase and stromelysin expression.	1 and 10 μM	(Jackson, Higo et al. 2006)
Stilbenes					
Resveratrol	Grapes	RA-FSL	Resveratrol induces apoptosis through caspase-3, caspase-9, caspase-8 and PARP cleavage,	100 μM	(Byun, Song et al. 2008)

			mitochondrial cytochrome c release and AIF translocation to the nucleus.		
		MH7A cells	Resveratrol induces apoptosis through activation of caspase-3 and caspase-9, disrupts mitochondrial membrane potentials as a result of decreased Bcl-XL expression, allowing cytochrome c from the mitochondria into the cytosol, in a sirtuin 1-dependent manner.	100 µM	(Nakayama, Yaguchi et al. 2012)
		RA-FSL	Resveratrol attenuates IL-1β-induced production of MMP-1, MMP-3 and MMP-9. qRT-PCR showed a significant reduction in the relative abundance of the transcripts of OPG and RANKL.	6.25, 12.5, 25 and 50 µM	(Glehr, Breisach et al. 2013)
		RA-FLS	Resveratrol attenuates TNF-α-induced production of IL-1β and MMP-3 via inhibition of PI3K-Akt signaling pathway in RA FLS.	100 µM	(Tian, Chen et al. 2013)
Other dietary polyphenols					
Oleocanthal	EVOO	ATDC-5	Oleocanthal inhibits LPS-induced NO production and iNOS expression	10 and 25 µM	(Iacono, Gomez et al. 2010)
		ATDC-5	Oleocanthal suppresses LPS-induced IL-6 and MIP-1α expression.	15 µM	(Scotece, Gomez et al. 2012)

Table 2. *In vivo* studies of dietary polyphenols in RA.

Polyphenol	Source	Experimental System	Mechanism/s of Action	Efficacious []	Ref
Phenolic acids					
Bezoic acid derived					
Protocatechuic acid	Onions, mushroom.	AIA in rats	Protocatechuic acid inhibits arthritis index, decreases LPO and NO and increases antioxidants, SOD, catalase and GSH in liver.	50 and 100 mg/Kg orally	(Lende, Kshirsagar et al. 2011)
Cinnamic acid derived					
p-Coumaric Acid	Peanuts, tomatoes, carrots and garlic.	AIA in rats	*p*-coumaric acid reduces the serum circulating immune complex levels. Synovial tissue of arthritic rat treated with *p*-coumaric acid showed a reduction in immunostaining for TNF-α.	100 mg/kg i.p.	(Pragasam, Venkatesan et al. 2013)
Chlorogenic Acid	Peach, prunes and green coffee	AIA in rats	Chlorogenic acid controls the total (CD3) and differentiated (CD4 and CD8) T cells count, suppresses CD80/86 and suppresses the Th1 cytokines but elevates Th2 cytokines.	2.5, 5, 10, 20 and 40mg/kg. orally	(Chauhan, Satti et al. 2012)
Rosmaric acid	Basil, rosemary, thyme and peppermint	CIA in mice	Rosmaric acid reduces the arthritic index, histological damage and COX-2 expression.	50 mg/Kg i.p.	(Youn, Lee et al. 2003)
Flavonoids					
Flavones					

Nobiletin	Tangerine	CIA in mice	Nobiletin suppresses arthritic index.	2 and 20 mg/Kg i.p.	(Murakami, Song et al. 2007)
		CIA in mice	Nobiletin improves arthritic progression and joint histology and exerts a chondroprotective action from the aggrecanase-mediated cartilage destruction according to the inhibition of gene expression and production of ADAMTS-4 and -5	15, 30 and 60 mg/Kg i.p.	(Imada, Lin et al. 2008)
Flavonols					
Quercetin	Onions	AIA in rats	Quercetin improves arthritic progression and joint histology, decreases lipid peroxidation and increases antioxidants, SOD, catalase, GSH and GPx.	0.2 and 0.4 mg/Kg into thioglycolica cid-cappedcadm iumtelluride quantumdot s. Orally	(Jeyadevi, Sivasudha et al. 2013)
		AIA in rats	Quercetin reduces MCP-1, IL-1β, C-reactive protein and 13/15 LOX. Upregulates HO-1 expression and inhibits ERK phosphorylation and NF-κB activation.	150 mg/Kg Orally	(Gardi, Bauerova et al. 2015)
Kaempferol	Apples, grapes, tomatoes, green tea, potatoes, onions, broccoli, Brussels sprouts, squash, cucumbers, lettuce, green beans, peaches, blackberries, raspberries, and spinach	CIA in rats	Kaempferol inhibits PIM1-mediated FOXP3 phosphorylation at S422 to promote FOXP3 transcriptional activity.	100 mg/Kg	(Lin, Luo et al. 2015)
Flavanones					
Hesperidin	Orange and lemon	CIA in mice	Hesperidin improves clinical socore, histological features and downregulates TNF-α mRNA expression.	150 mg/Kg Orally	(Kawaguchi , Maruyama et al. 2006)
		AIA in rats	Hesperidin reduces polyarthritis index and histological features. Enhances CoA-induced T-lymphocyte proliferation and recovers IL-2 production in solenocytes; also, inhibits IL-1, IL-6 and TNF-α level in peritoneal macrophages from treated rats.	40, 80 and 160 mg/Kg Intragastrica lly	(Li, Li et al. 2008)
		CIA in rats	Hesperidin improves clinical socore, histological features and decrases TBARS concentration by inhibition of lipid peroxidation in the cartilage. Upregulates GSH and SOD antioxidant enzymes and downregulates cartilage catalase activity and nitrite level.	160 mg/Kg Orally	(Umar, Kumar et al. 2013)
Naringin	Grapes	AIA in rats	Naringin suppresses paw swelling and histopathological changes of joint tissue. Downregulates TNF-α, IL-1β and IL-6 serum level	20 and 40 mg/Kg Orally	(Zhu, Wang et al. 2015)

			and induces apoptosis via regulation of Bcl-2 and Bax protein expression.		
Isoflavones					
Genistein	Lupin, fava beans, soybeans, kudzu, and psoralen	CIA in rats	Genistein modulates immune responses in CIA model. Suppresses the secretion of IFN-γ and augments the IL-4 production.	80 mg/Kg Orally	(Wang, Zhang et al. 2008)
		CIA in rats	Genistein improves disease symptoms and reduces TNF-α, IL-6, adiponectin and leptin serum concentration.	20 mg/Kg Orally	(Mohammad-Shahi, Haidari et al. 2011)
		CIA in rats	Genistein restores paraoxonase and arylesterase activity and decreases MDA levels.	20 mg/Kg Orally	(Mohammadshahi, Haidari et al. 2013)
Daidzein	Soybeans	CIA in rats	Daidzein improves disease symptoms and reduces TNF-α, IL-6, adiponectin and leptin serum concentration.	20 mg/Kg Orally	(Mohammad-Shahi, Haidari et al. 2011)
		CIA in rats	Daidzein restores paraoxonase and arylesterase activity and decreases MDA levels.	20 mg/Kg Orally	(Mohammadshahi, Haidari et al. 2013)
Antocyanins					
Malvidin	Red wine	AIA in rats	Malvidin is efficient in reducing arthritis sings and decreases nitrites level from peritoneal macrophages from treated rats.	25 mg/Kg Orally	(Decendit, Mamani-Matsuda et al. 2013)
Flavanols					
EGCG	Green tea	CIA in mice	EGCG-containing green tea extract reduces disease symptoms, inhibits COX-2, IFN-γ and TNF-α level in arthritic joint and decreases anti-CII IgG serum level.	2 g/L of green tea extract in water, ad libitum as the sole source of drinking water.	(Haqqi, Anthony et al. 1999)
		CIA in mice	EGCG ameliorates arthritis during the disease course and reduces histologic scores of arthritis. Also reduces the number of osteoclasts in the pannus of arthritic paw joint.	20 mg/Kg i.p.	(Morinobu, Biao et al. 2008)
		CIA in rats	EGCG reduces the severity of CIA and alleviates joint destruction. Moreover, diminishes the number of CCL2-producing osteoblasts and recruitment of CD68+ macrophages.	20 mg/Kg i.p.	(Lin, Chang et al. 2008)
		CIA in mice	EGCG ameliorates clinical symptoms, histological scores in arthritis, IL-1β, IL-6, TNF-α, IFN-γ, IL-10 and anti-CII IgG serum level. Also supresses T cell proliferation and increases Nrf2 and HO-1 levels.	10 mg/Kg Orally	(Min, Yan et al. 2015) Min 201
		CIA in mice	EGCG reduces histopathological cartilage degradation.	12 mg/Kg Intra-articular injection	(Natarajan, Madhan et al. 2015)

		AIA in rats	EGCG inhibits IL-6 and MMP-2production. EGCG enhances the synthesis of gp130 protein and endogenous inhibitor of IL-6 signalling.	100 mg/Kg i.p.	(Ahmed, Marotte et al. 2008)
		AIA in rats	Green tea extract ameliorates arthritic damage via inhibition of IL-17 and upregulation of IL-10 serum level.	8 and 12 g/L green tea extract in drinking warter	(Kim, Rajaiah et al. 2008)
Diarylheptanoids and arylalkanones					
Ginger	Ginger (*Zingiber officinale*)	CIA in rats	Ginger ameliorates the clinical score, cartilage destruction and decreases serum levels of IL-1β, TNF-α, IL-2, IL-6 and anti-CII IgG.	50, 100 and 200 mg/Kg i.p.	(Fouda and Berika 2009)
Curcumin	*Curcuma longa*	CIA in mice	Curcumin attenuates arthritic index and reduces BAFF, IFN-γ and IL-6 sera levels.	50 mg/Kg i.p.	(Huang, Xu et al. 2013)
		AIA in rats	Curcumin ameliorates symptoms of arthritis and preserves radiological alterations. Curcumin reduces TNF-α, MDA, nitrites C-reactive protein and citrullinated peptide antibody sera levels.	10 and 30 mg/Kg Curcumin loaded solid lipid nanoparticles. Orally	(Arora, Kuhad et al. 2015)
		AIA in rats	Curcumin improves histopathological changes, decreases IL-1β and TNF-α synovial and sera levels and inhibit NF-κB pathway.	50 mg/Kg i.v. or orally (nanoformulation)	(Zheng, Sun et al. 2015)
Stilbenes					
Resveratrol	Grapes	Intraarticular injection of LPS in rabbits	Resveratrol decreases cartilage destruction and loss of matrix proteoglycan content in the cartilage.	2.3 mg/Kg Intraarticular injection	(Elmali, Baysal et al. 2007)
		CIA in mice	Resveratrol attenuates clinical parameters and bone erosion; associated with markedly reduced serum levels of pro-inflammatory cytokines and collagen-specific IgG and with reduced numbers of Th17 cells and the production of IL-17 in DLN.	20 mg/Kg i.p.	(Xuzhu, Komai-Koma et al. 2012)
		AIA in rats	Resveratrol reduces cartilage degeneration, reduces the production of COX-2 and PGE_2 and inhibits the proliferation of Con-A spleen cells	10 and 50 mg/Kg Intragastrically	(Chen, Lu et al. 2014)
Other dietary polyphenols					
Punicalagin	Pomegranate	AIA in rats	Punicalagin diminishes disease development and paw edema	10 and 50 mg/Kg i.p.	(Jean-Gilles, Li et al. 2013)
Ellagic acid	Blackberries, cranberries, pecans, pomegranates, raspberries, strawberries, walnuts, wolfberries, and grapes	CFA-induced polyarthritis in rats	Ellagic acid reduces joint inflammation and histopathological changes; also suppresses TNF-α and IL-1β production.	0.13, 0.325 and 0.65% Topically	(Mo, Panichayupakaranant et al. 2013)

Oleuropein	EVOO	CIA in mice	Oleuropein ameliorates clinical symptoms and histological scores in arthritis; also decreases pro-inflammatory cytokines and chemokine plasma levels and MPo activity.	40 mg/Kg i.p.	(Impellizzeri, Esposito et al. 2011)
Hydroxytyrosol	EVOO	CIA in rats	Hydroxytyrosol decreases paw edema and histological damage and reduces COX-2 and iNOS expression.	5 mg/Kg Orally	(Silva, Sepodes et al. 2015)

JUSTIFICACIÓN Y OBJETIVOS

Según la Organización Mundial de la Salud, las enfermedades reumáticas son la causa más frecuente de incapacidad, de origen no mental, en el mundo. En España estas patologías están relacionadas con el 50.7% de las incapacidades laborales y son la principal causa de bajas laborales permanentes. Especial relevancia tiene la **artritis reumatoide** (AR), por sus repercusiones sobre la calidad de vida de los pacientes y el deterioro progresivo que produce esta enfermedad. Entre el 20% y el 30% de los pacientes con AR se convierten en incapacitados permanentes. Demográficamente, la AR es la forma más común de artritis inflamatoria y afecta aproximadamente al0.5 - 1% de la población mundial, con un impacto económico comparable con el de la enfermedad arterial coronaria. Esta patología puede aparecer en cualquier etapa de la vida, sin embargo la prevalencia aumenta con la edad de tal forma que el 80% de todos los pacientes inician la enfermedad entre los 35 y 50 años de edad y es unas 3 veces mayor en mujeres que en hombres (Gonzalez Cernadas, Rodriguez-Romero et al. 2014).

La AR es una enfermedad autoinmunológica inflamatoria, crónica y sistémica que se caracteriza por una sinovitis erosiva simétrica, en la cual el tejido de granulación de origen sinovial (*pannus*), invade y erosiona el cartílago y el hueso de las articulaciones diartrodiales. La enfermedad se desarrolla con cifras elevadas de la velocidad de sedimentación eritrocitaria, proteína C reactiva, presencia de inmunocomplejos y el factor reumatoide. Los pacientes con AR presentan afectación generalmente de las articulaciones diartrodiales de forma simétrica. Dicha enfermedad tiene, en ocasiones, un componente extraarticular, afectando a diversos órganos y sistemas. El deterioro progresivo, articular o extraarticular, acaba provocando que estos pacientes presenten deformidad, dolor, incapacidad funcional y disminución de la expectativa de vida (Fauci 2010).

La etiología de AR se desconoce aunque factores genéticos, infecciosos (infección viral, e.g. Virus Epsein-Barr, Proteus, rubeola,) ambientales (tabaco, situaciones de estrés y traumatismo, obesidad, dieta) y hormonales parecen estar involucrados en vías relacionadas y complejas (Suzuki and Yamamoto 2015). Se han identificado múltiples genes que contribuyen a la predisposición y susceptibilidad a la enfermedad fundamentalmente el gen *HLA-DRB1* del complejo mayor de histocompatibilidad, *PADI4, PTPN22, CCR6 y FCRL3,* entre otros (Yamamoto, Okada et al. 2015).

Aunque la AR ha sido considerada como una patología autoinmune, no existe consenso sobre la naturaleza del autoantígeno(s) o factores ambientales que inicia la desregulación de la respuesta inmune, mayormente orquestados por macrófagos, células T reguladoras (Treg), T

cooperadoras, (Th1 y Th17) y celulas T citotóxicas. Estos procesos incluyen el desbalance de la producción de citocinas Th1: interferón (IFN)-γ, (TNF)-α y las interleuquinas (IL)-1β, IL-2 , IL-4, IL-6, IL-13, IL-15, IL-6, IL-23e IL-27; Th17 (IL-17), de citocinas Th2: (IL-10, IL-20, IL-22), quimioquinas (CXCR4, IP-10, SDF1, MCP-1) y metaloproteasas (MMP-3 y MMP-9), capaces de digerir el cartílago (Brzustewicz and Bryl 2015). Entre las vías de señalización moleculares comprometidas se incluyen los factores nucleares de transcripción NF-κB y FOXP3 y las proteínas cinasas activadas por mitógenos (MAPK): c-Jun NH_2 –terminal cinasa (JNK), p38 cinasa y cinasa regulada por señal extracelular (ERK) fundamentalmente (Han, Boyle et al. 2001, McInnes and Schett 2011, Nie, Zheng et al. 2013).

La secreción de citocinas proinflamatorias a sangre amplifica la respuesta inmunológica, produciéndose una expresión clonal de linfocitos B sobre todo del tipo IgG y una intensa reacción inflamatoria vía receptores *Toll-like* acompañada de la liberación de citocinas, quimioquinas, mediadores lipídicos, como el factor activador de plaquetas (PAF), leucotrienos (LT), prostaglandinas (PG) y activación de polimorfonucleares (PMN) substancialmente, neutrófilos, los cuales secretan proteasas, radicales libres derivados del oxígeno (RLO) y mieloperoxidasa (MPO), contribuyendo de esta forma al daño celular (Anderson, Pratt et al. 2015).

El abordaje terapéutico de la AR es multidisciplinar, y está dirigido principalmente a la supresión inespecífica del proceso inflamatorio con el objetivo de mitigar los síntomas y signos de la enfermedad englobando tratamiento médico, fisioterápico y otro grupo de métodos dentro del cual se incluyen las medidas dietéticas (Fauci 2010).

En este contexto, la **terapia nutricional** en la actualidad, más allá de su soporte dietético, puede ejercer efectos profilácticos y terapéuticos carentes de los efectos indeseables que acompañan a la farmacoterapia clásica en determinadas enfermedades. En los últimos años, se ha puesto de manifiesto el potencial terapéutico de los alimentos funcionales o aquellos alimentos que, independientemente de sus propiedades nutritivas, poseen un efecto beneficioso para el organismo; es decir, su ingesta diaria, dentro de una dieta equilibrada, contribuye a mantener o mejorar el estado de salud y bienestar.

Diversos estudios epidemiológicos indican que la dieta Mediterránea, que posee al aceite de oliva (AO) como principal fuente de grasa, está asociada a una menor incidencia de un buen número de patologías. Efectos beneficiosos se han observado en particular en: enfermedades cardiovasculares, digestivas (Alarcon de la Lastra, Barranco et al. 2001, Alarcon

de la Lastra, Barranco et al. 2002, Perez-Jimenez, Alvarez de Cienfuegos et al. 2005, Motilva, Talero et al. 2008, Sanchez-Fidalgo, Villegas et al. 2010), neurodegenerativas, diversos tipos de cáncer, principalmente cáncer colorrectal, pulmón y estómago, pero también de mama, endometrio, ovario, y próstata, entre otros (La Vecchia 2009, Pelucchi, Bosetti et al. 2009). Igualmente, en diferentes ensayos clínicos se ha puesto de manifiesto una mejoría de la sintomatología clínica de los pacientes con patología reumática que siguieron una dieta con AO como fuente de triglicérido, así como una reducción del uso de antiinflamatorios y en consecuencia una menor incidencia de reacciones adversas (Smedslund, Byfuglien et al. 2010). Por tanto, el AO podría ser definido desde un punto de vista estrictamente inmunológico y a la luz de los resultados obtenidos, como un componente de la dieta capaz de ejercer un efecto inmunomodulador (Puertollano, Puertollano et al. 2010).

En el mercado nos encontramos con diferentes tipos de AO en virtud de unos determinados parámetros de calidad: acidez, índice de peróxidos, absorbancia en el ultravioleta, características organolépticas y contenidos en ceras. Según el reglamento (CE) nº 1989/2003 los AO se clasifican en aceite de oliva virgen, aceite de oliva virgen extra (AOVE), aceite de oliva virgen lampante, aceite de oliva refinado, aceite de oliva, aceite de orujo de oliva crudo, aceite de orujo de oliva refinado y aceite de orujo de oliva. Concretamente, el AOVE es obtenido de la aceituna tras aplicar solamente procedimientos mecánicos o medios físicos en condiciones sobre todo térmicas y cuya acidez libre expresada en ácido oleico es como máximo 0.8%.

Hasta hace relativamente poco tiempo, las propiedades beneficiosas del AOVE, han sido atribuidas casi exclusivamente a su fracción saponificable caracterizada por el alto contenido en ácidos grasos monoinsaturados (ácido oleico). Las fracciones insaponificable (FI) y polifenólica (FP), a pesar de ser minoritarias, están constituidas por un amplio número de compuestos de alto valor biológico. En concreto, la FI incluye en su composición: esteroles, alcoholes y dioles terpénicos (eritrodiol, uvaol, ácido oleanólico y maslínico) entre otros, alcoholes alifáticos (dicosanol, tetraconasol, etc.), tocoferoles (α, β, γ y δ), hidrocarburos esteroideos (etigmasta-3,5-dieno), hidrocarburos terpénicos (escualeno), 4,4-metil-esteroles (obtusifoliol, gramisterol, cicloeucalenol, etc.), pigmentos (clorofila, carotenos) y compuestos volátiles responsables de los aromas de los aceites (Sanchez-Fidalgo, Sanchez de Ibarguen et al. 2012).

La FP está constituida por más de 30 compuestos aunque su composición cuali- y cuantitativa depende de la variedad de la aceituna, el grado de maduración y la climatología.

Destacan la oleaceína y el oleocantal y los aglicones monoaldehídicos de la oleuropeína y del ligustrósido (Franco, Galeano-Diaz et al. 2014). Otros componentes fenólicos minoritarios incluyen el hidroxitirosol, el acetato de hidroxitirosol y el dihidroxifenilglicol (Medina, de Castro et al. 2006). Entre los efectos biológicos de los fenoles del AOVE destacamos sus propiedades antioxidantes, antiaterogénicas y antiinflamatorias relacionadas con la génesis de las patologías crónicas más relevantes como la enfermedad cardiovascular, la neurodegeneración, el síndrome metabólico o el cáncer, entre otras. Además, son partícipes de importantes actividades farmacológicas *i.e.* antiarrítmica, antiagregante plaquetaria, vasodilatadora, antimicrobiana y quimiopreventiva (Cardeno, Sanchez-Hidalgo et al. 2013, Aparicio-Soto, Sanchez-Fidalgo et al. 2015, Sanchez-Fidalgo, Villegas et al. 2015).

En estudios previos, hemos puesto de manifiesto, mediante modelos experimentales de enfermedad inflamatoria intestinal (EII) y cáncer colorrectal, un importante efecto beneficioso de dietas enriquecidas con AOVE. Se confirmó una reducción estadísticamente significativa del daño colónico, de la incidencia de tumores, de la liberación de citocinas y de la inmunoreactividad de las proteínas ciclooxigenasa 2 (COX-2), óxido nítrico sintasa indicible (iNOS) y β- catenina en las mucosas procedentes de animales alimentados con AOVE (Sanchez-Fidalgo, Villegas et al. 2010).

De forma similar, en posteriores investigaciones evaluamos el potencial antiinflamatorio e inmunomodulador de las fracciones FI y FP del AOVE y subfracciones de éstas, en modelos animales de inflamación colónica y en células inmunocompetentes humanas y de ratón, y se dilucidaron los mecanismos de acción implicados. Los resultados revelaron que ambas fracciones (FI y FP) inducían significativos efectos antiinflamatorios e inmunomoduladores en los modelos *in vitro* e *in vivo* experimentales ensayados. Pudimos constatar que dietas elaboradas con AOVE minimizaron el daño colónico, reduciendo la expresión de las proteínas pro-inflamatorias COX-2 y iNOS, y generando un aumento de la expresión del receptor activador de la proliferación de los peroxisomas gamma (PPAR-γ) hasta niveles basales a través de la inhibición de las vías del NF-kB y las MAPK cinasas. Además, estos efectos beneficiosos fueron potenciados tras el enriquecimiento del aceite con las FI y FP. Estos resultados sugerían que una dieta suplementada con FI y FP podría constituir una estrategia terapéutica novedosa para el tratamiento nutricional de la EII y otras patologías de etiología inmunoinflamatoria (Sanchez-Fidalgo, Sanchez de Ibarguen et al. 2012, Sanchez-Fidalgo, Cardeno et al. 2013, Aparicio-Soto, Sanchez-Fidalgo et al. 2015, Sanchez-Fidalgo, Villegas et al. 2015). Con la finalidad de extrapolar estos resultados a la clínica, nuestro grupo

puso de manifiesto actividad inmunomoduladora de la FI del AOVE en linfocitos aislados de sangre periférica procedente de pacientes con EII recidivante en comparación con un grupo de sujetos sanos (Cardeno, Magnusson et al. 2014) gracias a la colaboración con el Department of Microbiology and Immunology de Sahlgrenska Academy (Universidad de Gothenburg, Suecia) bajo la dirección de la Dra. Lena Ohman.

Como se ha comentado, existen datos epidemiológicos que relacionan una menor prevalencia de enfermedades reumáticas en países mediterráneos en comparación con los del norte de Europa. En consecuencia, estas evidencias sugieren un papel del AO de la dieta en el desarrollo y progresión de procesos inflamatorios reumáticos articulares.

