CLINICAL STUDY N° 1

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Supplementation with a rich-polyphenols olive tree powder reduces circulating inflammatory markers, disease activity, and pain intensity in patients with rheumatoid arthritis: a 9-week randomized, double-blind, placebo-controlled clinical trial

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ABSTRACT

Background- Notwithstanding the way that olive fruits polyphenols and olive leafs polyphenols have been known for their natural anti-inflammatory effect in the Mediterranean countries, there is little deep scientific study to confirm these benefits.

Objective- To assess the effect of rich-polyphenols olive tree powder (made of leafs, baby leafs, olive fruit and olive oil) made from olive trees planted in the middle of a

rocky desert on inflammatory process and pain intensity, a randomized, double-blinded, placebo-controlled trial was conducted on human subjects with rheumatoid arthritis (RA).

Methods- Seventy-nine RA patients were randomized to get either 1 g per day of olive tree powder or 1 g of placebo powder during 9 weeks. Laboratory analysis, questionnaires administration, pain intensity, disease activity score, and inflammatory biomarkers were determined at the baseline and at the end of the trial. Specialists have monitored eventual side effects and antagonistic impacts of taking the olive tree powder through the period of the study.

Results- Good compliance (over 95%) with the treatment was observed, without any side effect or study-intervention adverse. Significant decrease in disease activity score has shown at the end of intervention within the treated group, and between groups ($P<0.0001$). Compared with the placebo group, inflammatory biomarkers decreased significantly in treated participants ($P<0.0001$). Here are the changes noticed from baseline in treated group were -1.25 mg/L (CI, -1.75 to -0.75), -2.09 pg/mL (CI, -2.63 to -1.54), -0.82 pg/mL (CI, -1.14 to -0.49) and -1769 pg/mL (CI, -2254 to -1283) for hs-CRP, IL-6, TNF-α and PGE2 respectively. Additionally, it is important to note that pain relief and global participants satisfaction increased significantly ($P<0.0001$) after 9 weeks of olive tree powder supplementation.

Conclusion- A net improvement in circulating inflammatory markers, disease activity, and pain intensity was observed in RA patients allocated to rich-polyphenols olive tree powder food supplement.

Keywords: Rheumatoid arthritis; olive tree powder; Inflammatory biomarkers; Pain intensity; Disease activity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune disease associated with joint deterioration, pain, stiffness, and swelling. Fatigue, fever, and weight loss are also common symptoms. As the illness progresses, non-joint structures can also be affected including the skin, eyes, lungs, and heart. RA is the most common autoimmune