La efectividad de la dieta mediterránea en pacientes con AR se ha evaluado en varios ensayos y revisiones, aunque la evidencia todavía es inconclusa. En concreto, la evidencia obtenida a partir de los ensayos clínicos revisados permite afirmar que los principales beneficios clínicos de la dieta mediterránea en la AR están relacionados con el dolor, la rigidez matutina, el número de articulaciones inflamadas, una mejora de la percepción de salud de los pacientes (medida a través de cuestionarios como el SF-36 y el HAQ), y una disminución de la actividad de la enfermedad (valorada mediante escalas tipo EVA, o el cuestionario DAS2833-36) (Abendroth, Michalsen et al. 2010). En línea con estos hallazgos se encuentran las conclusiones de las revisiones sistemáticas donde se afirma que la dieta mediterránea reduce el dolor (Smedslund, Byfuglien et al. 2010) y produce una mejora en la función física (Gonzalez Cernadas, Rodriguez-Romero et al. 2014).

No obstante, no existen hasta la fecha, estudios experimentales *in vitro* e *in vivo* que validen el potencial antirreumático del AOVE, de los componentes bioactivos posibles responsables así como de los mecanismos bioquímicos y moleculares implicados.

Por ello el **objetivo general** de esta Tesis Doctoral ha sido investigar el funcionalismo del AOVE en la artritis reumatoide experimental.

En concreto, nos hemos centrado en los siguientes objetivos específicos:

1. Caracterización química cuali y cuantitativa del AOVE seleccionado: Extracción y análisis químico de la fracción polifenólica.

2. Valoración en un modelo experimental *in vivo* de AR inducida por colágeno tipo II en ratones DBA/1 los efectos de :

   2a. Diferentes dietas elaboradas con AOVE y aceite de girasol (AG) y explorar las rutas bioquímicas y vías de señalización intracelulares posiblemente comprometidas.

   2b. La administración oral de la FP del AOVE en estudio, estudiar las rutas bioquímicas y mecanismos de señalización moleculares implicados más significativos.

   2c. Dietas enriquecidas con dos compuestos fenólicos del AOVE: Hidroxitirosol y acetato de hidroxitirosol y dilucidar los mecanismos moleculares y vías de señalización intracelulares implicados.

Dietary extra-virgin olive oil prevents inflammatory response and cartilage matrix degradation in murine collagen-induced arthritis.

(Rosillo et al. Eur J Nutr. 2015 In press)

LA DIETA DE ACEITE DE OLIVE VIRGEN EXTRA PREVIENE LA RESPUESTA INFLAMATORIA Y LA DEGRADACIÓN DE LA MATRIZ DEL CARTÍLAGO EN UN MODELO DE ARTRITIS INDUCIDA POR COLÁGENO TIPO II EN RATONES

MªÁngeles Rosillo, Marina Sánchez-Hidalgo, Susana Sánchez-Fidalgo, Marina Aparicio-Soto, Isabel Villegas, Catalina Alarcón-de-la-Lastra

Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España

RESUMEN

Antecedentes del tema: Aunque la patogenia de la artritis reumatoide (AR) sigue sin conocerse en su totalidad, actualmente se ha descrito la existencia de una desregulación en el balance Th1/Th2 y Th17/células T reguladoras en pacientes con AR. En las articulaciones artríticas, se produce un incremento en la expresión de citocinas proinflamatorias, tales como TNF-α, IL-1β o IL-17, que juegan un papel destacado en el desarrollo y progresión de la enfermedad. Este aumento se atribuye, al menos en parte, a una desregulación en las rutas de señalización celular protagonizadas por las MAP cinasas (MAPKs), JAK/STAT y el factor nuclear NF-κB. Por otro lado, el factor nuclear Nrf2, es una pieza clave en la transcripción de distintas enzymas antioxidantes como la hemo oxigenasa 1 (HO-1), se ha demostrado que la activación de la expresión génica de esta proteína es capaz de modular la respuesta inflamatoria. El hecho de que el abordaje farmacológico de la AR implique severos efectos secundarios, ha generado en los últimos años un auge en el uso de otras estrategias terapéuticas, entre las que destaca la terapia nutricional. Recientes estudios epidemiológicos han confirmado que el consumo de aceite de oliva virgen extra (AOVE), dentro del contexto de la dieta Mediterránea, resulta eficaz en la prevención de ciertas patologías relacionadas con el estrés oxidativo, la inflamación y el sistema inmune. Por otro lado, recientes publicaciones de nuestro grupo de investigación, así como de otros autores, han demostrado las propiedades antiinflamatorias e inmunomoduladoras de la dieta de AOVE en distintos modelos experimentales de inflamación.

Objetivos: En base a los anteriores antecedentes, nos planteamos evaluar la influencia de una dieta de AOVE de la variedad picual, en un modelo de artritis experimental inducida por colágeno tipo II en ratones.

Material y Métodos: Ratones DBA-1/J recién destetados, fueron distribuidos en cuatro grupos experimentales: (1) Ratones sanos alimentados con dieta de aceite de girasol (CS-AG), (2) Ratones artríticos alimentados con dieta de aceite de girasol (CIA-AG), (3) Ratones sanos

alimentados con dieta de AOVE (CS-AOVE), (4) Ratones artríticos alimentados con dieta de AOVE. Después de seis semanas, la artritis fue inducida. El día 0, los ratones fueron inmunizados con una inyección en la base de la cola de 100 mg de colágeno II. El día 21, recibieron una inyección de refuerzo intraperitoneal, con la misma cantidad de colágeno. El grado de desarrollo de la enfermedad fue valorado de forma visual siguiendo una escala de 0-2, dónde 0=sin inflamación; 1=inflamación leve; 1.5=inflamación marcada; 2=inflamación severa y según el estudio histológico de las articulaciones. Los niveles en suero de la proteína oligomérica de la matriz del cartílago (COMP) y de metaloproteasa 3 (MMP-3) así como los niveles de citocinas proinflamatorias como TNF-α, IL-1β e IL-17 en el homogenado de pata fueron determinados mediante la técnica de ELISA. Los cambios en la expresion proteica de HO-1, Nrf2 y de las proteínas implicadas en las vías de señalización celular MAPKs, JAK/STAT y NF-κB fueron estudiadas mediante western blot.

Resultados: la dieta de AOVE redujo de forma significativa el edema articular y la destrucción del cartílago, previniendo el desarrollo de la AR. Los niveles séricos de COMP y MMP-3, los encontramos disminuidos en aquellos animales alimentados con la dieta de AOVE. La expresión de las citocinas proinflamatorias TNF-α, IL-1β e IL-17 fue menor el tejido articular de los ratones artríticos que recibieron la dieta de AOVE, en comparación con los CIA-AG. Además la activación de las vías de señalización MAPKs, JAK/STAT y NF-κB fue inhibida por la dieta de AOVE. De acuerdo con la expresión proteica de Nrf2 y HO-1, estuvo aumentada en las articulaciones de los ratones pertenecientes al grupo CIA-AOVE.

Conclusión: Estos resultados confirman, por primera vez, que el AOVE muestra un elevado efecto protector en la prevención y la progresión de la AR experimental, probablemente a través de la activación de la vía Nrf2/HO-1, e inactivación de las vías MAPKs, JAK/STAT y NF-κB, con los que podría ser considerado como un alimento funcional de primera magnitud dentro de la terapia nutricional de pacientes con AR, si futuros ensayos clínicos confirmasen estos resultados experimentales.

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ORIGINAL CONTRIBUTION

Dietary extra-virgin olive oil prevents inflammatory response and cartilage matrix degradation in murine collagen-induced arthritis

María Angeles Rosillo · Marina Sánchez-Hidalgo · Susana Sánchez-Fidalgo · Marina Aparicio-Soto · Isabel Villegas · Catalina Alarcón-de-la-Lastra

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Abstract
Purpose Current experimental studies support a beneficial role of extra-virgin olive oil (EVOO) in several inflammatory diseases. The present study was designed to evaluate the effects of dietary EVOO on type II collagen-induced arthritis (CIA) in mice.
Methods DBA-1/J mice were randomized in four experimental groups (10 or 15 animals per group): (1) Sham sunflower diet (SO-Sham), (2) CIA sunflower diet (SO-CIA), (3) Sham EVOO diet (EVOO-Sham) and (4) CIA EVOO diet (EVOO–CIA) group. After 6 weeks, arthritis was induced by type II collagen. Mice were sacrified 42 days after first immunization. In addition to macroscopic and histological analyses, serum levels of cartilage olimeric matrix protein (COMP), metalloproteinase-3 (MMP-3) and pro-inflammatory cytokines levels were evaluated by ELISA. The expressions of heme oxygenase-1 (HO-1), nuclear factor E2-related factor 2 (Nrf2), mitogen-activated protein kinases (MAPKs), Janus kinase-signal transducer and activator of transcription (JAK/STAT) and nuclear transcription factor-kappa B (NF-κB) pathways were studied by western blotting.
Results EVOO diet significantly reduced joint edema and cartilage destruction, preventing the arthritis development. Dietary EVOO significantly decreased serum COMP and MMP-3 levels, as well as, the pro-inflammatory cytokines levels (TNF-α, IL-1β and IL-17). Moreover, the activation of JAK/STAT, MAPKs and NF-κB pathways was drastically ameliorated. According to Nrf2 and HO-1, the protein expressions were up-regulated in those mice fed with EVOO.
Conclusion These results support the interest of EVOO as a beneficial functional food to prevent the development of the rheumatoid arthritis (RA).

Keywords CIA · EVOO · Inflammatory response · Olive oil · Rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovial membranes and proliferation of the synovial lining leading to synovial hyperplasia and massive infiltration of immune cells, vasculogenesis, cartilage and bone destruction and progressive joint damage [1]. Although the pathogenesis of RA is not fully understood, genetic and immunologic factors clearly contribute to the joint damage during RA [2]. In fact, dysregulated balances of Th1/Th2 and Th17/Treg cells have been demonstrated in RA patients [3].

RA synovium is characterized by up-regulated proinflammatory cytokines levels such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, the two key cytokines involved in destructive arthritis in addition to IL-17, IL-21, IL-23 and IL-6, among others. In fact, IL-17 plays important roles in the additive/synergistic effects induced together with TNF-α and IL-1β, and cartilage matrix breakdown induction through the chondrocyte metabolism dysregulation. Besides, cytokines contribute to the osteoclasts and macrophages activation and also promotion of recruitment of both neutrophils and monocytes

M. A. Rosillo (✉) · M. Sánchez-Hidalgo · S. Sánchez-Fidalgo · M. Aparicio-Soto · I. Villegas · C. Alarcón-de-la-Lastra
Department of Pharmacology, Faculty of Pharmacy, University of Seville, Profesor García González, 2, 41012 Seville, Spain
e-mail: rosillo@us.es

C. Alarcón-de-la-Lastra
e-mail: calarcon@us.es

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by means of inducing various chemokines. The infiltrated leukocytes produce more cytokines and tissue-destructive enzymes leading to chronic inflammation, cartilage damage and bone erosion [4].

The process of gene expression of these pro-inflammatory mediators involves multiple signal transduction pathways including mitogen-activated protein kinases (MAPKs) [5, 6] and nuclear transcription factor-kappa B (NF-κB) pathways [6, 7]. Actually, NF-κB activation results in metalloproteinases (MMPs) production, which degrade articular extracellular matrix constituents and subsequently cause articular destruction. In addition, the Janus kinase–signal transducer and activator of transcription (JAK-STAT) is another relevant inflammatory pathway activated in response to cytokines [8]. In particular, STAT-3 overexpression has been reported in synovial membranes from RA patients [9]. Likewise, nuclear factor E2-related factor 2 (Nrf2) is a key transcription factor orchestrator of the induction of several antioxidant enzymes, such as heme oxygenase (HO)-1. The activation of HO-1 gene expression is considered to be an adaptive cellular response to survive exposure to environmental stresses. More recently, HO-1 has also been reported to modulate inflammatory responses, cell proliferation and prevent apoptosis [10, 11].

Recent research has suggested that dietary patterns, such as the traditional Mediterranean diet, which is characterized by large amounts of foods naturally derived, in particular, vegetables, fruits, nuts, fish and grains, may confer protection from certain chronic diseases related to oxidative stress, inflammation and the immune system. In comparison with other healthy diets, Mediterranean diet has a high content of total fat as its most distinctive feature due to the usual high intake of olive oil from olive tree, *Olea europaea* [12].

Extra-virgin olive oil (EVOO) is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to oil alteration. Traditionally, the beneficial effects of EVOO have been attributed to its high MUFA content (oleic acid) as it protects lipoproteins and cellular membranes, from oxidative damage [13]; however, nowadays, there is increasing evidence to support the beneficial effects of EVOO intake that are due to its minor highly bioactive components (about 1–2 % of oil weight) including phenolic compounds, triterpene alcohols, sterols, hydrocarbons (squalene), vitamins (α- and γ-tocopherols), β-carotene, phytosterols, among others [12, 14]. Particularly, current experimental studies support a beneficial role of polyphenols from EVOO in several inflammatory diseases, including RA [15–18]. In fact, our research group has previously demonstrated that an EVOO diet enriched with hydroxytyrosol, a phenolic compound present in the oil, and a dietary EVOO-polyphenol extract supplementation could improve the inflammatory responses associated with a chronic experimental colitis model [19, 20]. Furthermore, we also confirmed beneficial effects of dietary EVOO-unsaponifiable supplementation on an acute experimental colitis model [21].

Although dietary EVOO has demonstrated immunomodulatory and antiinflammatory properties in several inflammation experimental models [22–24], nevertheless its possible contribution to RA remains unknown.

Taken this background into account, the present study was designed to evaluate the potential protective effects of dietary EVOO in collagen-induced arthritis (CIA) model, a well-studied animal model of RA useful in the development of new therapies for RA [10], and to identify its underlying molecular mechanisms. The severity of arthritis was assessed by macroscopic and histology parameters. Inflammatory mediators and the signaling pathways involved were also explored.

Materials and methods

Animals and diets

A total of 50 three-week-old male DBA-1/J mice (Janvier®, Le Genest St Isle, France) were maintained in our Animal Laboratory Center under standard conditions (temperature, 24–25 °C; humidity, 70–75 %; lighting regimen, 12L/12D). They were fed pellet diets and water ad libitum. Mice were randomized into four experimental groups (10 or 15 animals per group) during all experimental period: (1) Sham sunflower diet (SO-Sham) group received a diet elaborated with a marketable sunflower oil (Koipesol®, DEOLEO, Spain), (2) CIA sunflower diet (SO-CIA) group received a diet elaborated with a marketable sunflower oil (Koipesol®, DEOLEO, Spain), (3) Sham EVOO diet (EVOO-Sham) group were fed with a diet made with a marketable EVOO picual variety characterized by a high content of polyphenolic compounds (600 ppm) (Oleoestepa®, Seville, Spain) and (4) CIA EVOO diet (EVOO-CIA) group were fed with a diet made with a marketable EVOO picual variety, containing high levels of polyphenolic compounds (600 ppm) (Oleoestepa®, Seville, Spain). All diets were formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of various sources of carbohydrate (Table 1), at 10 % of total oil (Table 2) and were prepared by mixing the respective compounds under yellow light and stored at −80 °C. Fresh diets were provided daily. The animals were fed with the corresponding diet during 6 weeks previous to the CIA induction and during the experiment time. Experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European

Table 1 Composition of experimental diets (g/kg diet)

Ingredients	SO diet	EVOO diet
Casein	200	200
DL-methionine	3	3
Corn starch	150	150
Sucrose	447.3	447.3
Cellulose	50	50
Sunflower oil[a]	100	–
EVOO[b]	–	100
Mineral mix[c]	35	35
Vitamin mix[d]	10	10
Choline bitarate	2	2

Diet was formulated on the basis of the AIN standard reference diet with the modification of various sources of carbohydrate

[a] Sunflower oil from Koipesol® (Spain)

[b] EVOO from picual variety, Oleoestepa®, Seville, Spain

[c] Mineral mix provided the following (g/kg diet): calcium carbonate, 35.7; monopotassium phosphate, 25.0; sodium chloride, 7.4; potassium sulfate, 4.66; potassium citrate monohydrate, 2.8; magnesium oxide, 2.4; ferric citrate, 0.606; zinc carbonate, 0.165; manganese carbonate, 0.063; copper carbonate, 0.03; potassium iodate, 0.001; sodium selenate, anhydrous, 0.001025; ammonium molybdate-4H_2O, 0.000795; sodium metasilicate-9H_2O, 0.145; chromium potassium sulfate-12H_2O, 0.0275; boric acid, 0.00815; sodium fluoride, 0.00635; nickel carbonate, 0.00318; lithium chloride, 0.00174; ammonium vanadate

[d] Vitamin mix provided the following (g/kg diet): nicotinic acid, 30 mg; D-calcium pantothenate, 16 mg; pyridoxine HCL, 7 mg; thiamine HCL, 6 mg; riboflavin, 6 mg; folic acid, 2 mg; D-biotin, 0.2 mg; vitamin B12, 25 mg; α-tocopherol powder (250 U/g), 300 mg; vitamin A palmitate (250,000 U/mg), 16 mg; vitamin D3 (400,000 U/g), 2.5 mg; phylloquinone, 0.75 mg

Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU).

Induction of CIA

Arthritis was induced in 10-week-old male DBA-1/J mice. Bovine type II collagen (CII) 2 mg/mL in dilute acetic acid (MD Bioproducts®, Zürich, Switzerland) was emulsified in equal volumes of Freund's complete adjuvant (2 mg/mL *Mycobacterium tuberculosis*, strain H37Ra; Difco®, Detroit, Michigan, USA). On day 0, DBA-1/J mice were immunized at the base of the tail with 100 mg of bovine CII. On day 21, mice received an intraperitoneal booster injection of 100 mg of CII dissolved in phosphate-buffered saline (PBS). Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or in other parts of the paws. Joint inflammation was scored visually in each paw, using a scale of 0–2 where 0 = uninflamed, 1 = mild, 1.5 = marked and 2 = severe. Scoring was performed by two independent observers without knowledge of the experimental groups.

Table 2 Fatty acid, sterols, squalene, triterpene alcohols and tocopherol composition of EVOO and SO

Parameters EVOO	SO	
Fatty acid composition as determined by GC (% m/m methyl esters)		
Myristic C-14:0	0.01	0.08
Palmitic C-16:0	11.00	6.62
Palmitoleic C-16:1	0.90	0.12
Margaric C-17:0	0.10	0.04
Margaroleic C-17:1	0.10	0.03
Stearic C-18:0	3.10	4.27
Oleic C-18:1	79.40	24.83
Linoleic C-18:2	3.90	62.62
Linolenic C-18:3	0.70	0.17
Araquic C-20:0	0.40	0.27
Eicosenoic C-20:1	0.20	0.22
Behenic C-22:0	0.10	0.58
Lignoceric C-24:0	0.10	0.17
Sterols as determined by GC, relative amounts (%)		
Cholesterol	0.10	0.20
Brassicasterol	0.00	0.10
Campesterol	3.30	10.70
Stigmasterol	0.50	8.80
β-sitosterol	95.40	63.40
Δ^7-Stigmastenol	0.40	10.50
Δ^5-Avenasterol	0.00	6.2
Total sterols (mg/kg)	1,351	3,832
Squalene as determined by GC (mg/kg)	5,772	–
Triterpene alcohols (mg/kg)	542	–
Tocopherols and tocotrienols as determined by HPLC (mg/kg)		
α-Tocopherol (Vitamin E)	85	575

Animals were euthanized on day 42 by overdoses of pentobarbital.

Histological analysis

After mice were killed, knee joints were removed and fixed in 4 % formalin. After decalcification in 10 % EDTA, specimens were processed for paraffin embedding. Tissue sections (7 mm) were stained with hematoxylin and eosin (H&E) to perform histological analysis.

Measurement of inflammatory markers

Enzyme-linked immunoassay (ELISA) kits were used to determine serum levels of cartilage oligomeric matrix protein (COMP) levels, with sensitivity of 0.2 U/L (MD Biosciences®, Zurich, Switzerland) and matrix metalloproteinase-3 (MMP-3) (sensitivity of 10 pg/mL) (R&D Systems®, Abingdon, UK). Hind paws were amputated above the ankle and homogenized in 1 mL of 10 mM

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4. Supernatants were used for the determination of IL-17, TNF-α and IL-1β levels, using the ELISA kit with sensitivities of 4.6 (Diaclone®, Besancon Cedex, France), 25 (Diaclone®, Besancon Cedex, France) and 5.1 (R&D Systems®, Abingdon, UK) pg/mL, respectively.

Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Frozen hind paws were homogenized in liquid N_2. Isolation of cytoplasmic and nuclear proteins was performed as described by Rosillo et al. [25] with some modifications. Protein concentration of the homogenate was determined following Bradford's colorimetric method [26]. Aliquots of supernatant that contains equal amount of protein (50 µg) were evaluated to determine HO-1 (Enzo®, Madrid, Spain), Nrf2, p-p38, p-JNK, p-STAT3, p65 (Santa Cruz Biotecnology®, CA, USA) and IκB-α (Cell Signalling Technology®, MA, USA) proteins by Western blot as described by Rosillo et al. [25]. The signals were analyzed and quantified by a scientific imaging systems (ImageJ software®, Maryland, USA).

Data analysis

All values in the figures and text are expressed as arithmetic mean ± standard error (SEM). Data were evaluated with Graph Pad Prism version 5.04 software. The statistical significance was evaluated by two-way (time course) or one-way (rest of parameters) analysis of variance (ANOVA), followed by Newman–Keuls' test. P values < 0.05 were considered statistically significant.

Results

Effects of dietary EVOO on CIA model

The development of arthritis was monitored until day 42. The time course of arthritic score (Fig. 1a) indicates that control mice fed with SO presented a progressive development of arthritis observed from day 29. However, mice feeding with EVOO showed a significant ($p < 0.05$ vs. SO-CIA) delayed onset and decreased the disease severity of CIA reducing disease incidence, number of involved paws, footpad thickness and clinical index from days 30 to 42. These results suggested that dietary EVOO not only could retard the development but it also may have a therapeutic effect on ongoing inflammatory arthritis. Representative photographs of hind paws from the different experimental animal groups are shown in Fig. 1b.

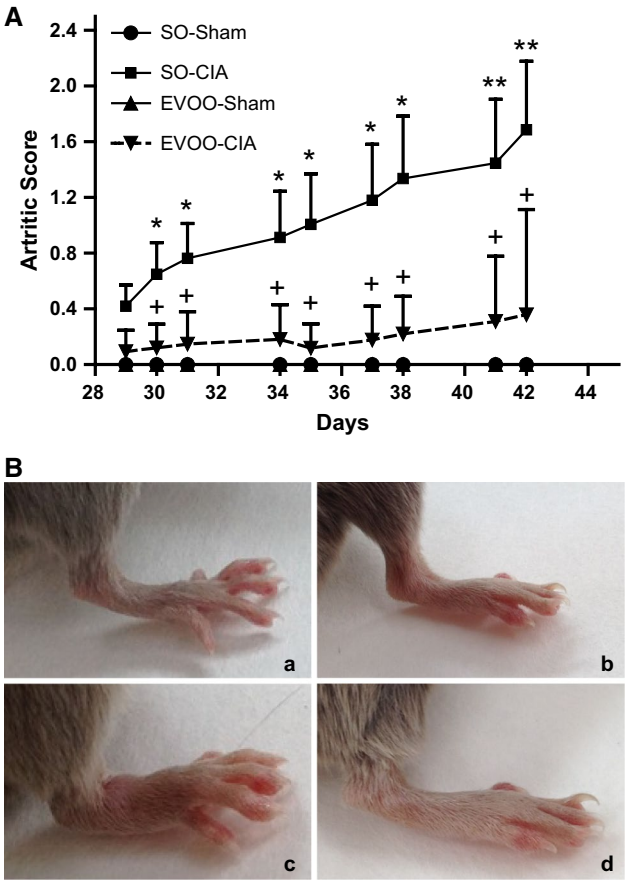

Fig. 1 Time course of the arthritis macroscopic score (**a**), representative photographs (**b**) of hind paws at the end of the experiment (day 42). SO-Sham (**a**), non-arthritic mice feed with SO diet; EVOO-Sham (**b**), non-arthritic mice feed with EVOO diet; SO-CIA (**c**), control arthritic group feed with SO diet; EVOO-CIA (**d**), arthritic mice feed with EVOO diet. Data represent mean ± SEM $n = 10$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs Sham (SO and EVOO); $^{+}p < 0.05$ vs SO-CIA

In addition, H&E staining revealed that histological features of the joint from sham animals were typical of normal structure with synovial membrane composed of synovial cells and collagen and a clear synovial space (Fig. 2a,b). On the contrary, joint from SO-CIA mice exhibited histological changes indicative of severe arthritis, characterized by an extensive infiltration of inflammatory cells into articular tissues, exudation into the synovial space, synovial hyperplasia and cartilage erosion (Fig. 2c). These histological features were less evident in EVOO-CIA group (Fig. 2d).

Effects of dietary EVOO on serum biomarkers

MMP-3 is well known as a predictor for joint destruction in early or established RA. In the present study, we determined serum MMP-3 levels, as synovial inflammatory biomarker, in CIA mice to investigate the potential beneficial effects of our nutritional therapeutic strategy. Circulating

MMP-3 level was markedly increased ($p < 0.001$ vs. Sham) in serum from CIA animal group fed with SO diet (Fig. 3). By contrast, a strong reduction in MMP-3 serum levels was observed in those arthritic animals feeding with EVOO ($p < 0.001$ vs. SO-CIA) reaching levels comparable to those described in sham animals. In addition, serum levels of the cartilage degradation marker, COMP, were significantly elevated in SO-CIA animals when compared to sham controls. On the contrary, dietary EVOO significantly decreased serum COMP levels in CIA mice in comparison with those SO-CIA animals ($p < 0.001$ vs. SO-CIA) (Fig. 3).

Effects of dietary EVOO on joint mediators

It has been reported that TNF-α, IL-1β and IL-17 are critical cytokines involved in the pathogenesis of RA [27]. To explore whether cytokines joint levels were paralleled to the disease severity of CIA, concentration of these cytokines was examined in paw homogenates by ELISA. As shown in Fig. 4, TNF-α, IL-1β and IL-17 levels were significantly increased in paw homogenates from SO-CIA animals when compared with sham mice ($p < 0.001$ and $p < 0.5$ vs. Sham), suggesting its relationship with the synovial tissue inflammation. Conversely, our results indicate that animals fed with EVOO diet showed a significant reduction in all those pro-inflammatory cytokines production in comparison with SO-CIA group (TNF-α: $p < 0{,}01$, IL-1β: $p < 0.001$ and IL-17: $p < 0.05$ vs. SO-CIA). Importantly, IL-1β and IL-17 cytokines values were similar to those obtained from control sham animals.

Effect of dietary EVOO on p-STAT3 protein expression

STAT-3 has been described as a critical transcription factor involved in the pathogenesis of RA by steering the abnormal activation, automaticity and prolonged survival of synovial cells. Besides, STAT-3 is a key transcription factor involved in Th17 cell differentiation [28]. We evaluated p-STAT3 protein expression by Western blot from hind paw homogenates. Statistical analysis revealed a significant p-STAT-3 overexpression in SO-CIA group when compared to the sham control groups ($p < 0.01$ vs. Sham), whereas nutritional therapy with EVOO significantly suppressed STAT3 phosphorylation in arthritic CIA mice ($p < 0.01$ vs. SO-CIA) (Fig. 5). These results are in accordance with those obtained in the measurement of pro-inflammatory cytokines levels suggesting that dietary EVOO may repress IL-17 production via negatively interfering with STAT3 signaling pathway.

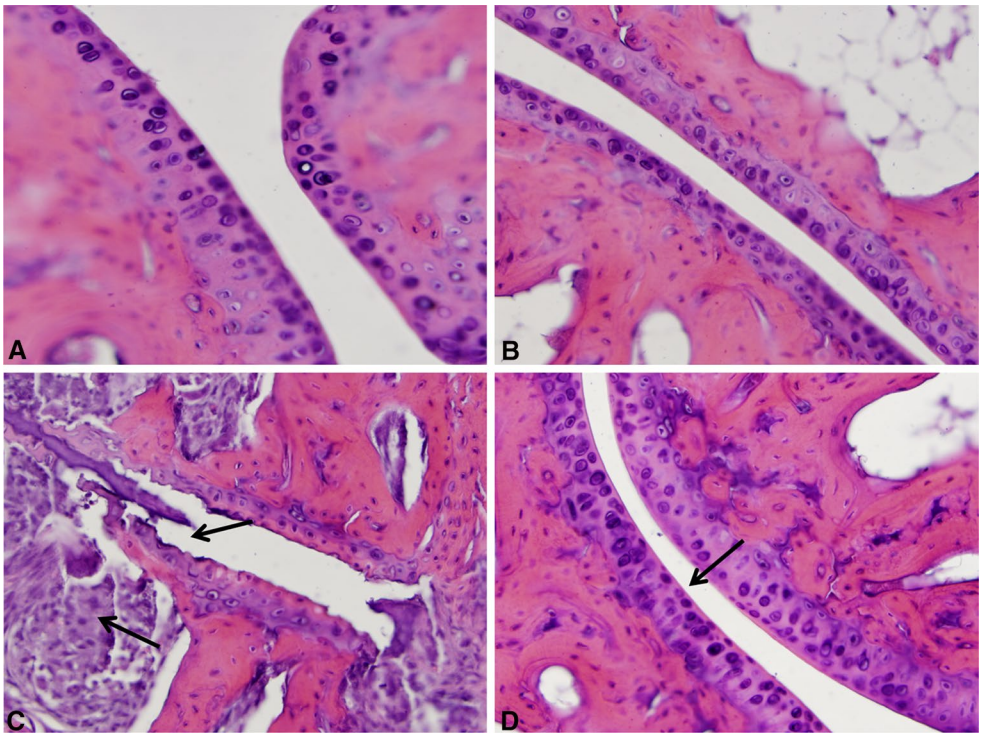

Fig. 2 Histological analysis of the frontal sections of knee joints on day 42. Sections were stained with hematoxylin and eosin. Original magnification x200. SO-Sham (**a**), non-arthritic mice feed with SO diet; EVOO-Sham (**b**), non-arthritic mice feed with EVOO diet; SO-CIA (**c**), control arthritic group feed with SO diet; EVOO-CIA. *Arrows* the infiltration of inflammatory cells and the cartilage erosion. **d** Arthritic mice feed with EVOO diet. The *arrow* less damage at the cartilage. The images are representative of at least six experiments

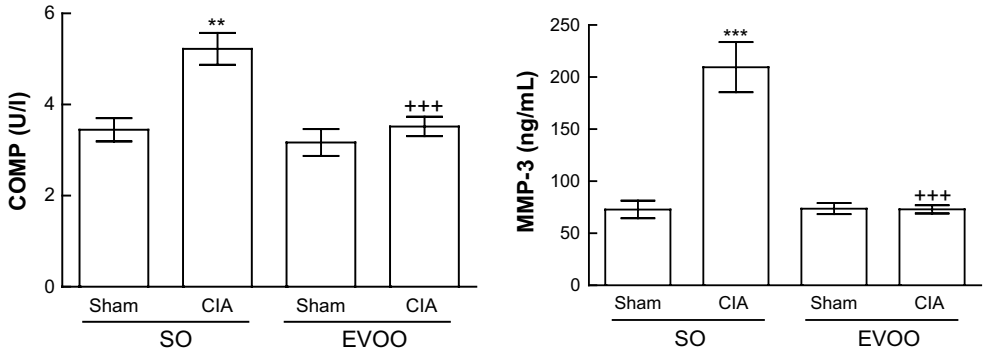

Fig. 3 Measurement of serum COMP and MMP-3 levels. These were measured by ELISA kits. Data represent mean ± SEM, $n = 10$. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs Sham (SO and EVOO); $^{+++}p < 0.001$ vs SO-CIA

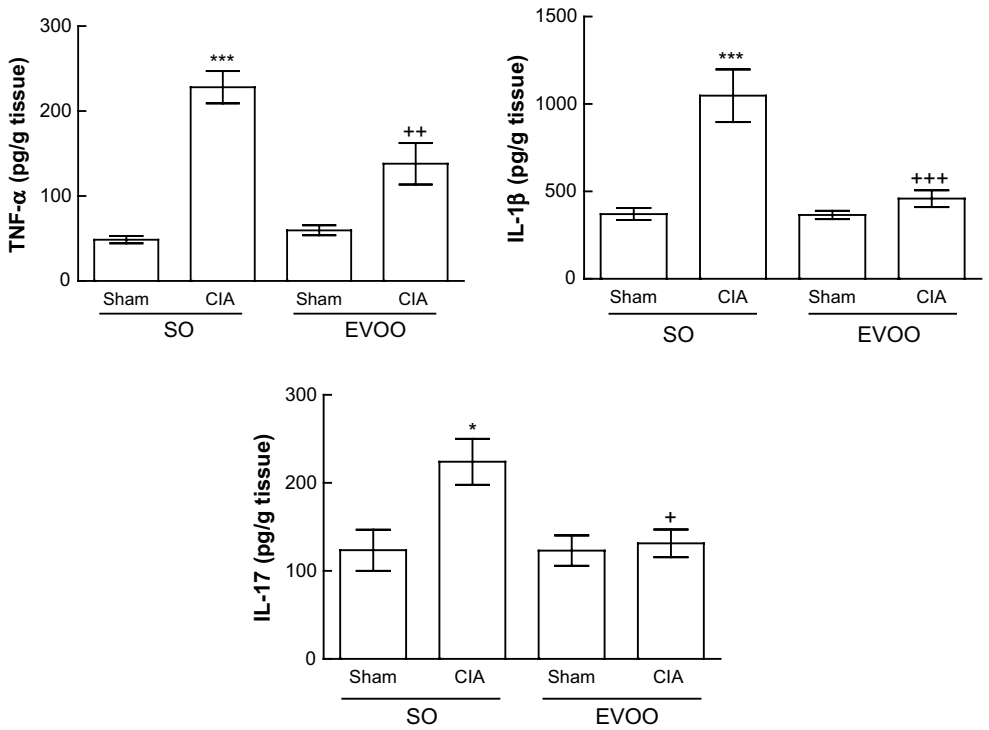

Fig. 4 Measurement of TNF-α, IL-1β and IL-17 levels in hind paw homogenates. These were determined by ELISA kits. Data represent mean ± SEM, $n = 10$. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs Sham (SO and EVOO); $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs SO-CIA

Effect of dietary EVOO on HO-1 and Nrf2 protein expression

The expression of the proteins HO-1 and Nrf2 was also evaluated in paw homogenates by western blotting. Our data show that HO-1 was significantly downregulated in SO-CIA mice during the maintenance of chronic inflammation ($p < 0.001$ vs. Sham); however, dietary EVOO treatment induced a strong HO-1 overexpression in comparison with SO-CIA arthritic control group ($p < 0.01$ vs. SO-CIA) (Fig. 6). Nrf2 activation has been reported to play an important role in HO-1 expression. According to our results, nuclear Nrf2 expression was reduced in those arthritic animals feed with SO diet ($p < 0.05$ vs. Sham), whereas a significant increase in nuclear Nrf2 protein levels was observed in EVOO-CIA animals ($p < 0.05$ vs. SO-CIA) (Fig. 6).

Effect of dietary EVOO on JNK and p38 MAPKs phosphorylation

MAPKs play a key role inducing the pro-inflammatory gene expression, which initiates inflammatory responses.

We investigated the effect of dietary EVOO on MAPKs (JNK and p38) signaling pathway activation in CIA mice. In the present study, phosphorylation of JNK and p38 proteins increased in cytosolic extracts from SO-CIA mice paw homogenates. Nonetheless, the protein expression of both p-JNK and p-p38 was significantly ameliorated in EVOO-CIA mice ($p < 0.05$ and $p < 0.01$ vs. CIA-SO) (Fig. 7).

Effects of dietary EVOO on NF-κB signaling pathway

We also investigated the effects of EVOO on IkB-α degradation in cytoplasmic extracts from mice joints. As shown in Fig. 8, IκB-α expression was significantly reduced in CIA-induced arthritis mice when compared with sham mice, in the SO treatment. The IκB-α expression observed in CIA mice is consistent with an increase in IκB-α degradation, thus allowing NF-κB translocation into the nucleus to bind specific DNA sequences leading to pro-inflammatory genes transcription. On the contrary, the effect observed in arthritic control group on IκB-α protein expression was prevented by dietary EVOO treatment. According to the results obtained, the nuclear p65 protein levels were increased in SO-CIA group ($p < 0.001$ vs. Sham), whereas dietary EVOO treatment prevented the CIA-induced nuclear translocation level of p65 in paw homogenate in comparison with those arthritic animals feed with SO ($p < 0.01$ vs. SO-CIA) (Fig. 8) avoiding the NF-κB-mediated transcriptional activation.

Discussion

The focus of this study has been to demonstrate the potential protective role of a diet elaborated with EVOO in the prevention and development of inflammatory arthritis and joint damage in a murine CIA-induced experimental arthritis model. This model is commonly used to investigate mechanisms relevant to RA as well as new antiarthritic treatments [10]. CIA induction resulted in the development of a pronounced synovitis associated with an autoimmune response against cartilage and production of matrix-degrading enzymes accompanying cartilage degradation and bone erosions [29]. The results of the present work clearly indicate, for the first time, that EVOO, as the lipid component of the diet, effectively exhibited preventive and therapeutic effects in the development of inflammatory arthritis and joint damage in CIA arthritic mice in comparison with those CIA mice fed with SO. This effect was well correlated to an improved arthritis score, a minor inflammatory cells infiltration into articular tissues, reduced exudation into the synovial space, synovial hyperplasia and cartilage erosion.

The pathogenesis of RA is closely related to an imbalance of cytokine network contributing to the development and progression of both CIA and RA [30]. Actually, overexpression of pro-inflammatory cytokines, such as IL-1β,

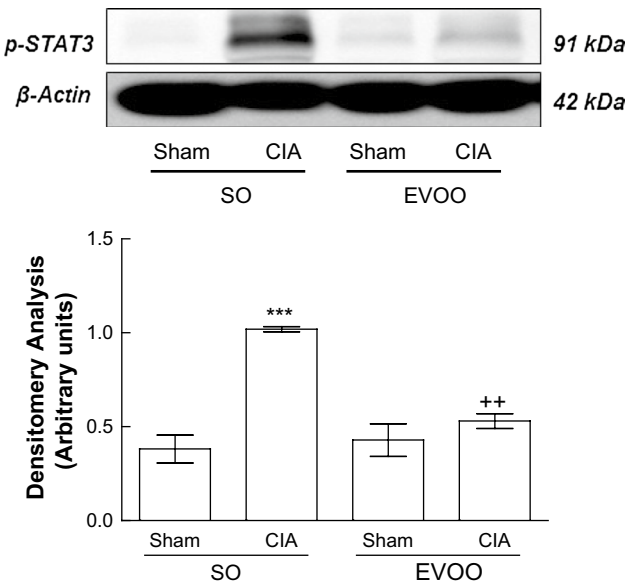

Fig. 5 Changes in p-STAT3 protein expression in hind paws homogenate. The expression of p-STAT3 was quantified by densitometry and normalized with respect to β-actin. Data represent mean ± SEM, $n = 4$.***$p < 0.001$ vs Sham (SO and EVOO); $^{++}p < 0.01$ vs SO-CIA

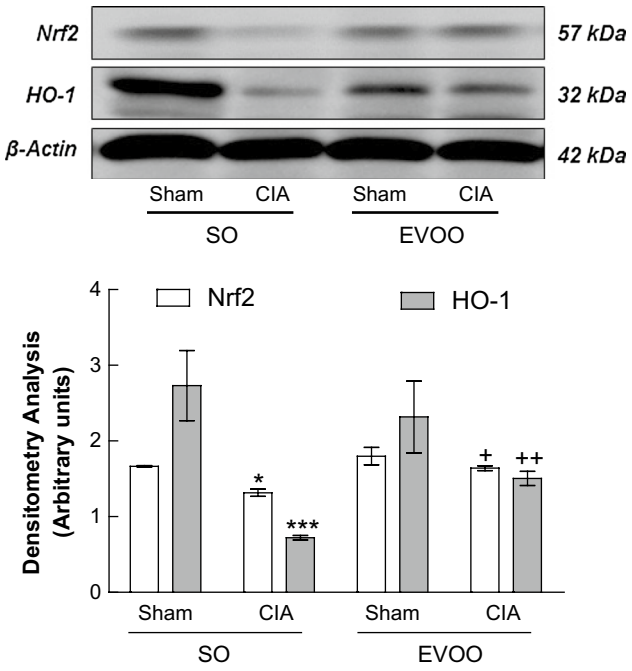

Fig. 6 EVOO dietary up-regulated HO-1 and Nrf2 protein expression in hind paws homogenate. The expression was quantified by densitometry and normalized with respect to β-actin. Data represent mean ± SEM, $n = 4$.*$p < 0.05$ and ***$p < 0.001$ vs Sham (SO and EVOO); $^{+}p < 0.05$ and $^{++}p < 0.01$ vs SO-CIA

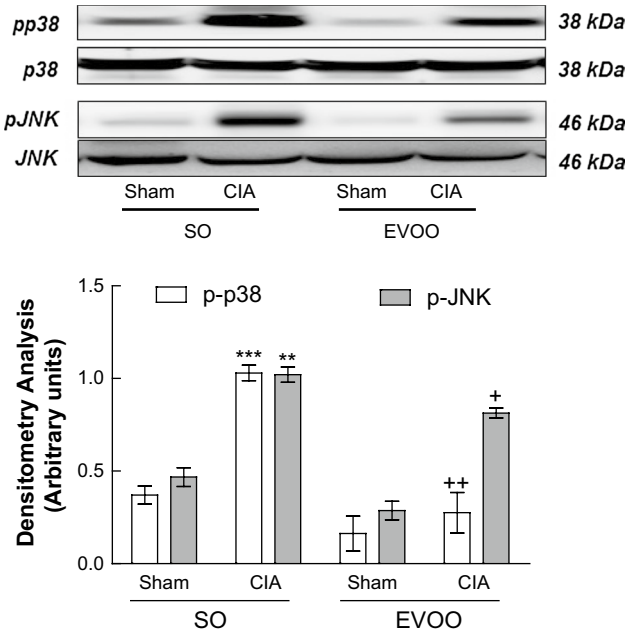

Fig. 7 Effect of dietary EVOO on JNK and p38 MAPKs phosphorylation in hind paws homogenate. The expression of phosphorylated proteins was expressed related to the expression of the corresponding total protein. Data represent mean ± SEM, $n = 4$. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs Sham (SO and EVOO); $^{+}p < 0.05$, $^{++}p < 0.01$ vs SO-CIA

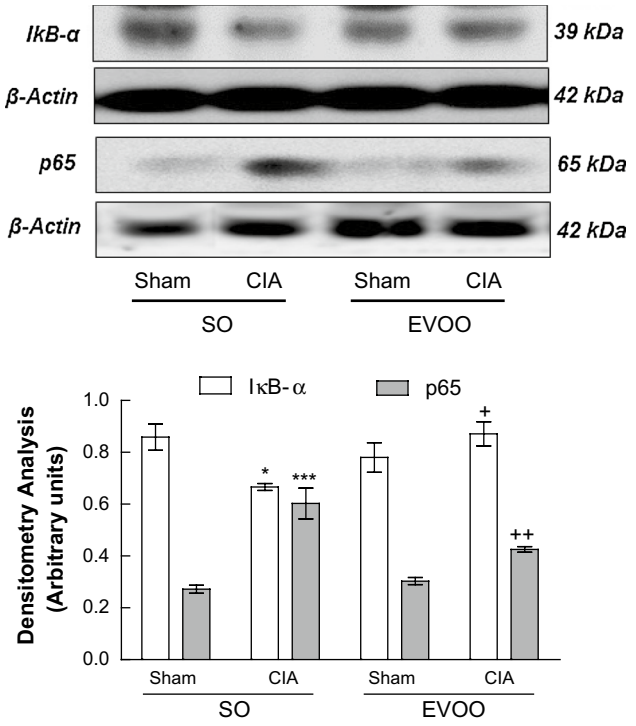

Fig. 8 Expression of IκB-α in cytoplasmic fraction and p65 NF-κB in nuclear fraction of homogenates from hind paws. The expression was quantified by densitometry and normalized with respect to β-actin. Data represent mean ± SEM, $n = 4$. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs Sham (SO and EVOO); $^{+}p < 0.05$ and $^{++}p < 0.01$ vs SO-CIA

TNF-α and IL-17, may activate osteoclasts and macrophages and also recruit leukocytes in inflamed joints. The infiltrated leukocytes produce cytokines, chemokines and tissue-destructive enzymes leading to chronic inflammation, cartilage damage and bone erosion. Besides, IL-17 is able to induce the release of IL-8 and IL-6, and plays a remarkable role in the additive/synergistic effects induced by TNF-α and IL-1β [31]. Importantly, targeting IL-17 has demonstrated therapeutic effects in animal models of inflammatory and autoimmune diseases, including RA [32]. Cytokines also up-regulate MMPs expression by chondrocytes and synoviocytes at the pannus cartilage junction leading to cartilage degradation [3, 4, 33]. Therefore, we examined TNF-α, IL-1β and IL-17 levels in paw homogenates from CIA animals in comparison with sham control group. Our results indicate that animals fed with EVOO diet showed a significant reduction in all pro-inflammatory cytokines levels. Consequently, EVOO intake would contribute to reduce the inflammatory response and joint damage by suppressing the production of the key Th17 polarization cytokines, TNF- α and IL-1β, in CIA arthritic mice.

COMP is a matrix protein with a great potential as a biological marker of cartilage metabolism in arthritis [34]. Increased fragments of COMP have been reported in patients with osteoarthritis, RA and joint injury; thus, the monitoring of COMP levels in synovial fluid or serum has been suggested to be a helpful method for assessing the presence and progression of the inflammatory disease. In this sense, serum COMP levels are reduced in RA patients in remission [34, 35]. In addition, COMP is a putative substrate for MMPs. Particularly, MMP-3 is a proteinase secreted by synovial fibroblasts and chondrocytes. Its activity results in degradation of aggrecan core protein, cartilage link protein, fibronectin and collagen types IV, VII, IX and XI. MMP-3 is present in RA synovial fluid, overexpressed in rheumatoid synovium and then associated with higher joint damage in RA [36]. Likewise, serum MMP-3 level is suggested as a predictor for joint destruction in early RA or established RA and should be used in association with usual inflammatory markers to follow therapy efficiency [37]. Our data are in agreement with above studies and showed that high serum COMP and MMP-3 levels were associated with disease activity and joint progression in CIA mice; by contrast, the production of both cartilage and synovial biomarkers was significantly inhibited by dietary EVOO treatment in CIA mice.

Abnormal signaling pathways play an important role in the inflammatory process and can lead to dysregulation of the inflammatory response being crucial in RA

pathogenesis. Signal transduction pathways closely involved in inflammation included the MAPKs, JAK-STAT and NF-κB pathways [9]. NF-κB is a crucial transcriptional activator for the expression of multiple proinflammatory genes in the microenvironment of the arthritic joints, including TNF-α, IL-1β, IL-6 and IL-17, that exert their influences on both osteoclasts and osteoblasts differentiation and thereby contributing to progressive joint destruction in RA patients and CIA mice [38, 39] as mentioned above. In fact, several anti-rheumatic drugs, currently in clinical use, inhibit NF-κB activation [40]. Interestingly, intra-articular or systemic blockade of NF-κB signaling is an effective target in the treatment of arthritis in animal models of RA [41]. Taken together, these studies confirm the importance of NF-κB activation in the development of RA. Consequently, NF-κB pathway suppression could be a novel strategy for delaying the progress of RA. Our results suggested that dietary EVOO treatment suppressed NF-κB activation, down-regulating pro-inflammatory cytokines and MMP-3 expression, thus minimizing joint destruction in CIA-induced arthritic mice.

MAPK family members, including p38 kinases, ERKs 1 and 2 and JNKs, play critical roles in many important cell processes, including cell division, differentiation, inflammation and apoptosis. They are especially drawing attention in RA because of the involvement in regulating both cytokine production and cytokine action [5, 6]. In fact, JNK MAPK regulates MMPs production by synovial fibroblasts and drives osteoclast differentiation in RA [42], and p38 MAPK regulates MMP-3 induction in fibroblasts [43] and osteoclast differentiation [44]. Moreover, MAPKs phosphorylate the JAK-STAT important in pro-inflammatory cytokine-mediated signaling pathway leading to STAT-3 activation [8]. STAT-3 has been described as a critical transcription factor involved in Th17 cell differentiation [28]. In this line, STAT-3 overexpression has been also detected in synovial membranes from RA patients and it has been reported to contribute to the chronicity of inflammation in a murine zymosan-induced arthritis model [45]. Recently, STA-21, a promising STAT-3 inhibitor, has shown antiarthritic effects in interleukin-1 receptor antagonist knockout (IL-1Ra-KO) mice, an animal model of RA [46]. Our study showed that p38 and JNK MAPKs phosphorylations were increased in SO-CIA mice. Similarly, STAT-3 overexpression was also evidenced in RA synovium of SO-CIA mice and was positively related to the severity of synovitis, whereas EVOO diet intake reduced significantly both MAPKs and STAT-3 activation at transcriptional level. Altogether, our results suggest that dietary EVOO may repress IL-17 production interfering negatively with JNK and p-38 MAPKs and STAT-3 signaling pathways.

HO-1 activity catabolizes heme to carbon monoxide, iron and biliverdin, and the latter being reduced to bilirubin by biliverdin reductase. Nrf2 plays a central role for expression of HO-1. In basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-likeECH-associated protein1 (Keap1) and degraded by the ubiquitin-dependent 26S proteasome system. Under activation, Nrf2 released from Keap1 inhibition, translocates to the nucleus, heterodimerizes with Maf and binds antioxidant response elements (AREs) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 [47]. In inflammatory and immune conditions, the expression of this protein could be part of an adaptative mechanism to limit cytotoxicity via several mechanisms including scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis [10]. Likewise, it has been reported that HO-1 deficiency in mice results in a chronic inflammatory state [48]. Our data showed that HO-1 could represent a potential molecular target susceptible to EVOO modulation, which has not been demonstrated previously, since dietary EVOO treatment strongly augmented Nrf2 and HO-1 expression conferring a role of HO-1 in the beneficial effects of EVOO in this murine model of chronic inflammation.

Since our results show that EVOO diet was more effective in reducing arthritis severity in comparison with SO diet, we could suggest that the responsibility for such beneficial properties could to be assigned to both an adequate fatty acid profile of EVOO and the presence of a high proportion of phenolic compounds. In this sense, a recent report showed that oleuropein aglycone, a polyphenol found in olive oil, exerted an anti-inflammatory effect and ameliorated the joint damage associated with CIA [15]. In addition, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it is not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action [14, 49]. Collectively, our results confirm, for the first time, that EVOO intake dramatically attenuated the progression and severity of arthritis in CIA DBA/1 J mice. The mechanisms underlying these protective effects involved JAK/STAT, NFκB and MAPK signaling pathways inhibition, which decreased the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-17 accompanied by an important reduction of the serum synovial and cartilage biomarkers COMP and MMP-3 and a significant Nrf2 and HO-1 up-regulation, conferring a role of HO-1 in the beneficial effects of EVOO in this murine model of chronic inflammation. We concluded that EVOO diet could be a beneficial functional food on RA if subsequent randomized trials in humans confirm the present results in animal models.

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Conflict of interest The authors declare that they have no conflict of interest.

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EFECTO ANTIINFLAMATORIO Y PROTECTOR DE LAS ARTICULACIONES DE UN EXTRACTO POLIFENÓLICO DEL ACEITE DE OLIVA VIRGEN EXTRA EN ARTRITIS EXPERIMENTAL

MªÁngeles Rosillo[1], María José Alcaraz[2], Marina Sánchez-Hidalgo[1], José G. Fernández-Bolaños[3], Catalina Alarcón-de-la-Lastra[1], María Luisa Ferrandiz[2]

[1]Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España

[2]Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, España

[3]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, España

RESUMEN

Antecedentes del tema: La AR es una enfermedad autoinmune caracterizada por la inflamación sinovial y la formación de pannus, que provoca daños en el cartílago y en el hueso. Aunque la patogenia de la AR sigue sin conocerse en su totalidad, está ampliamente demostrado, que se produce un aumento de los niveles de citocinas proinflamatorias, que contribuyen a la cronicidad y al daño tisular en la AR. Este aumento se atribuye, al menos en parte, a una desregulación en las rutas de señalización celular protagonizadas por las MAPKs, JAK/STAT y el factor nuclear NF-κB. El modelo de artritis experimental inducida por colágeno tipo II en ratones, es un modelo ampliamente usado para determinar el potencial antiartrítico de nuevas moléculas. La terapia biológica, ha supuesto un gran avance en el tratamiento de la AR, sin embargo, este tipo de medicamentos sólo son efectivos en una parte de los pacientes y tienen otras limitaciones como su alto coste económico o el requerimiento de ser administrados por vía parenteral. Es por ello, por lo que en los últimos años se ha generado un auge en el uso de otras estrategias terapéuticas, entre las que destaca la terapia nutricional. El AOVE en los países Mediterráneos ha demostrado efectos beneficiosos en la prevención de ciertas enfermedades crónicas. Tradicionalmente, el efecto beneficioso del AOVE ha sido atribuido a su alto contenido en ácidos grasos monoinsaturados, como el ácido oleico; sin embargo, estudios recientes han puesto de manifiesto que los componentes minoritarios, el especial la fracción polifenólica, del AOVE juegan un papel fundamental.

Objetivos: En base a los anteriores antecedentes, nos planteamos evaluar los efectos de la administración oral de un extracto polifenólico (PE) procedente de AOVE de la variedad picual, en un modelo de artritis experimental inducida por colágeno tipo II en ratones.

Material y Métodos: La artritis fue inducida en ratones DBA-1/J de 11 semanas de edad. El día 29, después de la primera inmunización, los ratones ya presentaban signos de inflamación en sus extremidades; éste día fueron separados en 4 grupos: (1) Controles sanos (CS); (2) Controles artritis (CIA); (3) Ratones artríticos que recibían 100mg/Kg PE de forma oral (PE100); (4) Ratones artríticos que recibían 200 mg/Kg PE de forma oral (PE200). El grado de desarrollo de la enfermedad fue valorado de forma visual siguiendo una escala de 0-2, dónde 0=sin inflamación; 1=inflamación leve; 1.5=inflamación marcada; 2=inflamación severa y según el estudio histológico de las articulaciones. Los niveles de citocinas proinflamatorias como TNF-α, IL-1β e IL-6, así como los niveles del ecosanoide PGE_2, en el homogenado de pata fueron determinados mediante la técnica de ELISA. La expresión de COX-2 fue analizada por inmunohistoquímica y los cambios en la expresión proteica de m-PGES1 y el papel de las vías de señalización celular de MAPKs, JAK/STAT y NF-κB fueron estudiadas mediante western blot.

Resultados: El tratamiento con PE fue capaz de reducir los signos y síntomas de la enfermedad, disminuyendo la inflamación articular, la migración celular, la degradación del cartílago y la erosión ósea. Tras el tratamiento con PE a ambas dosis, se redujeron significativamente los niveles de las citocinas proinflamatorias TNF-α, IL-1β e IL-6 y del ecosanoide PGE_2 en el las patas de los animales artríticos, del mismo modo, los niveles de expresión de las proteínas proinflamatorias COX-2 y mPGES-1 también se encontraron disminuidas en las patas de los animales tratados con los polifenoles. El PE fue capaz de inhibir la fosforilación de la proteína STAT3, así como de las proteínas MAPKs JNK y p38. Finalmente tras la administración del extracto, los niveles de la proteína inhibitoria IκB-α se encontraron aumentados en comparación con los animales pertenecientes al grupo CIA, impidiendo la translocación al núcleo de la proteína p65 NF-κB.

Conclusión: Estos resultados ponen de manifiesto, por primera vez, el efecto antiinflamatorio y protector de las articulaciones del extracto polifenólico procedente del AOVE, que puede estar relacionado con la inhibición de las principales vías de señalización celular implicadas en la inflamación, como son NF-κB, JAK/STAT o MAPKs, controlando la producción de mediadores proinflamatorios. Estos datos demuestran el interés de los compuestos naturales, como los polifenoles del AOVE, en el desarrollo de nuevos productos terapéuticos para el tratamiento de la AR.

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Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis☆

María Ángeles Rosillo[a], María José Alcaraz[b], Marina Sánchez-Hidalgo[a], José G. Fernández-Bolaños[c], Catalina Alarcón-de-la-Lastra[a], María Luisa Ferrándiz[b,*]

[a]Department of Pharmacology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain
[b]Department of Pharmacology and IDM, University of Valencia, 46100 Burjasot, Valencia, Spain
[c]Department of Organic Chemistry, Faculty of Chemistry, University of Seville, 41012 Seville, Spain

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Abstract

The consumption of extra virgin olive oil (EVOO) in Mediterranean countries has shown beneficial effects. A wide range of evidence indicates that phenolic compounds present in EVOO are endowed with anti-inflammatory properties. In this work, we evaluated the effects of EVOO-polyphenol extract (PE) in a model of rheumatoid arthritis, the collagen-induced arthritis model in mice. On day 0, DBA-1/J mice were immunized with bovine type II collagen. On day 21, mice received a booster injection. PE (100 and 200 mg/kg) was orally administered once a day from days 29 to 41 to arthritic mice. We have demonstrated that PE decreases joint edema, cell migration, cartilage degradation and bone erosion. PE significantly reduced the levels of proinflammatory cytokines and prostaglandin E_2 in the joint as well as the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1. Our data indicate that PE inhibits c-Jun N-terminal kinase, p38 and signal transducer and activator of transcription-3. In addition, PE decreases nuclear factor κB translocation leading to the down-regulation of the arthritic process. These results support the interest of natural diet components in the development of therapeutic products for arthritic conditions.

Keywords: Rheumatoid arthritis; CIA; EVOO; Polyphenols; Inflammatory response

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation and pannus leading to cartilage and bone damage. The earliest event is activation of the innate immune by exogenous and autologous antigens and thus auto-antibodies and later high levels of cytokines can be detected years before clinical symptoms [1]. At a joint level, there are synovial hyperplasia and massive infiltration of immune cells, including $CD4^+$ T cells, B cells, natural killer cells, macrophages, dendritic cells, neutrophils and mast cells [2,3]. A wide range of evidence has demonstrated the contribution of cytokines to the chronicity of inflammation and tissue damage in RA. Therefore, the importance of proinflammatory cytokines has been confirmed by the success of cytokine-directed therapies in RA [4]. Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor α (TNFα) activate many signal transduction pathways involved in inflammation. The mitogen-activated protein kinase (MAPK) pathway includes extracellular signal-regulated kinases (ERK1/2 or p42/p44), c-Jun N-terminal kinases (JNK)1/2/3 and p38, which are activated in the synovium of patients with RA [5]. The janus kinase-signal transducer and activator of transcription (JAK-STAT) is another relevant inflammatory pathway activated in response to cytokines. In particular, overexpression of STAT-3 has been reported in synovial membranes from RA patients [6].

Proinflammatory cytokines activate different transcription factors to induce the expression of inflammatory and catabolic mediators [7]. Nuclear factor κB (NF-κB) and activating protein-1 (AP-1) are activated in RA synovium and play an important role in metalloproteinase induction [8]. NF-κB regulates a wide range of genes that contribute to inflammation, such as IL-1β, TNFα, IL-6, chemokines and microsomal prostaglandin E synthase-1 (mPGES-1), an efficient downstream enzyme functionally coupled with cyclooxygenase-2 (COX-2).

Biological therapies have improved the treatment of chronic inflammatory diseases such as RA. Nevertheless, these drugs are effective in a fraction of patients only and have other limitations including a high cost, the requirement for parenteral administration and important side effects. Therefore, new therapeutic strategies are under investigation. Epidemiological studies about consumption of functional foods, particularly extra virgin olive oil (EVOO) in Mediterranean countries, have showed important beneficial effects.

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* Corresponding author. Department of Pharmacology, Faculty of Pharmacy, Av. Vicent Andrés Estellés s/n, 46100 Burjasot, Valencia, Spain. Tel.: +34 963543284.
E-mail address: luisa.ferrandiz@uv.es (M.L. Ferrándiz).

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EVOO, obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to oil alteration, has shown antioxidant, anti-inflammatory, immunomodulatory, antiproliferative and anti-apoptotic effects [9–15]. Although these health-protective effects have been traditionally ascribed to its high monounsaturated fatty acid content [15,16] a wide range of evidence indicates that many of the beneficial effects of EVOO intake are due to its minor highly bioactive components. Among them, phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein have shown anti-inflammatory and antioxidant effects [17]. Olive oil polyphenols have also been associated with neuroprotective, antiaging and antiatherogenic effects and the regulation of important cellular signaling pathways. On the other hand, because of their ability to modulate cell death, olive polyphenols have been proposed as chemopreventive and therapeutic anti-cancer agents [18–21]. Our research group has previously demonstrated that a diet made with EVOO enriched with hydroxytyrosol, could improve inflammatory processes in a chronic colitis model [22]. In addition, we evaluated the protective effect of dietary EVOO-polyphenol extract (PE) supplementation in the inflammatory response associated to a chronic colitis model [23].

Collagen-induced arthritis (CIA) in DBA/1 mice is an animal model of RA widely used to test potential therapeutic agents [24]. The pathogenesis of CIA is dependent on the host's response to type II collagen challenge and the subsequent generation of antibodies that recognize collagen-rich joint tissue [25]. Joint inflammation in this model is characterized by significant cellular exudate and infiltrate, synovitis, and articular degradation. High levels of proinflammatory cytokines are detected at the onset of the disease, leading to the production of a wide range of mediators relevant in CIA pathogenesis [26,27]. Taken into account the anti-inflammatory properties of olive oil constituents, we hypothesized that PE may control the development of inflammation and articular lesion. In fact, intraperitoneal administration of oleuropein aglycone, a major constituent of the leaves and unprocessed olive drupes, has shown beneficial effects in CIA [28], and a recent study has reported that EVOO diet in conjunction with physical activity is able to preserve the articular cartilage in an osteoarthritis model [29].

The present study was designed to evaluate the effects of the oral administration of PE in the arthritis model of CIA in mice. In addition to macroscopic and histological analyses, we have determined the effects of PE on the production of inflammatory mediators. In order to gain a better insight into its mechanisms of action, signaling pathways were also explored.

2. Materials and methods

2.1. Extraction of PE

PE was obtained by the method described by Vazquez Roncero et al. [30] with some modifications [31]. Fifty grams of EVOO (Oleoestepa, Seville, Spain) was extracted with methanol/water (80:20, vol/vol, 125 ml). The mixture was mixed with a vortex at 5000 g for 1 min and sonicated for 15 min. After decantation, the methanolic extract was concentrated in vacuum under a stream of nitrogen at <35°C until it reached a syrup consistency; finally, it was lyophilized and stored at −80°C.

2.2. Characterization of phenolic compounds in PE

PE (70–75 mg) extracted from EVOO (75 g) was dissolved in $CDCl_3$ (750 μl) or in DMSO-d6 (750 μl) and an precisely measured volume of the solution (550 μl) was placed in a 5 mm NMR tube for the detection and quantification of phenolic compounds. The phenolic mixture to be analyzed in DMSO-d6 was previously dissolved in a MeOD-D_2O 1:1 (1 ml) and concentrated to dryness at reduced pressure in order to exchange hydroxyl protons with deuterium nuclei. The NMR solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). NMR experiments were conducted on a Bruker Avance III 700 spectrometer, operating at 700.25 M Hz. The probe temperature was 24.8±1°C. All chemical shifts were given in ppm, and the J couplings in Hz. The solvent itself was used as a chemical shift reference (7.26 ppm for $CHCl_3$, and 2.50 for DMSO-d6). ^{1}H NMR spectra were acquired with the following acquisition parameters: acquisition time 2.3s, relaxation delay 5s, spectral width of 0–20 ppm, data points 32k, number of scans 32, and line broadening of 0.3 Hz. Postacquisition processing included zero-filling to 64k. The spectra were phase corrected automatically using TOPSPIN and integration was performed manually.

We have determined the levels of oleocanthal and oleacein following a modification of the method developed by Karkoula et al. [32] for direct measurement of both dialdehydes in olive oil by quantitative high resolution ^{1}H NMR in $CDCl_3$. The levels of other phenolic compounds have been determined by the procedure described by Christophoridou and Dais [33].

2.3. Induction of CIA

Arthritis was induced in male DBA-1/J mice (Janvier, Le Genest St Isle, France) of 11 weeks of age. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee (University of Valencia, Spain). Bovine type II collagen (CII; 2 mg/ml in dilute acetic acid; MD Biosciences, Zürich, Switzerland) was emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml *Mycobacterium tuberculosis*, strain H37Ra; Difco, Detroit, MI, USA). On day 0, DBA-1/J mice were immunized at the base of the tail with 100 μg of bovine CII. On day 21, mice received an intraperitoneal booster injection of 100 μg of CII dissolved in phosphate-buffered saline. Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or in other parts of the paws. Joint inflammation was scored visually in each paw, using a scale of 0–2 where 0=uninflamed, 1=mild, 1.5=marked and 2=severe. Scoring was performed by two independent observers without knowledge of the experimental groups. In addition, photographs and X-ray (Carestream MS FX, Gainesville, FL, USA) images of hind paws were obtained at the end of the experiment (day 42).

2.4. Treatment groups

On day 29, animals were randomized into control and treatment groups. The study was performed in four groups of mice (n=10): naive group (NA), CIA group (CIA) and two treatment groups: CIA mice with PE treatment (PE 100 and 200 mg/kg, orally, once a day from days 29 to 41). On day 42 after immunization mice were anesthetized, blood samples were taken by intracardiac puncture and animals were killed by cervical dislocation. Paws were amputated and processed for either histological analysis or homogeneization and assessment of inflammatory mediators and mechanisms.

2.5. Histological and immunohistochemical analyses

Knee joints were fixed in 4% formalin. After decalcification in 10% EDTA, specimens were processed for paraffin embedding. Tissue sections (7 μm) were stained with hematoxylin and eosin to perform histological analyses. For immunohistochemistry, the endogenous peroxidase activity was inhibited with hydrogen peroxide and then the sections were incubated in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) for 20 min to reduce nonspecific staining and successively incubated with rabbit anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA) at dilution 1:250 overnight at 4°C. Later on, slides were treated with antirabbit IgG antibody (Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min and incubated with the streptavidin–peroxidase complex of the kit for 30 min, at room temperature. After incubation with the peroxidase substrate 3,3′-diaminobenzidine and washing with water, the sections were counterstained with hematoxylin. Negative control sections were treated in the same way but in the absence of primary antibody. Positive cells and total cells were counted in five random high-power fields by two independent observers.

2.6. Enzyme-linked immunosorbent assay

Hind paws (knees) were homogenized in liquid N_2 with 1 ml of A buffer pH 7.46 (10 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 5 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 0.1 μg/ml aprotinine, and 0.5 mM phenylmethyl sulfonyl fluoride (PMSF)). The tissue homogenates were sonicated (10 s three times at 20% with a 10-s incubation on ice between bursts) in an ultrasonic processor (VC130PB, Sonics & Materials Inc., Newtown, CT, USA) and centrifuged at 600×g, 15 min at 4°C. Supernatants were removed and used for determination of inflammatory mediators by enzyme-linked immunosorbent assay (ELISA): PGE_2 (Cayman Chemical, Michigan, USA), TNFα and IL-1β (R&D Systems Inc., Minneapolis, MN, USA), and IL-6 (Diaclone, Besançon Cedex, France).

2.7. Western blot

Hind paws were homogenized in liquid N_2 with 1 ml of ice-cold phosphate buffer (0.01 mM K_2HPO_4, KH_2PO_4 and 0.15 M NaCl) and centrifuged (800×g, 10 min at room temperature). Pellets were resuspended in hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1 μg/ml leupeptin, 20 mM NaF, 0.5 mM DTT, 1 mM EDTA and 10 mM HEPES, pH 7.9], incubated for 10 min on ice and centrifuged (14,000×g, 30 s at room temperature). Supernatants (cytoplasmic proteins) and pellets (nuclear proteins) were collected. Pellets were resuspended in ice-cold low-salt buffer and nuclear proteins were released by adding a

high-salt buffer (20% vol/vol glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 420 mM NaCl, 0.2 mM EDTA, 20 mM NaF, 1 μg/ml leupeptin and 20 mM HEPES, pH 7.9). Samples were incubated on ice for 30 min. Soluble nuclear proteins were recovered by centrifugation (14,000×*g*, 10 min, 4°C) and proteins were stored at −80°C. Protein concentration was determined by the Bradford's colorimetric method [34]. Nuclear fractions were used to study p65 protein expression and cytoplasmic fractions were used for the rest of determinations. Aliquots containing equal amount of protein (50 mg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). The proteins were then electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit polyclonal anti-mPGES-1 (1:200; Cayman Chemical, USA) rabbit polyclonal anti-IκB-α (1:1000; Cell Signalling Technology, USA), rabbit polyclonal p65 (1:200, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p-JNK and rabbit polyclonal anti-JNK (1:1000; Santa Cruz Biotechnology, Inc.), mouse polyclonal anti-p-p38 and rabbit polyclonal anti-p38 (1:1000; Santa Cruz Biotechnology, Inc.) and mouse polyclonal anti-p-STAT3 (1:200; Santa Cruz Biotechnology, Inc.), overnight at 4°C. Membranes were washed three times for 15 min and incubated with anti-rabbit (Pierce Biotechnology, IL, USA) or anti-mouse (Dako, Carpinteria, CA, USA) horseradish peroxidase-labeled secondary antibody, for 1–2 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma–Aldrich). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (SuperSignal West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data were studied following normalization to the control (housekeeping gene). The signals were analyzed and quantified by ImageJ software (Bethesda, MD, USA).

2.8. Data analysis

All values in the figures and text are expressed as arithmetic means±standard error (S.E.M.). Data were evaluated with Graph Pad Prism Version 5.04 software. The statistical significance was evaluated by two-way (time course) or one-way (rest of parameters) analysis of variance (ANOVA), followed by Bonferroni's test. *P* values <.05 were considered statistically significant.

3. Results

3.1. Chemical composition of PE

Table 1 shows the result of qualitative and quantitative analyses of PE. Nine different phenolic compounds have been identified. Oleocanthal and oleacein are the main components of PE.

3.2. Effect of PE on established CIA

The time-course of arthritic score (Fig. 1A) indicates that control CIA mice showed a progressive development of clinical symptoms. The severity of arthritis in the groups treated with PE (100 and 200 mg/kg) was lower than that of the arthritic control group with significant results for the highest dose on days 39 and 41. Fig. 1B shows representative photographs of hind paws from the different groups. In addition, X-ray images (Fig. 1C) revealed the presence of joint edema and focal bone erosions in hind paws of CIA mice, whereas in animals treated with PE, the inflammation was lower. Interestingly, in the PE200 group, the integrity of bone was similar to that of naïve mice. Control CIA mice exhibited histological changes indicative of severe arthritis, with extensive infiltration of inflammatory cells into articular tissues, exudation into the synovial space, synovial hyperplasia and cartilage erosion (Fig. 2). These histological features were less apparent in mice treated with PE (200 mg/kg).

3.3. Effect of PE on joint inflammatory mediators

We also explored whether PE could modify the production of proinflammatory cytokines and PGE_2. At the end of the experimental period (day 42), the levels of IL-1β, TNFα and IL-6 were determined in hind paw homogenates. As shown in Fig. 3, animals treated with PE (100 or 200 mg/kg) showed a significant reduction in joint levels of all proinflammatory cytokines. In addition, a significant increase in PGE_2 levels in the paws of arthritic control animals with respect to naïve mice was found (Fig. 3). Treatment with PE reduced the levels of this eicosanoid, which reached statistical significance at both doses.

3.4. Effect of PE on COX-2 and mPGES-1 expression

The effect of PE on COX-2 protein expression was determined by immunohistochemistry of knee joint sections (Fig. 4A). We observed a significant increase in the percentage of COX-2-positive cells in cartilage of arthritic control group (CIA), with respect to naïve animals. In the group treated with PE (200 mg/kg), the immunoreactivity for this protein was significantly reduced. COX-2 expression was accompanied by a significant increase in mPGES-1 protein expression, which was determined by Western blot, in the paws of CIA control mice with respect to naïve animals (Fig. 4B). Treatment with PE significantly decreased the expression of this protein.

3.5. Effect of PE on p38 and JNK phosphorylation

p38 and JNK play an important role in inflammation and tissue damage in RA [5,35]. We investigated the effect of PE on the activation of JNK and p38 in the CIA model by western blot using phosphospecific antibodies. The development of CIA induced strong MAPK phosphorylation in joints (Fig. 5). However, PE at both doses significantly decreased the phosphorylation of JNK and p38.

3.6. Effect of PE on p-STAT3 protein expression in paw homogenate

p-STAT3 protein expression was evaluated by Western blot analysis of hind paw homogenates. Fig. 6 shows that arthritis strongly induced STAT3 phosphorylation, as observed in CIA control mice with respect to naïve animals. Treatment with PE at 200 mg/kg and, to a lower extent, at 100 mg/kg, significantly reduced the expression of p-STAT3 in arthritic mice.

3.7. Effects of PE on NF-κB signaling pathway

We investigated the effect of PE on IκB-α expression in cytoplasmic extracts of mice joints. As shown in Fig. 7, the expression of IκB-α was significantly reduced in arthritic control mice (CIA) compared with naïve mice. The effect of arthritis on IκB-α was blocked by PE treatment at the dose of 200 mg/kg. The reduction in IκB-α expression observed in CIA mice is consistent with an increase in IκB-α degradation thus allowing the translocation of NF-κB into the nucleus to bind specific DNA sequences leading to the transcription of proinflammatory genes. Therefore, CIA mice showed an increased expression of p65 NF-κB in nuclear extracts of joints which was significantly inhibited by PE at 200 mg/kg.

4. Discussion

In this study, we have demonstrated that oral administration of PE is able to down-regulate the arthritic process in the CIA model of RA. PE showed anti-inflammatory effects with reductions in joint edema and migration of inflammatory cells. In addition, PE protected joints against cartilage alterations and bone erosion. According to previous

Table 1
Chemical constituents of PE

Phenolic compound	μmol/100 g	ppm
Free hydroxytyrosol	2.25	3.47
Free tyrosol	5.21	7.20
Oleacein	9.62	30.82
Oleocanthal	10.43	31.74
Oleuropein aglycone monoaldehyde (5*R*, 8*S*, 9*R*)	1.49	5.63
Ligstroside aglycone monoaldehyde (5*R*, 8*S*, 9*R*)	3.88	14.06
Apigenin	0.56	1.52
Luteolin	1.26	3.61
Acetoxypinoresinol	1.92	8.00

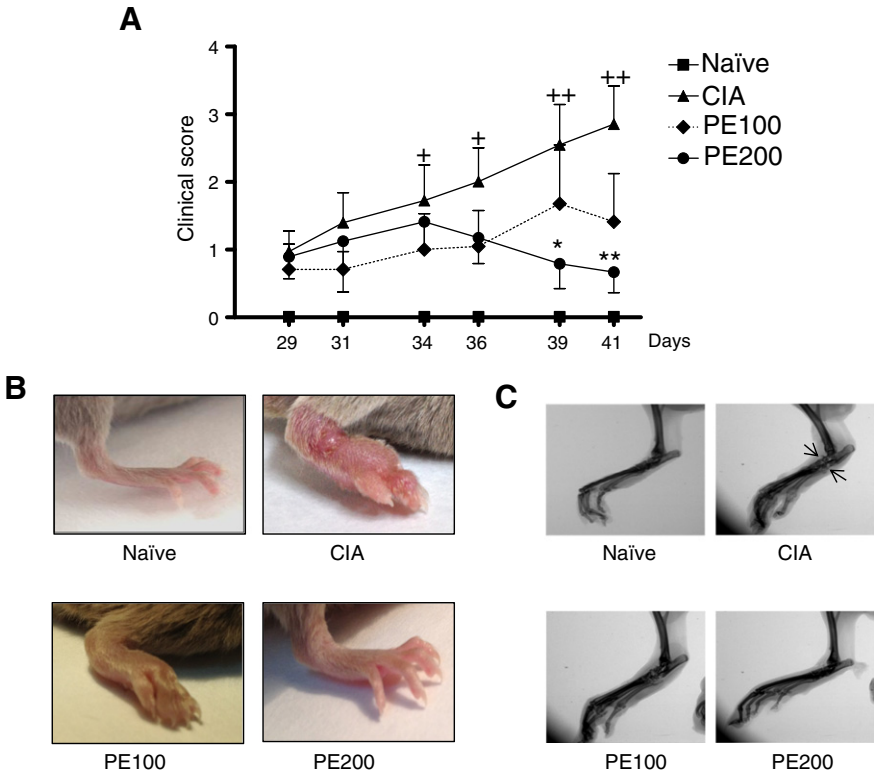

Fig. 1. Time course of the arthritis macroscopic score (A), representative photographs (B) and X-ray images (C) of hind paws at the end of the experiment (day 42). Naïve, non-arthritic mice; CIA, control arthritic group; PE 100, arthritic mice treated with PE (100 mg/kg/day p.o. from days 29 to 41 after immunization); PE 200, arthritic mice treated with PE (200 mg/kg/day p.o. from days 29 to 41 after immunization). Data represent mean±S.E.M. $n=10$. $+P<.05$, $++P<.01$ vs. naïve; $^{*}P<.05$, $^{**}P<.01$ vs. CIA. Arrows indicate focal bone erosion.

reports on the biological properties of olive oil constituents [17,18,28,9], the anti-inflammatory effects of PE are likely dependent on the mixture of different phenolic compounds.

The progression of arthritis is associated with sustained production of proinflammatory cytokines [4]. We have shown in the CIA model that PE controls the local levels of these cytokines driving the production of different inflammatory and catabolic mediators. Proinflammatory cytokines induce further cytokines, chemokines, eicosanoids and reactive oxygen species (ROS) amplifying the inflammatory response [36]. Therefore, COX-2 and mPGES-1, enzymes responsible for the overproduction of PGE_2 in inflammation, are up-regulated [37] contributing to the progression of RA through EP_4 receptor activation [38]. We have shown that PE reduces PGE_2 levels which would be dependent on the down-regulation of COX-2 and mPGES-1 expression in the joint. Cytokines also induce the expression of matrix metalloproteinases at the pannus cartilage junction leading to cartilage degradation [39,40]. In addition, TNFα stimulates osteoclastogenesis [41], suppresses the recruitment of osteoblasts and inhibits the expression of matrix genes [42], whereas IL-6 increases osteoclast numbers in trabecular bone [43]. Therefore, inhibition of proinflammatory cytokine production by PE would result in the reduction of the inflammatory response and tissue damage.

MAPKs regulate the synthesis of chemokines, cytokines, adhesion molecules and PGs involved in inflammation. In addition, they mediate the induction of matrix metalloproteinases responsible for cartilage breakdown. JNK1/2 and p38 are also involved in osteoclast differentiation and thus the activation of these enzymes may contribute to bone destruction [35]. In particular, p38 plays a key

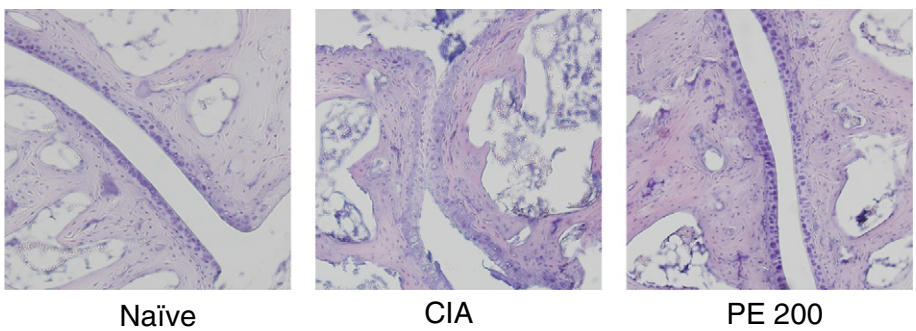

Fig. 2. Histological analysis of the frontal sections of knee joints on day 42. Sections were stained with hematoxylin and eosin. Original magnification ×100. Naïve, nonarthritic mice; CIA, control arthritic group; PE 200, arthritic mice treated with PE (200 mg/kg/day p.o. from days 29 to 41 after immunization). The images are representative of at least six experiments performed on different days.

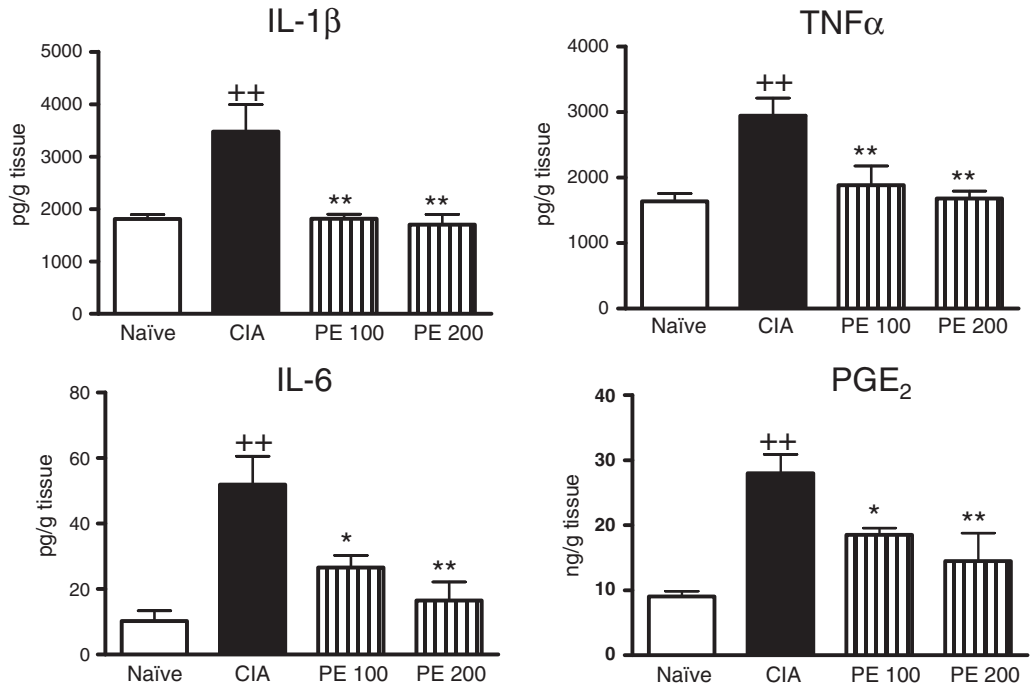

Fig. 3. Levels of TNFα, IL-1β, IL-6 and PGE_2 in paw homogenates. These inflammatory mediators were determined by ELISA. Data represent mean±S.E.M., $n=6$. ++$P<.01$ vs. naïve; *$P<.05$, **$P<.01$ vs. CIA.

role in RA [44]. JAKs bind to intracellular domains of IL-6 or interferon receptors and catalyze ligand-induced phosphorylation of themselves and of intracellular tyrosine residues. Phosphorylation of STATs on tyrosine residues results in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences. This pathway can be regulated by different protein kinases such as MAPK that phosphorylate STATs on serine residues to potentiate STAT-activating stimuli [45]. STAT3 may contribute to the chronicity of inflammation in experimental arthritis [46]. Our data indicate that PE administration is able to reduce the phosphorylation of JNK, p38 and STAT3, suggesting that PE could control the activation of these important signaling pathways during the arthritic process.

The NF-κB pathway is involved in the transcription of many inflammatory genes [47]. Cellular stimulation by proinflammatory cytokines induces the recruitment of costimulatory molecules, such as TNF receptor-associated factor (TRAF) leading to the activation of NF-κB-inducing kinase. This protein induces IκB kinase activation resulting in the phosphorylation of IκB [6], which is followed by ubiquitination and proteolytic degradation thus allowing the release of NF-κB to enter the nucleus and regulate gene transcription [48].

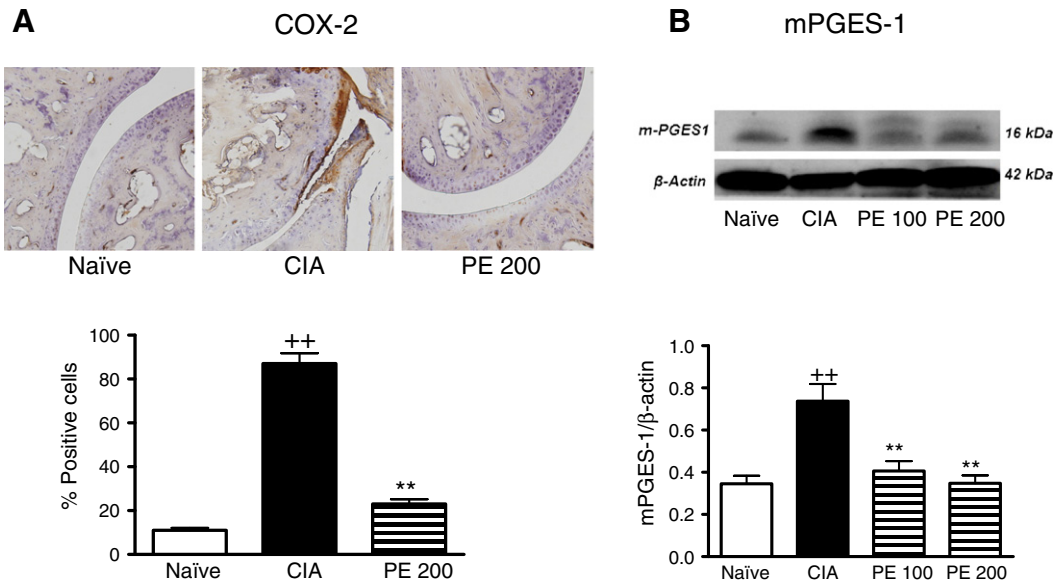

Fig. 4. Expression of COX-2 (A) and mPGES-1 (B) in hind paws. COX-2 was determined by immunohistochemistry and expressed as percentage of positive cells. mPGES-1 was determined by Western blot, quantified by densitometry and normalized with respect to β-actin. Data represent mean±S.E.M., $n=4$. ++$P<.01$ vs. naïve; **$P<.01$ vs. CIA.

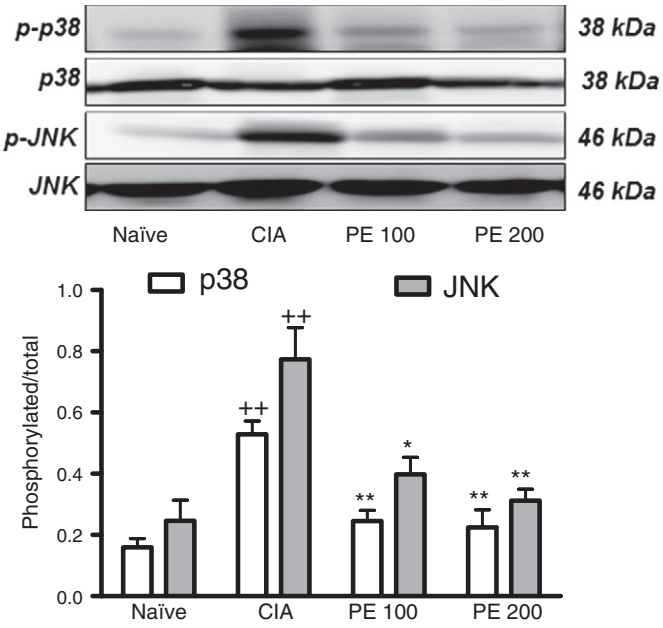

Fig. 5. Expression of p-p38 and p-JNK in hind paws. The expression of phosphorylated proteins was expressed related to the expression of the corresponding total protein. Data represent mean±S.E.M., $n=4$. ++$P<.01$ vs. naïve; *$P<.05$, **$P<.01$ vs. CIA.

Our results indicate that PE enhances the levels of the inhibitory protein IκB-α leading to the reduction of NF-κB nuclear translocation. This mechanism may be responsible for the down-regulation of proinflammatory cytokines in the joint during the CIA process in the animals treated with PE. Proinflammatory cytokines and ROS could act as mediators of NF-κB activation [49]. As demonstrated in previous studies, PE inhibits the generation of ROS [50] besides its effect on proinflammatory cytokines, suggesting that both mechanisms could be involved in the control of NF-κB translocation.

In conclusion, or study has demonstrated the anti-inflammatory and joint protective effects of PE from EVOO, which would be related to the inhibition of relevant signaling pathways such as NF-κB, STAT-3, JNK and p38 controlling the production of inflammatory mediators.

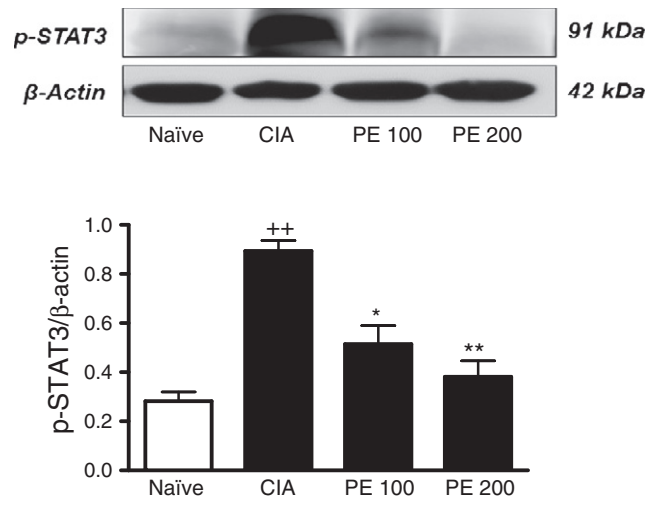

Fig. 6. Expression of p-STAT3 in hind paws. The expression of p-STAT3 was quantified by densitometry and normalized with respect to β-actin. Data represent mean±S.E.M., $n=4$. ++$P<.01$ vs. naïve; *$P<.05$, **$P<.01$ vs. CIA.

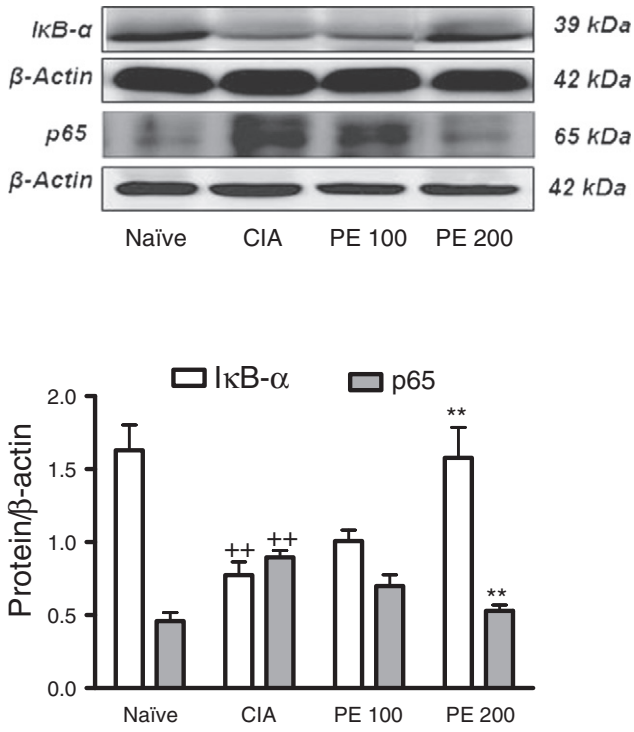

Fig. 7. Expression of IκBα in cytoplasmic fraction and p65 NF-κB in nuclear fraction of homogenates from hind paws. The expression was quantified by densitometry and normalized with respect to β-actin. Data represent mean±S.E.M., $n=4$. ++$P<.01$ vs. naïve; **$P<.01$ vs. CIA.

Our data also show the interest of natural diet components in the development of therapeutic products for arthritic conditions.

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M.A.R. and M.L.F. performed the experiments and data analysis. M.S.H. and J.G.F.B. performed part of the analysis. C.A. and M.J.A. designed the study and prepared the manuscript. All authors read and approved the final content of the manuscript. The authors declare that they have no conflicts of interest.The authors gratefully acknowledge the assistance of Center for Technology and Innovation Research, University of Seville (CITIUS) and the I+D+i Oleoestepa SAC Department who gave us the EVOO.

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Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis.

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EFECTO PREVENTIVO DE UNA DIETA DE ACETATO DE HIDROXITIROSOL, UN POLIFENOL DEL ACEITE DE OLIVA VIRGEN EXTRA, EN UN MODELO DE ARTRITIS INDUCIDA POR COLÁGENO II EN RATONES

Mª Ángeles Rosillo[1], Marina Sánchez-Hidalgo[1], Alejandro González-Benjumea[2], José G. Fernández-Bolaños[2], Erik Lubberts[3], Catalina Alarcón-de-la-Lastra[1]

[1]Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España

[2] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, España

[3]Departamento de Reumatología, Erasmus MC, Universidad Erasmus, Rotterdam, Holanda

RESUMEN

Antecedentes del tema: En la AR, a nivel de las articulaciones se produce hiperplasia sinovial y una infiltración masiva de células inmunes, incluyendo células T, células B, macrófagos, neutrófilos, células dendríticas, entre otras. Aunque la patogenia de la AR sigue sin conocerse en su totalidad, avances en la investigación de la enfermedad han demostrado que el aumento de los niveles de citocinas proinflamatorias como TNF-α, IL-1β, IL-6 e IFN-γ y el aumento de los niveles de metaloproteasas (MMP) en el sinovio de las articulaciones de los pacientes artríticos, juegan un papel fundamental en la erosión del cartílago articular y ósea. En la expresión génica de estos mediadores proinflamatorios, participan diversas vías de señalización celular entre las que cabe destacar las MAPKs, JAK/STAT o NF-κB, las cuales se encuentran activadas en el sinovio de pacientes con AR. La activación de estas vías de señalización, también están implicadas en la expresión de genes de proteínas proinflamatorias, como COX-2 o m-PGES-1, ambas relacionadas con el aumento de la expresión del ecosanoide proinflamatorio PGE_2. Por otro lado, el factor nuclear Nrf2, es una pieza clave en la transcripción de distintas enzimas antioxidantes como la hemo oxigenasa 1 (HO-1), se ha demostrado que la activación de la expresión génica de esta proteína es capaz de modular la respuesta inflamatoria. A pesar de los avances en la terapia farmacológica para el tratamiento de la AR, supone ciertas limitaciones, como sus efectos adversos, es por lo que en los últimos años ha crecido el interés en la utilización de suplementos dietéticos y nutracéuticos, carentes de los efectos adversos que acompaña a la farmacoterapia clásica. Estudios epidemiológicos y experimentales han puesto de manifiesto el papel beneficioso que ejercen distintos polifenoles de la dieta en la AR. AOVE, es rico en una variedad de compuestos fenólicos, entre los que se encuentra el acetato de hidroxitirosol (Ac-HT), que ha demostrado tener efectos antioxidantes y antiinflamatorios.

Capítulo III

Objetivos: En base a los anteriores antecedentes, nos planteamos evaluar la influencia de una dieta enriquecida con Ac-HT, en un modelo de artritis experimental inducida por colágeno tipo II en ratones.

Material y Métodos: Ratones DBA-1/J recién destetados, fueron distribuidos en cuatro grupos experimentales: (1) Ratones sanos alimentados con dieta estándar (CS), (2) Ratones artríticos alimentados con dita estándar (CIA), (3) Ratones artríticos alimentados con dieta enriquecida con hidroxitirosol (HT) (CIA-HT), (4) Ratones artríticos alimentados con dieta enriquecida con Ac-HT (CIA- Ac-HT). Después de seis semanas, la artritis fue inducida. El día 0, los ratones fueron inmunizados con una inyección en la base de la cola de 100 mg de colágeno II. El día 21, recibieron una inyección de refuerzo intraperitoneal, con la misma cantidad de colágeno. El grado de desarrollo de la enfermedad fue valorado de forma visual siguiendo una escala de 0-2, dónde 0=sin inflamación; 1=inflamación leve; 1.5=inflamación marcada; 2=inflamación severa y según el estudio histológico de las articulaciones. Los niveles en suero de IgG1, IgG2a, COMP y MMP-3 así como los niveles de citocinas proinflamatorias como TNF-α, IL-1β, IFN-γ, IL-6 e IL-17 en el homogenado de pata fueron determinados mediante la técnica de ELISA. Los cambios en la expresión proteica de HO-1, Nrf2 y de las proteínas implicadas en las vías de señalización celular MAPKs, JAK/STAT y NF-κB fueron estudiadas mediante western blot.

Resultados: la dieta enriquecida con Ac-HT, redujo de forma significativa el edema articular y la destrucción del cartílago, previniendo el desarrollo de la AR. Los niveles séricos de las IgG1 y 2 a tanto murinas como bobinas, así como los niveles de COMP y MMP-3, los encontramos disminuidos en aquellos animales alimentados con la dieta enriquecida con Ac-HT. La expresión de las citocinas proinflamatorias TNF-α, IL-1β, IFN-γ, IL-6 e IL-17 fue menor el tejido articular de los ratones artríticos que recibieron la dieta enriquecida con el acetato. El Ac-HT produjo una inhibición en la fosforilación de la proteína STAT-3, así como de las proteínas MAPKs e inhibió la translocación al núcleo de la proteína p 65 NF-κB, previniendo la degradación de la proteína inhibitoria IκB-α. Además, Ac-HT fue capaz de aumentar la expresión proteica de Nrf2 y HO-1.

Conclusión: Estos resultados ponen de manifiesto, por primera vez, el efecto antiinflamatorio de una dieta enriquecida con Ac-HT en un modelo de artritis experimental en ratones, que estuvo acompañado por una reducción de los marcadores COMP y MMP-3 en suero. Los mecanismos implicados podrían estar relacionados con una regulación de la respuesta de las células B, una activación de la vía Nrf2/HO-1 y la inhibición de la activación de distintas vías de señalización, como son JAK/STAT, MAPKs y NF- κB, implicadas en la producción de mediadores inflamatorios como citocinas y PGE_2. Concluyendo que un suplemento de AC-HT en la dieta,

podría servir de base para el desarrollo de una nueva estrategia nutricional para la prevención de la AR.

RESEARCH ARTICLE

Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis

María Angeles Rosillo[1], Marina Sánchez-Hidalgo[1], Alejandro González-Benjumea[2], José G. Fernández-Bolaños[2], Erik Lubberts[3] and Catalina Alarcón-de-la-Lastra[1]

[1] Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain
[2] Department of Organic Chemistry, Faculty of Chemistry, University of Seville, Spain
[3] Department of Rheumatology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

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Scope: Hydroxytyrosol acetate (HTy-Ac), an extra virgin olive oil (EVOO) polyphenol, has recently been reported to exhibit antioxidant and anti-inflammatory effects on LPS-stimulated macrophagesand ulcerative colitis. This study was designed to evaluate dietary HTy-Ac supplementation effects on collagen-induced arthritis (CIA) in mice.
Methods and results: DBA-1/J mice were fed from weaning with 0.05% HTy-Ac. After 6 weeks, arthritis was induced by type II collagen. Mice were sacrificed 42 days after first immunization. Blood was recollected and paws were histological and biochemically processed. HTy-Ac diet significantly prevent edarthritis development and decreased serum IgG1 and IgG2a, cartilage olimeric matrix protein (COMP) and metalloproteinase-3 (MMP-3) levels, as well as, pro-inflammatory cytokines levels (TNF-α, IFN-γ, IL-1β, IL-6 and IL-17A). The activation of Janus kinase-signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinases (MAPKs) and nuclear transcription factor-kappa B (NF-κB) pathways were drastically ameliorated whereas nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) protein expressions were significantly up-regulated in those mice fed with HTy-Ac.
Conclusion: HTy-Ac improved the oxidative events and returned pro-inflammatory proteins expression to basal levels probably through JAK/STAT, MAPKs and NF-κB pathways. HTy-Ac supplement might provide a basis for developing a new dietary strategy for the prevention of rheumatoid arthritis.

Keywords:
CIA / EVOO / Hydroxytyrosol acetate / Inflammation / Rheumatoid arthritis

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by inflammation of multiple joints and destruction of cartilage and bone. The earliest event is the development of systemic autoimmunity by exogenous and autologous antigens and thus auto-antibodies and later high levels of cytokines can be detected years before clinical symptom [1, 2]. At a joint level, there are synovial hyperplasia and massive infiltration of immune cells, including $CD4^+$ T-cells, B-cells, natural killer cells, macrophages, dendritic cells, neutrophils and mast cells. Although the cause of RA remains

Correspondence: Catalina Alarcón de la Lastra
E-mail: calarcon@us.es

Abbreviations: **COMP**, cartilage olimeric matrix protein; **COX-2**, cyclooxygenase-2; **ERK**, extracellular signal-regulated kinases; **EVOO**, extra virgin olive oil; **H&E**, hematoxylin and eosin; **HO-1**, heme oxygenase-1; **HTγ**, hydroxytyrosol; **HTy-Ac**, Hydroxytyrosol acetate; **JAK/STAT**, Janus kinase-signal transducer and activator of transcription; **JNK**, c-Jun N-terminal kinase; **MAPKs**, mitogen-activated protein kinases; **MMPs**, metalloproteinases; **MMP-3**, metalloproteinase-3; **mPGES-1**, prostaglandin E synthase-1; **NF-κB**, nuclear transcription factor-kappa B; **Nrf2**, nuclear factor E2-related factor 2; **RA**, rheumatoid arthritis; **SD**, standard diet; **STAT-3**, signal transducer and activator of transcription; **TNF-α**, tumor necrosis factor α

Colour online: See the article online to view Fig. 1 in colour.

unclear, increasing evidences indicate that pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-1β, IL-6, IFN-γ and metalloproteinases (MMPs) produced from RA synovium play an important role in the erosion of articular cartilage and subchondral bones [3, 4].

The process of gene expression of these pro-inflammatory mediators involves multiple signal transduction pathways including mitogen-activated protein kinases (MAPKs) which comprises extracellular signal-regulated kinases (ERK1/2 or p42/p44), c-Jun N-terminal kinases (JNK)1/2/3 and p38, and the nuclear transcription factor-kappa B (NF-κB) which are activated in the synovium of patients with RA [5, 6]. In particular, NF-κB plays an important role in MMPs induction and also regulates a wide range of genes that contribute to inflammation, such IL-1β, TNFα, IL-6, chemokines and microsomal prostaglandin E synthase-1 (mPGES-1), an efficient downstream enzyme co-localized and functionally coupled with the inducible enzymes cyclooxygenase-2 (COX-2). Both, COX-2 and mPGES-1, are up-regulated and responsible for the overproduction of prostaglandin E_2 (PGE_2) which may affect joint integrity through EP_4 receptor activation [7].

The signal transducer and activator of transcription-(STAT)-3 is another critical transcription factor inflammatory pathway activated in response to cytokines involved in the pathogenesis of RA by steering the abnormal activation, automaticity and prolonged survival of synovial cells. Specially, overexpression of STAT-3 has been reported in synovial membranes from RA patients correlating with paired serum IL-6 [8]. Likewise, nuclear factor E2-related factor 2 (Nrf2) is a key transcription factor orchestrator of the induction of several antioxidant enzymes, such as heme oxygenase (HO)-1. The activation of HO-1 in inflammatory conditions could be part of an adaptive mechanism to limit cytotoxicity. In fact, it has been reported that HO-1 deficiency in mice results in a chronic inflammatory state [9].

Despite significant advances in therapies for the treatment of many autoimmune diseases such as RA, a persistent unmet need still exists for more effective, durable and convenient treatment options. In this sense, the interest by dietary supplements and nutraceuticals without undesirable effects that accompany the classical pharmacotherapy is growing. Current epidemiological and experimental studies support a beneficial role of dietary polyphenols in several inflammatory diseases, including RA. In this regard, we have previously demonstrated that oral administration of a phenolic extract from extra virgin olive oil (EVOO) was able to down-regulate the arthritic process in the collagen-induced arthritis (CIA) model of RA [10].

EVOO is rich in a variety of phenolic compounds, mainly constituted by secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy) and of 2-(4-hydroxyphenyl)ethanol (tyrosol), and hydroxytyrosyl acetate (HTy-Ac), along with minor amounts of free HTy [11]. To date, HTy-Ac has shown protection effects against oxidative DNA damage in blood cells [12], as well as against iron-induced oxidative stress in human cervical cells (HeLa) [13]. In addition, a study from González-Correa et al. [14] showed a neuroprotective effect of HTy-Ac in a model of hypoxia–reoxygenation in rat brain slices, both in vitro and after oral administration. A greater antiplatelet aggregating activity than HTy has also been demonstrated [15]. More recently, we have showed that HTy-Ac, exerted an anti-inflammatory effect on acute ulcerative colitis. This effect involves a decrease in COX-2 and iNOS protein expression probably trough JNK MAPK and NF-κB signaling pathways [16]. In addition HTy-Ac was able to modulate inflammatory response in murine peritoneal macrophages [17].

Taken this background into account, the present study was designed to evaluate the effects of HTy-Ac dietary supplementation, in the arthritis model of CIA in mice. In addition to macroscopic and histological analyses, we have determined the effects of HTy-Ac on the production of inflammatory mediators. In order to gain a better insight into mechanisms of action, signaling pathways were also explored.

2 Material and methods

To evaluate the beneficial effects of HTy-Ac on CIA model, DBA 1J mice were used.

Mice were randomized in four experimental groups (10 animals per group): (i) naïve group, (ii) Control group (CIA), (iii) HTy diet group (CIA-HTy) and (iv) HTy-AC diet group (CIA-HTy-Ac). After 6 weeks, arthritis was induced by type II collagen. Mice were sacrificed 42 days after first immunization. Blood was recollected and paws were histological and biochemically processed.

ELISA, histopathological analysis and immunoblotting were performed as indicated in Supporting Information Material and Methods.

3 Results

3.1 Effects of dietary HTy-Ac on CIA-induced AR model

The development of arthritis was monitored until day 42. The time-course of arthritic score indicates that control CIA mice developed a progressive development of arthritis observed from day 34 (Fig. 1A). However, mice fed with HTy-Ac diet showed a significant delayed onset ($p<0.05$ and $p<0.01$ versus CIA) and decreased the disease severity of CIA reducing disease incidence, number of involved paws, footpad thickness and clinical index from days 37 to 42. Surprisingly, HTy dietary did not modify the development of arthritis. These results suggested that dietary enriched with HTy-Ac not only could retard the development but it also may have a therapeutic effect on ongoing inflammatory arthritis. Representative

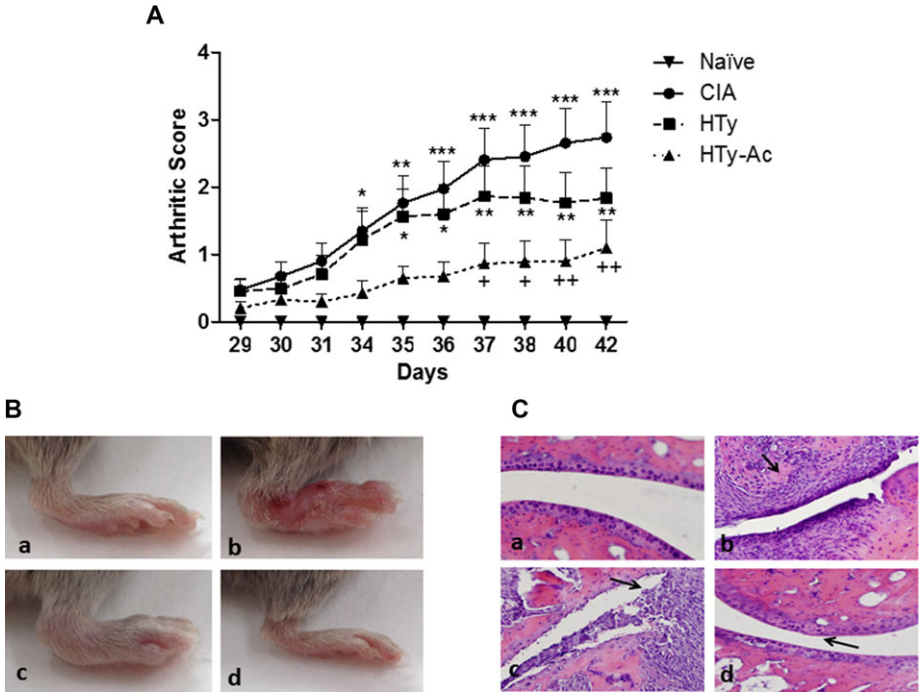

Figure 1. Time course of the arthritis macroscopic score (A), representative photographs (B) of hind paws at the end of the experiment (day 42). Naïve (B, a), non-arthritic mice fed with standard diet; CIA (B, b), control arthritic group feed with standard diet; HTy-CIA (B, c), arthritic mice fed with HTy diet; HTy-Ac-CIA (B, d), arthritic mice fed with HTy-Ac diet. (C) Histological analysis of the frontal sections of knee joints on day 42. Sections were stained with hematoxylin and eosin. Original magnification x200. Naïve (C, a), non-arthritic mice fed with standard diet; CIA (C, b), control arthritic group feed with standard diet; HTy-CIA (C, c), arthritic mice fed with HTy diet; HTy-Ac-CIA (C, d), arthritic mice fed with HTy-Ac diet. The images are representative of at least six experiments performed on different days. Data represent mean ± SEM. $n = 10$. $^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$ versus naïve; $^{+}p<0.05$ and $^{++}p<0.01$ versus CIA.

photographs of hind paws from the different experimental animal groups are shown in Fig. 1B.

In addition, H&E staining revealed that histological features of the joint from naïve animals were typical of normal structure with synovial membrane composed of synovial cells and collagen and a clear synovial space (Fig. 1C, a). On the contrary, joint from control CIA mice exhibited histological changes indicative of severe arthritis, characterized by an extensive infiltration of inflammatory cells into articular tissues, exudation into the synovial space, synovial hyperplasia and cartilage erosion (Fig. 1C, b). These histological features were less evident in CIA-HTy-Ac group (Fig. 1C, d).

3.2 HTy-Ac diet reduced levels of CII-specific antibodies

To determine the effect of HTy-Ac dietary on autoantibody production, serum was collected at day 42 and anti-CII specific IgG antibodies were measured. As shown in Fig. 2A, bovine IgG1 and IgG2a levels were significantly lower in dietary HTy-Ac feed group in comparison with CIA control ($p<$ 0.01 versus CIA) and HTy fed group ($p<0.01$ and $p<0.05$ versus HTy group). Similarly, mouse IgG1 and IgG2a levels were also significantly ameliorated in mice fed with HTy-Ac diet ($p<0.01$ and $p<0.05$ versus CIA and $p<0.05$ versus HTy group).

3.3 HTy-Ac diet decreased serum MMP-3 and COMP levels in CIA-induced RA

In the present study we determined serum MMP-3 levels, as synovial inflammatory biomarker, in CIA mice to investigate the potential beneficial effects of our nutritional therapeutic strategy. Circulating MMP-3 levels were markedly increased ($p<0.01$ versus naïve) in serum from arthritic CIA control group (Fig. 2C). By contrast, a reduction in MMP-3 serum levels was observed in those arthritic animals feeding with HTy-Ac ($p<0.05$ versus CIA). In addition, serum levels of COMP were significantly elevated in CIA animals when compared to naïve controls ($p<0.001$ versus naïve). On the contrary, CIA-HTy-Ac animal group exhibited significantly decreased COMP levels in comparison with CIA group ($p<0.001$ versus CIA) reaching levels comparable to those described in naïve animals (Fig. 2C); whereas dietary HTy treatment was ineffective.

3.4 Effects of dietary HTy-Ac on joint mediators

It has been reported that TNF-α, IL-1β, IL-6, IFN-γ and IL-17A are critical cytokines involved in the pathogenesis of RA [1]. To explore whether cytokines joint levels were paralleled to the disease severity of CIA, concentration of these cytokines were examined in paw homogenates by ELISA. As shown in Fig. 3, TNF-α, IL-1β, IL-6, IFN-γ and IL-17A levels were significantly increased in paw homogenates from CIA animals when compared with naïve mice ($p<0.01$ and $p<0.01$ versus naïve) suggesting its relationship with the synovial tissue inflammation. Conversely, our results indicate that animals fed with HTy-Ac diet showed a significant reduction in all those pro-inflammatory cytokines production in comparison with CIA group (TNF-α: $p<0.05$, IL-1β: $p<0.001$,IL-6: $p<0.01$, IFN-γ: $p<0.01$ and IL-17A: $p<0.001$ versus CIA).

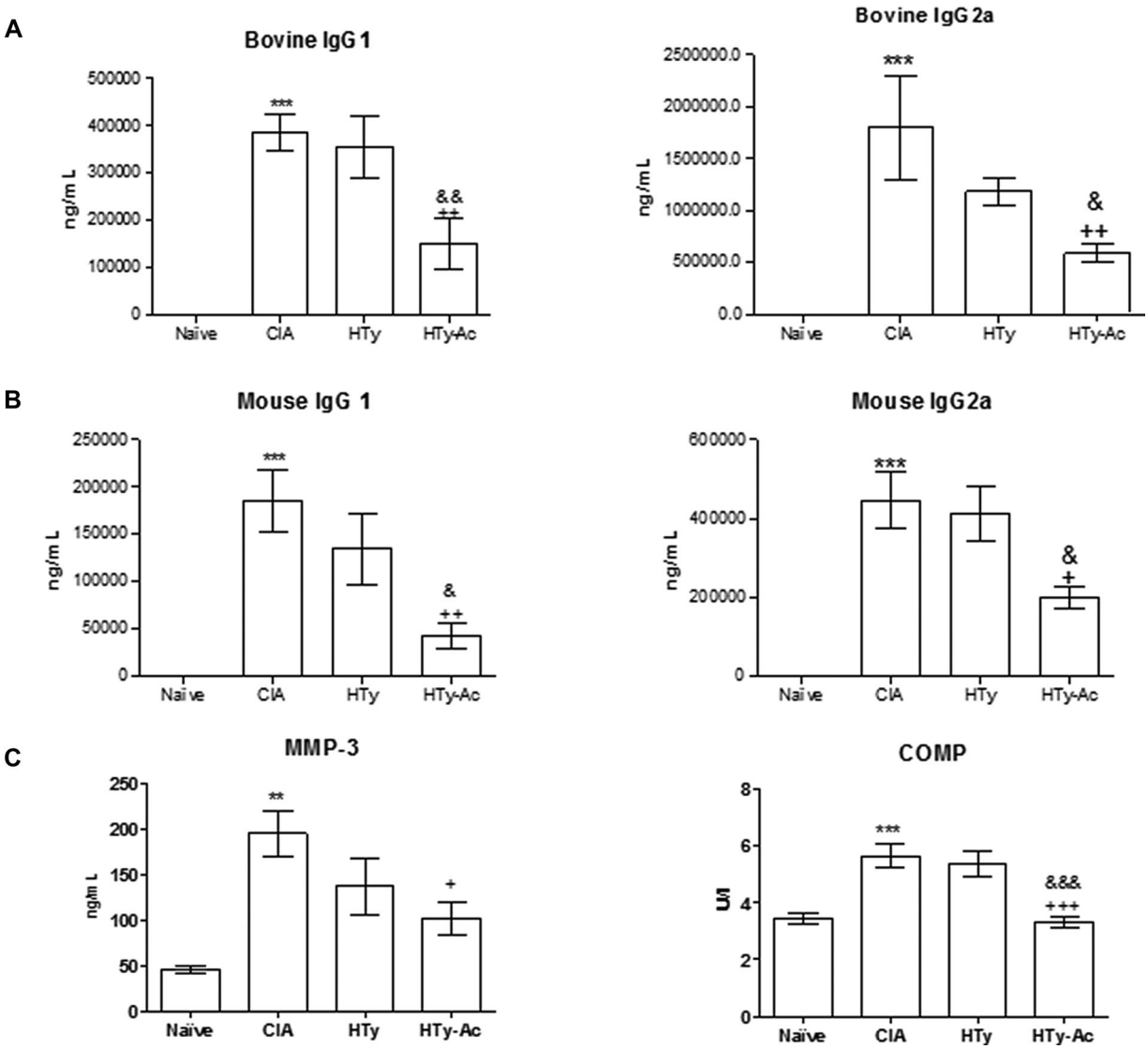

Figure 2. (A) Measurement of serum anti-bovine CII IgG1and IgG2a levels and (B) anti–mouse CII IgG1 and IgG2a levels. (C) Measurement of serum MMP-3 and COMP levels. These were measured by ELISA kits. Data represent mean ± SEM, $n = 10$. $^{**}p<0.01$ and $^{***}p<0.001$ versus naïve; $^{+}$ $p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus CIA; $^{\&}p<0.05$, $^{\&\&}$ $p<0.01$ and $^{\&\&\&}$ $p<0.001$ versus CIA-HTy.

3.5 Effect of dietary HTy-Ac on COX-2 and mPGES-1 expression

COX-2 and mPGES1 expressions were determined by western blot in paw homogenate (Fig. 4A). Arthritic control animal group showed an overexpression of both these pro-inflammatory enzymes (COX-2: $p<0.01$ and mPGES1: $p<0.001$ versus naïve) whereas HTy-Ac diet was able to reduce the protein expression levels of both them ($p<0.01$ versus CIA) (Fig. 4B). By contrast, dietary HTy treatment failed to reduce significantly the protein expression levels of both COX-2 and mPGES1 in CIA mice. On the other hand, levels of PGE_2 were measured on the paw homogenate, HTy-Ac was capable to reduce the levels of these eicosanoid (Fig. 4B).

3.6 Effect of dietary HTy-Ac on p-STAT-3 protein expression

STAT-3 has been described as a critical transcription factor involved in the pathogenesis of RA by steering the abnormal activation, automaticity and prolonged survival of synovial cells. It has been also described that IL-6 active the JAK/STAT pathway and mainly culminates in the activation of the STAT-3 transcription factor [18]. We evaluated p-STAT-3 protein expression by western blot from hind paw homogenates. Statistical analysis revealed a significant p-STAT-3 overexpression in CIA group when compared to the naïve control groups ($p<0.001$ versus naïve) whereas nutritional therapy with HTy-Ac significantly suppressed STAT3

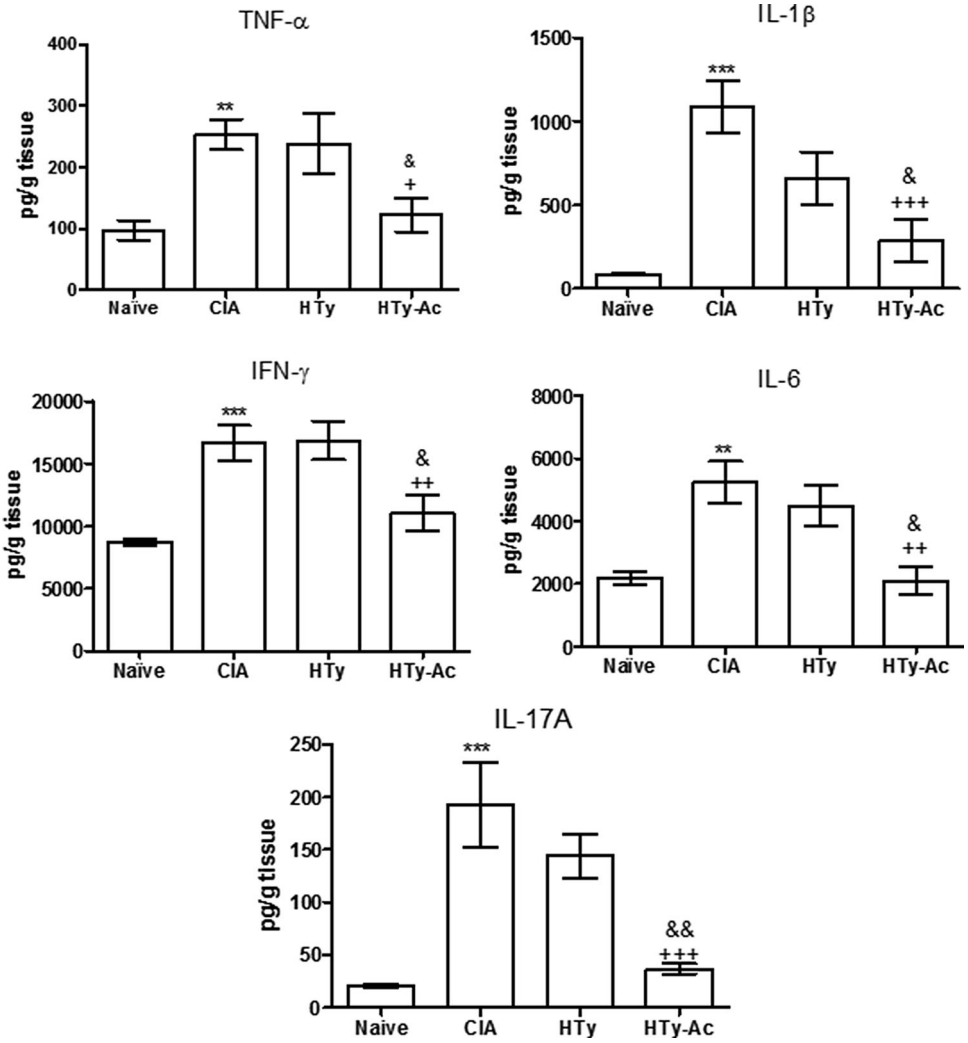

Figure 3. Measurement of TNF-α, IL-1β, IFN-γ, IL-6 and IL-17A levels in hind paw homogenates. These were determined by ELISA kits. Data represent mean ± SEM, $n = 10$. $^{**}p<0.01$ and $^{***}p<0.001$ versus naïve; $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus CIA; $^{\&}p<0.05$, $^{\&\&}p<0.01$ and $^{\&\&\&}p<0.001$ versus CIA-HTy.

phosphorylation in arthritic CIA mice ($p<0.01$ versus CIA), this suppression was also significant in comparison with HTy-CIA group ($p<0.01$ versus CIA-HTy) (Fig. 5A). These results are in accordance with those obtained in the measurement of pro-inflammatory cytokines levels suggesting that dietary HTy-Ac may repress STAT-3 activation reducing IL-6 levels in CIA mice.

3.7 Dietary HTy-Ac induces Nrf2/HO-1 antioxidant pathway activation

The expression of the proteins HO-1 and Nrf2 were also evaluated in paw homogenates by western blotting. Our data show that HO-1 was significantly downregulated in CIA mice during the maintenance of chronic inflammation ($p<0.001$ versus naïve); however, dietary HTy-Ac treatment induced a HO-1 overexpression in comparison with CIA arthritic control group ($p<0.05$ versus CIA) (Fig. 5B). Nrf2 activation has been reported to play an important role in HO-1 expression. According to our results, Nrf2 expression was similarly reduced in those arthritic animals fed with standard diet (SD; $p<0.001$ versus naïve) whereas a significant increase in Nrf2 protein levels was observed in CIA-HTy-Ac animals ($p<0.05$ versus CIA and $p<0.05$ versus CIA-HTy) (5B).

3.8 Effect of dietary HTy-Ac on MAPKs signaling pathway

MAPKs play a key role inducing the pro-inflammatory gene expression which initiates inflammatory responses. We investigated the effect of dietary HTy-Ac on MAPKs (JNK and p38) signaling pathway activation in CIA mice. In the present study, phosphorylation of JNK, p38 and ERK (1/2) proteins increased significantly in cytosolic extracts from CIA mice paw homogenates. Nonetheless, the proteins expression of all phosphorylated MAPKs proteins, p-JNK, p-p38 and p-ERK (1/2) was significantly ameliorated after dietary HTy-Ac treatment ($p<0.05$ and $p<0.01$ versus CIA) (Fig. 6A). Again, the

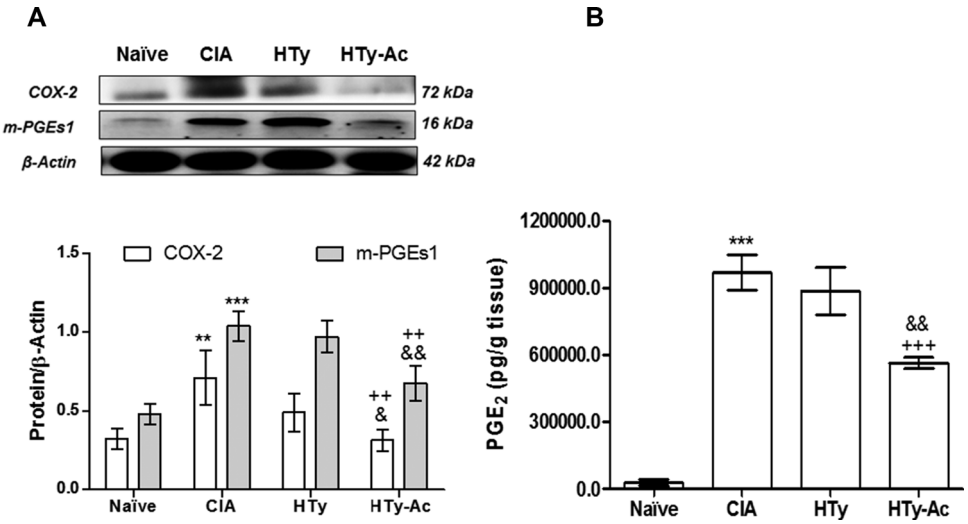

Figure 4. (A) Expression of COX-2 and mPGES-1 in hind paws. COX-2 and mPGES-1 were determined by western blot, quantified by densitometry and normalized with respect to β-actin. (B) Measurement of PGE2 levels in hind paw homogenates. These were determined by ELISA kits. Data represent mean ± SEM, $n = 4$. $^{**}p<0.01$ and $^{***}p<0.001$ versus naïve; $^{++}p<0.01$ and $^{+++}p<0.001$ versus CIA; $^{\&\&}p<0.01$ versus CIA-HTy.

HTy diet showed its ineffectiveness along the experimental period.

3.9 Effects of dietary HTy-Ac on NF-κB signaling pathway

We also investigated the effects of HTy-Ac on IkB-α degradation in cytoplasmic extracts from mice joints. As shown in Fig. 6B, IκB-α expression was significantly reduced in CIA-induced arthritis mice when compared with naïve mice ($p<0.001$ versus naïve). The IκB-α expression observed in CIA mice was consistent with an increase in IκB-α degradation thus allowing NF-κB translocation into the nucleus to bind specific DNA sequences leading to pro-inflammatory genes transcription. According to the results obtained, the effect observed in arthritic control group on IκB-α protein degradation was prevented by dietary HTy-Ac treatment. On the contrary, the nuclear p65 protein levels were significantly increased in CIA group ($p<0.001$ versus naïve) whereas

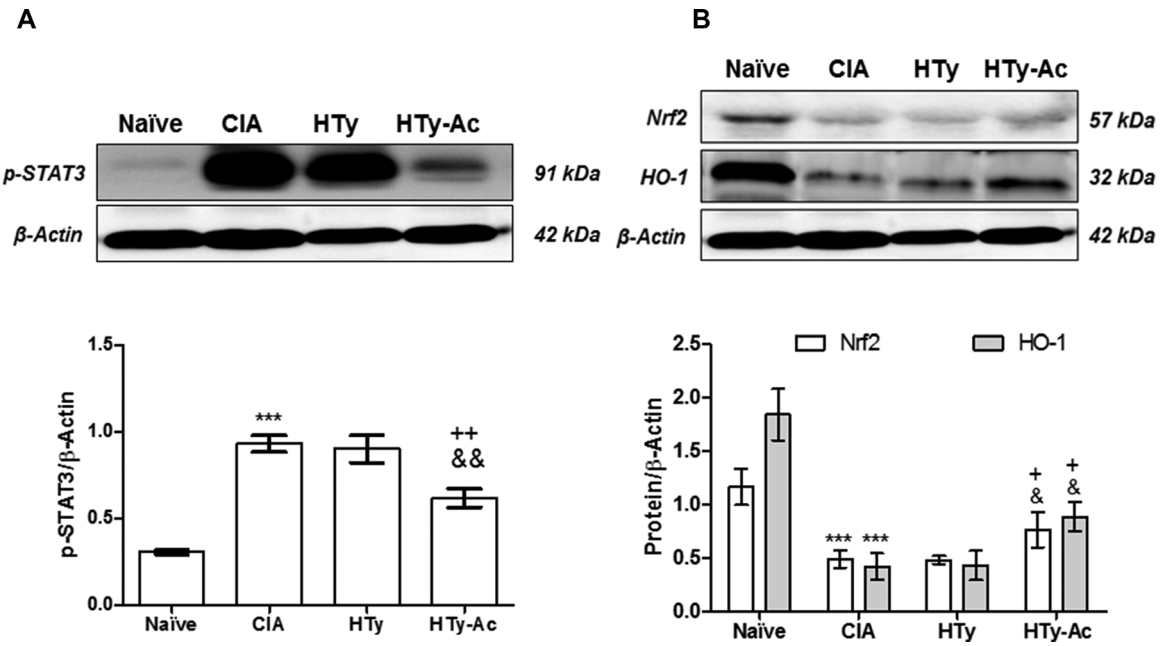

Figure 5. (A) Changes in p-STAT3 protein expression in hind paws homogenate. The expression of p-STAT3 was quantified by densitometry and normalized with respect to β-actin. (B) HTy-Ac dietary upregulated HO-1 and Nrf2 protein expression in hind paws homogenate. The expression was quantified by densitometry and normalized with respect to β-actin. Data represent mean ± SEM, $n = 4$. $^{***}p<0.001$ versus naïve; $^{+}p<0.05$ and $^{++}p<0.01$ versus CIA; $^{\&}p<0.05$ and $^{\&\&}p<0.01$ versus CIA-HTy.

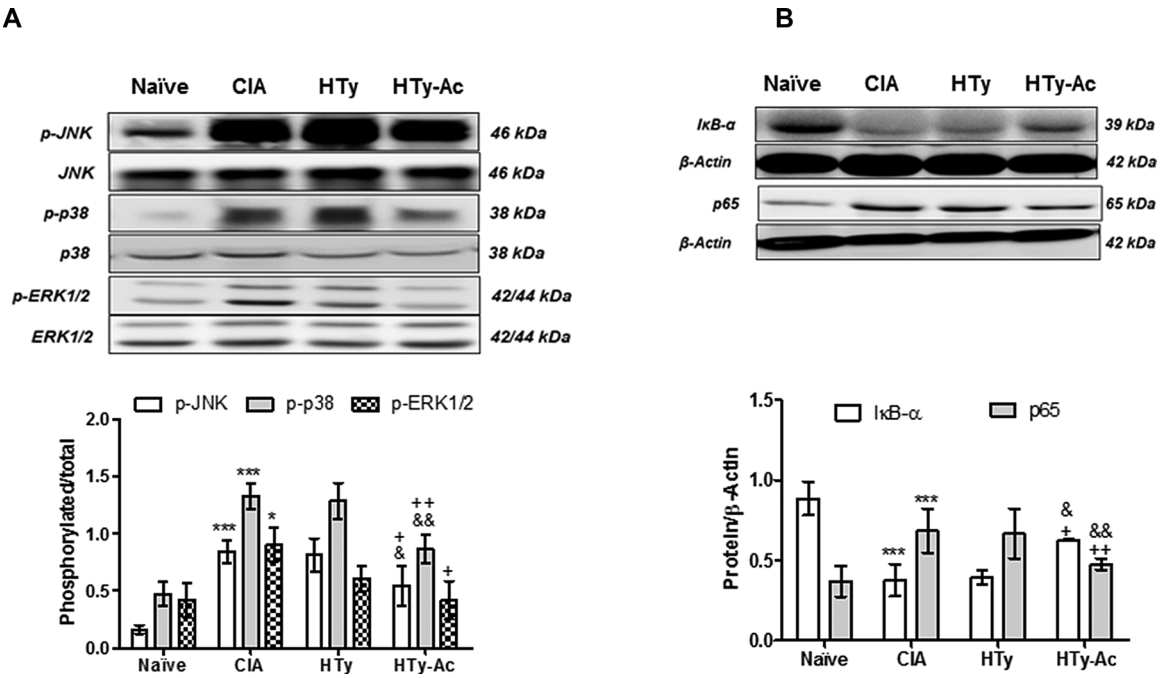

Figure 6. (A) Effect of dietary HTy-Ac on JNK, p38 and ERK (1/2) MAPKs phosphorylation in hind paws homogenate. The expression of phosphorylated proteins was expressed related to the expression of the corresponding total protein. (B) Expression of IκB-α in cytoplasmic fraction and p65 NF-κB in nuclear fraction of homogenates from hind paws. The expression was quantified by densitometry and normalized with respect to β-actin. Data represent mean ± SEM, $n = 4$. *$p<0.05$ and ***$p<0.001$ versus naïve; $^{+}p<0.05$, $^{++}p<0.01$ versus CIA; $^{\&}p<0.05$ and $^{\&\&}p<0.01$ versus CIA-HTy.

dietary HTy-Ac treatment prevented the CIA-induced nuclear translocation level of p65 in paw homogenate in comparison with those arthritic animals fed with SD ($p<0.01$ versus CIA) (Fig. 6B) avoiding the NF-kB-mediated transcriptional activation. HTy diet failed to modify IκB-α degradation and nuclear p65 translocation in CIA mice.

4 Discussion

Our findings, have shown, for the first time, that dietary HTy-Ac enrichment was able to prevent and down-regulate the arthritic process in the CIA model of RA. This model is commonly used to investigate relevant pathogenic mechanisms of RA as well as new antiarthritic treatments [19]. HTy-Ac was described for the first time in olive oil by Brenes et al. [20] and is found in most Spanish virgin olive oils. Moreover, recently, it was reported by Mateos et al. [21] that HTy-Ac is more soluble in the lipophilic phases than HTy, due to the presence of the ester group, which was demonstrated in a Caco-2 cell model. Thus, this increased lipophilicity suggests that HTy-Ac is better absorbed across intestinal epithelial cell monolayers than free HTy [22]. CIA induction resulted in the development of a pronounced synovitis associated with cartilage degradation and bone erosion [23]. However, dietary HTy-Ac supplementation could improve the arthritis score which was correlated with a minor migration of inflammatory cells into articular tissues in addition to a marked reduction of joint edema, synovial hyperplasia and cartilage erosion in comparison with those animals fed with SD and HTy enriched diets.

A role of humoral immunity in the pathogenesis of autoimmune arthritis is suggested by evidence derived from animal models as well as patients with RA. Antibody/antigen complexes are abundantly found in the joints of RA patients and are believed to play a role in triggering the joint inflammation [24]. CIA pathogenesis is characterized by the generation of anti-CII anti-bodies. All isotypes (IgG1, IgG2a and IgG2b) of anti-CII antibodies were arthritogenic, with the IgG1 and IgG2b isotypes as the dominating arthritogenic antibodies [25]. In the present study, dietary HTy-Ac supplementation significantly decreased serum levels of IgG1-and IgG2a-anti-CII antibodies in CIA mice. These results suggest a potential role for HTy-Ac in regulating B cell responses, which may be in part, responsible for its anti-arthritic effects.

The development and progression of RA is closely related to an imbalance of cytokine network. In RA synovium, elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-17 and INF-γ are produced by macrophages and synovial fibroblasts. These pro-inflammatory cytokines both directly and indirectly exert their effects through the production of additional pro-inflammatory cytokines, chemokines and MMPs at the pannus cartilage junction leading to cartilage degradation [26–28]. In addition, TNF-α stimulates

osteoclastogenesis [29], suppresses the recruitment of osteoblasts and inhibit the expression of matrix genes [30], whereas IL-6 increases osteoclast numbers in trabecular bone [31]. Besides, IL-17 is a T cell-derived cytokine able to induce the release of IL-8 and IL-6, and plays a considerable role in the additive/synergistic effects induced by TNF-α and IL-1β [32].

The present study showed that arthritic mice fed with HTy-Ac enriched diet had decreased IL-1β, IL-6, IFN-γ, IL-17 and TNF-α levels in paw homogenate in comparison with CIA mice fed with SD and HTy enriched diet. These results indicate that dietary HTy-Ac exerts anti-inflammatory activities by the blockage of IL-1β, IL-6, IFN-γ, IL-17 and TNF-α production.

COMP, a prominent non-collagenous component of cartilage, accounts for approximately 1% of the wet weight of articular tissue and shows great potential as a biological marker of cartilage metabolism in arthritis [33]. Increased fragments of COMP have been reported in patients with osteoarthritis, RA and joint injury, thus and the monitoring of COMP levels in synovial fluid or serum has been suggested to be a helpful method for assessing the presence and progression of arthritis. In fact, serum COMP is reduced in RA patients in remission [33]. In addition, COMP is a putative substrate for MMPs. In particular, MMP-3, is a proteinase secreted by synovial fibroblasts and chondrocytes. Its synthesis and activation is induced by various factors, including pro-inflammatory cytokines and Toll-like receptor ligand. Its activity is associated with higher joint damage in RA [34] and CIA [4] and results in degradation of aggrecan core protein, cartilage link protein, fibronectin and collagen types IV, VII, IX and XI. Likewise, serum MMP-3 level is suggested as a predictor for joint destruction in early RA or established RA and should be used in association with usual inflammatory markers to follow therapy efficiency [35]. Our data are in agreement with above studies and showed that high serum COMP and MMP-3 levels were associated with disease activity and joint progression in CIA mice, by contrast the production of both cartilage and synovial biomarkers were significantly inhibited by the HTy-Ac enriched diet in CIA mice.

COX-2 and mPGES-1, enzymes responsible for the overproduction of PGE_2 in inflammation, are up-regulated [36] contributing to the progression of RA through EP_4 receptor activation [7]. We have shown that dietary HTy-Ac supplementation reduced PGE2 levels which could be possibly due to decreased expression of both COX-2 and mPGES-1 in the joint. Therefore, regulation of these pro-inflammatory biomarkers by HTy-Ac could represent a potential molecular target susceptible to HTy-Ac modulation, which has not been demonstrated previously.

Signal transduction pathways closely involved in inflammation include the MAPKs, JAK-STAT and NF-κB pathways [37,38]. In fact, NF-κB nuclear transcription factor plays a pivotal role in the development and activation of Th-1 responses [39] and is responsible in addition to MAPKs for COX-2 up-regulation [40]. We investigated whether the activation of NF-κB signaling pathway was inhibited by HTy-Ac diet. Our data are in agreement with Sanchez-Fidalgo et al. that showed that HTy-Ac increased the inhibitory protein IkB-α and reduced p65 translocation, indicating that dietary HTy-Ac inhibited the NF-κB activation by blocking IκB-α degradation in dextran sulfate sodium -induced colitis in mice [16].

MAPK family members, including p38 kinases, ERKs 1 and 2 and JNKs, are involved in many important cell processes, mainly the regulation of the synthesis of chemokines, cytokines, adhesion molecules and PGs involved in RA [6,41]. Besides JNK MAPK modulates MMPs production by synovial fibroblasts and drives osteoclast differentiation in RA [42]. In particular, p38 MAPK regulates MMP-3 induction in fibroblasts [43] and osteoclast differentiation [44]. Moreover, MAPKs phosphorylate the JAK-STAT important in pro-inflammatory cytokine-mediated signaling pathways such as Th17 cell differentiation, leading to STAT-3 activation by phosphorylation on tyrosine residues resulting in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences [45]. In this line, STAT-3 overexpression has been also detected in synovial membranes from RA patients and it has been reported to contribute to the chronicity of CIA induced arthritis model [4, 46]. Our findings are in concordance with above reports and showed that p38 and JNK MAPKs phosphorylation were increased in CIA control mice. Similarly, STAT-3 overexpression was also evidenced in RA synovium of control CIA mice and was positively related to the severity of synovitis, whereas dietary HTy-Ac supplementation reduced significantly both MAPKs and STAT-3 activation at transcriptional level. Collectively, our data suggest that dietary HTy-Ac may repress IL-17 production interfering negatively with JNK, p-38, p-ERK MAPKs and STAT-3 signaling pathways.

Nrf2, is a redox-sensitive transcription factor and binds to antioxidant response elements (ARE) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 [47]. In inflammatory conditions, HO-1 expression protein could be part of an adaptive mechanism to limit cytotoxicity via several mechanisms including scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis. Likewise, it has been reported that HO-1 deficiency in mice results in a chronic inflammatory state [9]. Thus, Nrf2 regulates redox status and plays key roles in cellular defense by enhancing the removal of reactive oxygen species [48]. Furthermore, it has been documented that deficiency of Nrf2 accelerates the effector phase of arthritis and aggravates joint disease [49]. In the present study, in concordance with Karatas et al. [50], expressions of Nrf2 and HO-1 were decreased in the arthritis group, by contrast dietary HTy-Ac could restore Nrf2 and HO-1 expressions conferring a role of Nrf2/HO-1 signaling in the beneficial effects of HTy-Ac in this murine model of RA.

Collectively, our study has demonstrated for the first time the anti-inflammatory effects of dietary HTy-Ac in the CIA model, which were accompanied by an important reduction of the serum synovial and cartilage biomarkers COMP and

MMP-3. The mechanisms underlying these protective effects could be related to the regulation of B cell responses, activation of the Nrf2/HO-1 signaling and the inhibition of relevant signaling pathways such as JAK-STAT, MAPKs and NF-κB thus controlling the production of inflammatory mediators such as Th1 cytokines and PGE_2. We concluded that HTy-Ac supplement might provide a basis for developing a new dietary strategy for the prevention of RA.

M.A.R. performed the experiments and data analysis. A.G.B, J.G.F.B. and E.L. performed part of the analysis. M.S.H. and C.A. designed the study and prepared the manuscript. All authors read and approved the final content of the manuscript.

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The authors have declared no conflicts of interest.

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RESULTADOS/DISCUSIÓN GENERAL

The focus of the **chapter I** has been to demonstrate the potential protective role of a diet elaborated with EVOO in the prevention and development of inflammatory arthritis and joint damage in a murine CIA-induced experimental arthritis model. This model is commonly used to investigate mechanisms relevant to RA as well as new antiarthritic treatments (Ferrandiz, Maicas et al. 2008). CIA induction resulted in the development of a pronounced synovitis associated with an autoimmune response against cartilage and production of matrix degrading enzymes accompanying cartilage degradation and bone erosions (Schurgers, Billiau et al. 2011). The results of the present work clearly indicate, for the first time, that EVOO, as the lipid component of the diet, effectively exhibited preventive and therapeutic effects in the development of inflammatory arthritis and joint damage in CIA arthritic mice in comparison with those CIA mice fed with SO **(Chapter I. Figure 1 A and B)**. This effect was well correlated to an improved arthritis score, a minor inflammatory cells infiltration into articular tissues, reduced exudation into the synovial space, synovial hyperplasia and cartilage erosion **(Chapter I. Figure 2)**.

The pathogenesis of RA is closely related to an imbalance of cytokine network contributing to the development and progression of both CIA and RA (Komatsu and Takayanagi 2012). Actually, overexpression of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-17 may activate osteoclasts and macrophages and also recruit leukocytes in inflamed joints. The infiltrated leukocytes produce cytokines, chemokines and tissue destructive enzymes leading to chronic inflammation, cartilage damage and bone erosion. Besides, IL-17 is able to induce the release of IL-8, IL-6 and plays a remarkable role in the additive/synergistic effects induced by TNF-α and IL-1β (Jeong, Kim et al. 2004). Importantly, targeting IL-17 has demonstrated therapeutic effects in animal models of inflammatory and autoimmune diseases, including RA (Miossec and Kolls 2012). Cytokines also up-regulate MMPs expression by chondrocytes and synoviocytes at the pannus cartilage junction leading to cartilage degradation (Fossiez, Djossou et al. 1996, Azizi, Jadidi-Niaragh et al. 2013, Noack and Miossec 2014). Therefore, we examined TNF-α, IL-1ß and IL-17 levels in paw homogenates from CIA animals in comparison with naïve control group. Our results indicate that animals fed with EVOO diet showed a significant reduction in all pro inflammatory cytokines levels. Consequently, EVOO intake would contribute to reduce the inflammatory response and joint damage by suppressing the production of the key Th17 polarisation cytokines, TNF- α, and IL-1β, in CIA arthritic mice **(Chapter I. Figure 4)**. COMP, is a matrix protein with a great potential as a biological marker of cartilage metabolism in arthritis (Saxne and Heinegard 1992). Increased fragments of COMP have been reported in patients with osteoarthritis, RA and joint injury; thus, the monitoring of COMP levels in synovial

fluid or serum has been suggested to be a helpful method for assessing the presence and progression of the inflammatory disease. In this sense, serum COMP levels are reduced in RA patients in remission (Saxne and Heinegard 1992, Lai, Yu et al. 2012). In addition, COMP is a putative substrate for MMPs. Particularly, MMP-3, is a proteinase secreted by synovial fibroblasts and chondrocytes. Its activity results in degradation of aggrecan core protein, cartilage link protein, fibronectin, and collagen types IV, VII, IX, and XI. MMP-3 is present in RA synovial fluid and overexpressed in rheumatoid synovium and then, associated with higher joint damage in RA (Ally, Hodkinson et al. 2013). Likewise, serum MMP-3 level is suggested as a predictor for joint destruction in early RA or established RA and should be used in association with usual inflammatory markers to follow therapy efficiency (Denarie, Constant et al. 2014). Our data are in agreement with above studies and showed that high serum COMP and MMP-3 levels were associated with disease activity and joint progression in CIA mice, by contrast the production of both cartilage and synovial biomarkers were significantly inhibited by dietary EVOO treatment in CIA mice **(Chapter I. Figure 3)**.

Abnormal signalling pathways play an important role in the inflammatory process and can lead to dysregulation of the inflammatory response being crucial in RA pathogenesis. Signal transduction pathways closely involved in inflammation included the MAPKs, JAK-STAT and NF-κB pathways (Morel and Berenbaum 2004). NF-κB is a crucial transcriptional activator for the expression of multiple proinflammatory genes in the microenvironment of the arthritic joints, including TNF-α, IL-1β, IL-6 and IL-17, that exert their influences on both osteoclasts and osteoblasts differentiation and thereby contributing to progressive joint destruction in RA patients and CIA mice (Okamoto, Yoshio et al. 2010, Molinero, Cubre et al. 2012) as mentioned above. In fact, several anti-rheumatic drugs, currently in clinical use, inhibit NF-κB activation (Hwang, Noh et al. 2013). Interestingly, intra-articular or systemic blockade of NF-κB signalling is an effective target in the treatment of arthritis in animal models of RA (Min, Yan et al. 2015). Taken together, these studies confirm the importance of NF-κB activation in the development of RA. Consequently, NF-kB pathway suppression could be a novel strategy for delaying the progress of RA. Our results suggested that dietary EVOO treatment suppressed NF-κB activation **(Chapter I. Figure 8)**, down-regulating pro-inflammatory cytokines and MMP-3 expression thus minimizing joint destruction in CIA-induced arthritic mice. MAPK family members, including p38 kinases, ERKs 1 and 2, and JNKs, play critical roles in many important cell processes, including cell division, differentiation, inflammation, and apoptosis. They are especially drawing attention in RA because of the involvement in regulating both cytokine production and cytokine action (Criado, Risco et al. 2014, Li, Li et al. 2014). In fact, JNK MAPK regulates MMPs production by

synovial fibroblasts and drives osteoclast differentiation in RA (Han, Boyle et al. 2001) and p38 MAPK regulates MMP-3 induction in fibroblasts (Suzuki, Tetsuka et al. 2000) and osteoclast differentiation (Matsumoto, Sudo et al. 2000). Moreover, MAPKs phosphorylate the janus kinase signal transducer and activator of transcription (JAK-STAT) important in proinflammatory cytokine-mediated signalling pathway leading to STAT-3 activation (Aaronson and Horvath 2002). STAT-3 has been described as a critical transcription factor involved in Th17 cell differentiation (Park, Lim et al. 2013). In this line, STAT-3 overexpression has been also detected in synovial membranes from RA patients and it has been reported to contribute to the chronicity of inflammation in a murine zymosan-induced arthritis model (de Hooge, van de Loo et al. 2004). Recently, STA-21, a promising STAT-3 inhibitor has shown antiarthritic effects in interleukin-1 receptor antagonist-knockout (IL-1Ra-KO) mice, an animal model of RA (Park, Kwok et al. 2014). Our study showed that p38 and JNK MAPKs phosphorylations were increased in SO-CIA mice. Similarly, STAT-3 overexpression was also evidenced in RA synovium of SO-CIA mice and was positively related to the severity of synovitis. Whereas EVOO diet intake reduced significantly both MAPKs and STAT-3 activation at transcriptional level **(Chapter I. Figures 5 and 7)**.

Altogether, our results suggest that dietary EVOO may repress IL-17 production interfering negatively with JNK and p-38 MAPKs and STAT-3 signalling pathways. HO-1 activity catabolises heme to carbon monoxide, iron and biliverdin, the latter being reduced to bilirubin by biliverdin reductase. Nrf2 plays a central role for expression of HO-1. In basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch like ECH-associated protein1 (Keap1) and degraded by the ubiquitin dependent 26S proteasome system. Under activation, Nrf2 released from Keap1 inhibition, translocate to the nucleus, heterodimerizes with Maf, and binds antioxidant response elements (AREs) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 (Bang, Kim et al. 2012). In inflammatory and immune conditions the expression of this protein could be part of an adaptative mechanism to limit cytotoxicity via several mechanisms including scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis (Ferrandiz, Maicas et al. 2008). Likewise, it has been reported that HO-1 deficiency in mice results in a chronic inflammatory state (Minamino, Christou et al. 2001). Our data showed that HO-1 could represent a potential molecular target susceptible to EVOO modulation, which has not been demonstrated previously, since dietary EVOO treatment strongly augmented Nrf2 and HO-1 expression conferring a role of HO-1 in the beneficial effects of EVOO in this murine model of chronic inflammation **(Chapter I. Figure 6)**.

Since our results show that EVOO diet was more effective in reducing arthritis severity in comparison with SO diet, we could suggest that the responsibility for such beneficial

properties could to be assigned to both an adequate fatty acid profile of EVOO and the presence of a high proportion of phenolic compounds. In this sense, a recent report showed that oleuropein aglycone, a polyphenol found in olive oil, exerted an anti-inflammatory effect and ameliorated the joint damage associated with CIA (Impellizzeri, Esposito et al. 2011). In addition, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it is not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action (Lopez-Miranda, Perez-Jimenez et al. 2010, de la Lastra Romero 2011).

In the **chapter II,** we elucidated the effects of oral PE from EVOO treatment in murine CIA and study, the biochemical routes and signalling pathway involved. According to our results, we have demonstrated that oral administration of PE is able to down-regulate the arthritic process in the CIA model of RA **(Chapter II. Figure 1 A and B)**. PE showed anti-inflammatory effects with reductions in joint oedema and migration of inflammatory cells. In addition, PE protected joints against cartilage alterations and bone erosion **(Chapter II. Figure2)**. The progression of arthritis is associated with sustained production of proinflammatory cytokines (Goronzy and Weyand 2009). We have shown in the CIA model that PE controls the local levels of these cytokines driving the production of different inflammatory and catabolic mediators **(Chapter II. Figure 3)**. These findings are in agreement with ours previous results evaluated in CIA mice fed with dietary EVOO.

Particularly, proinflammatory cytokines induce further cytokines, chemokines, eicosanoids and reactive oxygen species amplifying the inflammatory response (Abramson and Amin 2002). Therefore, COX-2 and mPGES-1, enzymes responsible for the overproduction of PGE2 in inflammation, are up-regulated (Lazarus, Kubata et al. 2002) contributing to the progression of RA through EP4 receptor activation (McCoy, Wicks et al. 2002). We have shown that PE reduces PGE_2 levels **(Chapter II. Figure 3)**, which would be dependent on the down-regulation of COX-2 and mPGES-1 expression in the joint **(Chapter II. Figure 4 A and B)**. Cytokines also induce the expression of matrix metalloproteinases at the pannus cartilage junction leading to cartilage degradation (Okada, Nagase et al. 1987, Ribbens, Martin y Porras et al. 2002). In addition, TNFα stimulates osteoclastogenesis (Cenci, Weitzmann et al. 2000), suppresses the recruitment of osteoblasts and inhibits the expression of matrix genes (Nanes 2003) whereas IL-6 increases osteoclast numbers in trabecular bone (Jilka, Hangoc et al. 1992). Therefore, inhibition of proinflammatory cytokine production by PE would result in the reduction of the inflammatory response and tissue damage. MAPK regulate the synthesis of chemokines, cytokines, adhesion molecules and PGs involved in inflammation. In addition, they mediate the

induction of matrix metalloproteinases responsible for cartilage breakdown. JNK1/2 and p38 are also involved in osteoclast differentiation and thus the activation of these enzymes may contribute to bone destruction (Thalhamer, McGrath et al. 2008). In particular, p38 plays a key role in RA (Hammaker and Firestein 2010). JAKs bind to intracellular domains of IL-6 or interferon receptors and catalyze ligand-induced phosphorylation of themselves and of intracellular tyrosine residues. Phosphorylation of STATs on tyrosine residues results in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences. This pathway can be regulated by different protein kinases such as MAPK that phosphorylate STATs on serine residues to potentiate STAT-activating stimuli (Aaronson and Horvath 2002). STAT3 may contribute to the chronicity of inflammation in experimental arthritis (de Hooge, van de Loo et al. 2004). Our data indicate that PE administration is able to reduce the phosphorylation of JNK, p38 and STAT3, suggesting that PE could control the activation of these important signalling pathways during the arthritic process **(Chapter II. Figures 5 and 6)**.

The NF-κB pathway is involved in the transcription of many inflammatory genes (Foxwell, Browne et al. 1998). Cellular stimulation by proinflammatory cytokines induces the recruitment of costimulatory molecules, such as TNF receptor-associated factor (TRAF) leading to the activation of NF-κB-inducing kinase. This protein induces IκB kinase activation resulting in the phosphorylation of IκB (Morel and Berenbaum 2004), which is followed by ubiquitination and proteolytic degradation thus allowing the release of NF-κB to enter the nucleus and regulate gene transcription (Karin and Ben-Neriah 2000). Our results indicate that PE enhances the levels of the inhibitory protein IκB-α leading to the reduction of NF-κB nuclear translocation **(Chapter II. Figure 7)**. This mechanism may be responsible for the down-regulation of proinflammatory cytokines in the joint during the CIA process in the animals treated with PE.

Finally, in **chapter III**, we tried to deep insight into the effects of phenolic isolated compounds from minor PF from EVOO (HTy and HTy-Ac) in CIA model of RA, showing that dietary HTy-Ac enrichment was able to prevent and down-regulate the arthritic process. HTy-Ac was described for the first time in olive oil by Brenes et al. (Brenes, Garcia et al. 1999) and is found in most Spanish virgin olive oils. Moreover, recently, it was reported by Mateos et al. (Mateos, Trujillo et al. 2008) that HTy-Ac is more soluble in the lipophilic phases than HTy, due to the presence of the ester group, which was demonstrated in a Caco-2 cell model. Thus, this increased lipophilicity means that HTy-Ac is better absorbed across intestinal epithelial cell monolayers than free HTy (Rubio, Macia et al. 2012). CIA induction resulted in the development of a pronounced synovitis associated with cartilage degradation and bone erosion (Schurgers, Billiau et al. 2011).

Parallel to our results mentioned above in CIA animals treated with PE (Chapter II) or fed with EVOO diet (Chapter I), dietary HTy-Ac supplementation improved the arthritis score, which was correlated with a minor migration of inflammatory cells into articular tissues in addition to a marked reduction of joint oedema, synovial hyperplasia and cartilage erosion in comparison with those animals fed with SD and HTy-enriched diets **(Chapter III. Figure 1 A, B and C)**.

A role of humoral immunity in the pathogenesis of autoimmune arthritis is suggested by evidence derived from animal models as well as patients with RA. Antibody/antigen complexes are abundantly found in the joints of RA patients and are believed to play a role in triggering the joint inflammation (Mullazehi, Mathsson et al. 2007). CIA pathogenesis is characterized by the generation of anti-CII anti-bodies. All isotypes (IgG1, IgG2a and IgG2b) of anti-CII antibodies were arthritogenic, with the IgG1 and IgG2b isotypes as the dominating arthritogenic antibodies (Nandakumar, Andren et al. 2003). In the present study, dietary HTy-Ac supplementation significantly decreased serum levels of IgG1-and IgG2a-anti-CII antibodies in CIA mice **(Chapter III. Figure 2 A and B)**. These results suggest a potential role for HTy-Ac in regulating B cell responses, which may be in part, responsible for its anti-arthritic effects.

As mentioned above, the development and progression of RA is closely related to an imbalance of cytokine network. In fact, in RA synovium, elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-17 and INF-γ are produced by macrophages and synovial fibroblasts. These pro-inflammatory cytokines both directly and indirectly exert their effects through the production of additional pro-inflammatory cytokines, chemokines and MMPs at the pannus cartilage junction leading to cartilage degradation (Komatsu and Takayanagi 2012, Azizi, Jadidi-Niaragh et al. 2013, Noack and Miossec 2014). In the present chapter, we showed that arthritic mice fed with HTy-Ac enriched diet had decreased IL-1β, IL-6, IFN-γ, IL-17 and TNF-α levels in paw homogenate in comparison with CIA mice fed with SD and HTy-enriched diet **(Chapter III. Figure 3)**. These results indicate that dietary HTy-Ac exerts anti-inflammatory activities by the blockage of IL-1β, IL-6, IFN-γ, IL-17 and TNF-α production.

Serum MMP-3 level is suggested as a predictor for joint destruction in early RA or established RA and should be used in association with usual inflammatory markers to follow therapy efficiency (Denarie, Constant et al. 2014). Our data are in agreement with numerous studies (Saxne and Heinegard 1992, Ally, Hodkinson et al. 2013) showing that high serum COMP and MMP-3 levels were associated with disease activity and joint progression in CIA mice, whereas, the production of both cartilage and synovial biomarkers were significantly inhibited by the HTy-Ac enriched diet in CIA mice **(Chapter III. Figure 2 C)**.

It is well-known that both COX-2 and mPGES-1 are up-regulated in RA joint patients (Lazarus, Kubata et al. 2002) contributing to the progression of the disease through EP4 receptor activation (McCoy, Wicks et al. 2002). In this sense, we have shown that dietary HTy-Ac supplementation reduced PGE_2 levels, which could be possibly due to decreased expression of both COX-2 and mPGES-1 in the joint **(Chapter III. Figure 4)**. Therefore, regulation of these pro-inflammatory biomarkers by HTy-Ac could represent a potential molecular target susceptible to HTy-Ac modulation, which has not been demonstrated previously.

Signal transduction pathways closely involved in inflammation include the MAPKs, JAK-STAT and NF-κB pathways (Morel and Berenbaum 2004, Feldmann and Maini 2008). We investigated whether the activation of NF-κB signalling pathway was inhibited by HTy-Ac diet. Our data are in agreement with Sanchez-Fidalgo et al. that showed that HTy-Ac increased the inhibitory protein IkB-α and reduced p65 translocation **(Chapter III. Figure 6 B)**, indicating that dietary HTy-Ac inhibited the NF-κB activation by blocking IκB-α degradation in dextran sulfate sodium-induced colitis in mice (Sanchez-Fidalgo, Villegas et al. 2015).

Our findings are in concordance with above reports (de Hooge, van de Loo et al. 2004, Rosillo, Sanchez-Hidalgo et al. 2015) and showed that p38 and JNK MAPKs phosphorylation were increased in CIA control mice. Similarly, STAT-3 overexpression was also evidenced in RA synovium of control CIA mice and was positively related to the severity of synovitis, whereas dietary HTy-Ac supplementation reduced significantly both MAPKs and STAT-3 activation at transcriptional level **(Chapter III. Figures 5 A and 6 A)**. Collectively, our data suggest that dietary HTy-Ac may repress IL-17 production interfering negatively with JNK, p-38, p-ERK MAPKs and STAT-3 signalling pathways.

In inflammatory conditions, it has been documented that deficiency of Nrf2 accelerates the effector phase of arthritis and aggravates joint disease (Maicas, Ferrandiz et al. 2011). In the present study, in concordance with Karatas et al. (Karatas, Koca et al. 2015), expressions of Nrf2 and HO-1 were decreased in the arthritis group, by contrast dietary HTy-Ac could restore Nrf2 and HO-1 expressions conferring a role of Nrf2/HO-1 signalling in the beneficial effects of HTy-Ac in this murine model of RA **(Chapter III. Figure 5 B)**.

Altogether, our results suggest that EVOO exerts preventive effects in the development of experimental RA, playing its PE and isolated HTy-Ac compound a key role in these healthy benefits. Thus, EVOO may be considered as supportive nutritional therapy for RA patients as well as its PE and/or HTy-Ac supplement might provide an attractive nutraceutical complement in management of RA, mainly when the effects of inexpensive, side effect free therapies based

on dietary EVOO might suppose an improvement in public health on the prevention of high prevalence chronic pathologies.

RESUMEN/ABSTRACT

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by inflammation of the synovial membrane and progressive destruction of the articular cartilage and bone*. (Salgado and Maneiro 2014).*

RA patients exhibit an inflammatory chronic condition, which usually affects symmetrically arthrodial and small joints of hands and feet

Global prevalence of RA has been estimated to be around 0.5-1.0% of adults in developed countries with a large variation across regions and approximately three-times more common in the female gender. The disease may begin at any age, but around 80% of all patients initiate the disease between the ages of 35 and 50 years *(Rudan et al. 2015).*

Although the specific triggers and exact mechanisms of tissue damage in RA is still unknown, an increase in inflammatory mediators as well as a dysregulation of the immune system with uncontrolled T cell activity, play a remarkable role in its pathogenesis. Pharmacological treatment in AR including nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying antirheumatic drugs (DMARDs) and biological agents have improved the signs and symptoms of RA, but these drugs are only effective in a fraction of patients and have other limitations including a high cost, the requirement for parenteral administration and important side effects. Therefore, new therapeutic strategies are under investigation including nutritional therapy. The beneficial effects of the Mediterranean diet have been proven not only in cardiovascular diseases but also in diabetes, obesity, arthritis and cancer *(Cardeno, Sanchez-Hidalgo, and Alarcon-de-la-Lastra 2013)*. Evidence points out that Mediterranean diet decreases both pain and disease activity leading to better outcomes, and decreasing the doses of anti-inflammatory drugs, which exhibit important secondary effects *(Smedslund et al. 2010).*

Olive Oil is the characteristic culinary fat of the Mediterranean area being described as a key bio-active food *(Puertollano et al. 2010).* Extra virgin olive oil (EVOO) is obtained from the fruit of the olive tree (*Olea europea L.*) solely by mechanical or other physical means under conditions that do not alter its natural composition.

Traditionally the beneficial effects of EVOO have been ascribed to its monounsaturated fatty acid (MUFA) *(Bermudez et al. 2011)*. However, a wide range of evidence indicates that

many of the beneficial effects of EVOO intake are due to its minor highly bioactive components (about 1–2 % of oil weight) *(Alarcon de la Lastra et al. 2001)*

Among them, phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein have shown anti-inflammatory and antioxidant effects *(Omar et al. 2010).* Current experimental studies support a beneficial role of polyphenols from EVOO in several inflammatory diseases, including RA *(Martinez-Dominguez, de la Puerta, and Ruiz-Gutierrez 2001; Impellizzeri et al. 2011; Gong et al. 2009)*. Although EVOO has demonstrated anti-inflammatory effects, it has not reported so much evidence of its possible immunomodulatory effects.

Therefore, the **objectives** of this thesis were:

1. To determinate the possible protective effect of dietary extra virgin olive oil (EVOO) in collagen-induced arthritis (CIA) in DBA 1/J mice, an experimental model of RA and explore the biochemical routes and possibly intracellular signalling pathways.

2. To evaluate the oral polyphenolic extract from EVOO treatment in murine collagen-induced arthritis and study the biochemical routes and signalling pathway involved.

3. To investigate the effects of hydroxytyrosol (HTy) or hydroxytyrosol acetate (HTy-Ac), polyphenolics compounds from EVOO enriched-diets in experimental arthritis model in mice and elucidate the molecular mechanisms and signalling pathways involved.

Results and Discussion

1. **Dietary extra-virgin olive oil prevents inflammatory response and cartilage matrix degradation in murine collagen-induced arthritis**. *(Rosillo et al. Eur J Nutr. 2015 In press)*

Three-week-old male DBA-1/J mice were randomized into four experimental groups: (1) Sham sunflower diet (SO-Sham) group received a diet elaborated with a marketable sunflower oil; (2) CIA sunflower diet (SO-CIA) group received a diet elaborated with a marketable sunflower oil; (3) Sham EVOO diet (EVOO-Sham) group were fed with a diet made with a marketable EVOO picual variety and (4) CIA EVOO diet (EVOO-CIA) group were fed with a diet made with a marketable EVOO picual variety. After 6 weeks, arthritis was induced by type II collagen.

Experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU)

Our results revealed, that EVOO, as the lipid component of the diet, effectively exhibited preventive and therapeutic effects in the development of inflammatory arthritis and joint damage in CIA arthritic mice in comparison with those CIA mice fed with SO. This effect was correlated to an improved arthritis score, a minor inflammatory cells infiltration into articular tissues, reduced exudation into the synovial space, synovial hyperplasia and cartilage erosion. Overexpression of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-17 may activate osteoclasts and macrophages and recruit leukocytes in inflamed joints. Besides, it is well-known that IL-17 is able to induce the release of IL-8 and IL-6, and plays a remarkable role in the additive/synergistic effects induced by TNF-α and IL-1β *(Jeong et al. 2004).* Our results indicate that animals fed with EVOO diet showed a significant reduction in IL-1β, TNF-α and IL-17 pro-inflammatory cytokines levels in paw homogenates.

Cartilage oligomeric matrix protein (COMP) is a matrix protein with a great potential as a biological marker of cartilage metabolism in arthritis *(Saxne and Heinegard 1992).* In addition, COMP is a putative substrate for metalloproteinases (MMPs). Particularly, MMP-3 is a proteinase secreted by synovial fibroblasts and chondrocytes and its activity results in degradation of aggrecan core protein, cartilage link protein, fibronectin and collagen. Our data showed that the production of both cartilage (COMP) and synovial (MMP-3) biomarkers was significantly inhibited by dietary EVOO treatment in CIA mice.

Abnormal signalling pathways play an important role in the inflammatory process and can lead to a dysregulation of the inflammatory response being crucial in RA pathogenesis. Nuclear factor κB (NF-κB) is a crucial transcriptional activator for the expression of multiple pro-inflammatory genes involved in the microenvironment of the arthritic joints, playing an important role in the development of RA*. (Morel and Berenbaum 2004; Okamoto et al. 2010).* Our results suggested that dietary EVOO treatment suppressed NF-κB activation in CIA-induced arthritic mice. Similarly, the mitogen-activated protein kinase (MAPK) family also plays critical roles in RA pathogenesis *(Han et al. 2001; Suzuki et al. 2000)* regulating cytokine production, and activating the janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway through STAT-3 phosphorylation *(Aaronson and Horvath 2002).* Our results demonstrated that EVOO diet intake reduced significantly both MAPKs and STAT-3 activation. On the other hand, nuclear factor E2-related factor 2 (Nrf2) plays a central role for expression

of heme oxigenase 1 (HO-1), antioxidant enzyme. Our data showed that HO-1 could represent a potential molecular target susceptible to EVOO modulation, since dietary EVOO treatment strongly augmented Nrf2 and HO-1 protein expression conferring a role of HO-1 in the beneficial effects of EVOO in this murine model of chronic inflammation.

Altogether, our results confirm, for the first time, that EVOO intake dramatically attenuated the progression and severity of arthritis in CIA DBA/1 J mice through Nrf2/HO-1 upregulation and NF-κB, MAPKs and JAK-STAT signalling pathway inhibition, decreasing the inflammatory cascade induced by CIA.

2. **Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis.** *(Rosillo et al. Pharmacol Res. 2012;66(3):235-42.)*

CIA was induced in nine-weeks-old male DBA-1/J mice. On day 29, animals were randomized in four groups of mice: naïve group (NA), CIA group (CIA) and two treatment groups: CIA mice with polyphenolic extract (PE) treatment (PE 100 and 200 mg/kg, orally, once a day from days 29 to 41).

In this study, we have demonstrated that oral administration of PE was able to down-regulate the arthritic process in the CIA model of RA. PE showed anti-inflammatory effects with reductions in joint edema and migration of inflammatory cells. In addition, PE protected joints against cartilage alterations and bone erosion. The progression of arthritis is associated with sustained production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 *(Goronzy and Weyand 2009).* Our data shown that PE treatment controls the local levels of these cytokines, driving the production of different inflammatory and catabolic mediators in CIA model.

In this sense, COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1), enzymes responsible for the overproduction of PGE_2 in inflammation, are dramatically up-regulated *(Lazarus et al. 2002)* contributing to the progression of RA through EP_4 receptor activation *(McCoy, Wicks, and Audoly 2002).* Our data suggest that PE reduces PGE_2 levels, which would be dependent on the down-regulation of COX-2 and mPGES-1 protein expression in the joint.

In addition, PE treatment inhibits the phosphorilation of JNK and p38 MAPK and transcription factor STAT-3. Similarly, PE addition decreases NF-κB nuclear translocation leading to the down-regulation of the arthritic process.

In summary, our study has demonstrated, for the first time, the anti-inflammatory and joint protective effects of PE from EVOO in a CIA-induced experimental model, which would be related to the inhibition of relevant signalling pathways such as NF-κB, JAK/STAT and MAPKs controlling the production of inflammatory mediators.

3. **Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis.** *(Rosillo et al. Mol Nutr Food Res. 2015. In press)*

Three-weeks-old male DBA-1/J mice, were randomized into four experimental groups during all experimental period: (i) naïve group received a standard diet (SD), (ii) CIA group received a SD, (iii) CIA-HTy group received a 0.05% HTy diet and (iv) CIA-HTy-Ac group received a 0.05% HTy-Ac diet. After 6 weeks, arthritis was induced by type II collagen.

HTy-Ac was described for the first time in olive oil by Brenes et al. *(Brenes et al. 1999)* and is found in most Spanish virgin olive oils. Moreover, recently, it was reported that HTy-Ac is more soluble in the lipophilic phases than HTy, due to the presence of the ester group. Thus, this increased lipophilicity suggests that HTy-Ac is better absorbed across intestinal epithelial cell monolayers than free HTy *(Rubio et al. 2012).*

Our findings have revealed that dietary HTy-Ac supplementation could improve the arthritis score which was correlated with a minor migration of inflammatory cells into articular tissues in addition to a marked reduction of joint edema, synovial hyperplasia and cartilage erosion in comparison with those animals fed with standard diet (SD) and hydroxytyrosol (HTy) enriched diets.

CIA pathogenesis is characterized by the generation of anti-CII anti-bodies *(Nandakumar et al. 2003).* All isotypes (IgG1, IgG2a and IgG2b) of anti-CII antibodies were arthritogenic, with the IgG1 and IgG2b isotypes as the dominating arthritogenic antibodies *(Nandakumar et al. 2003)*. Our results demonstrated that dietary HTy-Ac supplementation significantly decreased serum levels of IgG1-and IgG2a-anti-CII antibodies in CIA mice. These results suggest a potential role for HTy-Ac in regulating B cell responses, which may be in part, responsible for its anti-arthritic effects.

Our results shown that arthritic mice fed with HTy-Ac enriched diet had decreased serum COMP and MMP-3 levels, as well as, pro-inflammatory cytokines such as IL-1β, IL-6, IFN-γ, IL-17 and TNF-α levels in paw homogenate in comparison with CIA mice fed with SD and HTy

enriched diet. Moreover, the activation of JAK/STAT, MAPKs and NF-κB pathways were drastically ameliorated whereas Nrf2 and HO-1 protein expressions were significantly up-regulated in those mice fed with HTy-Ac.

As a conclusion, our study reveals, for the first time, the anti-inflammatory effects of dietary HTy-Ac in the CIA model. The mechanisms underlying these protective effects could be related to the regulation of B cell responses, activation of the Nrf2/HO-1 signalling pathway and the inhibition of relevant signalling pathways such as JAK-STAT, MAPKs and NF-κB controlling the production of inflammatory mediators.

Conclusion

Altogether, our results suggest that EVOO exerts preventive effects in the development of experimental RA, playing its multiple minor components a key role in these healthy benefits. Thus, EVOO may be considered as supportive nutritional therapy for RA patients as well as its poliphenolic fraction might provide an attractive nutraceutical complement in management of RA, mainly when the effects of inexpensive, side effect-free therapies based on dietary EVOO might suppose an improvement in public health on the prevention of chronic pathologies with high prevalence in the population.

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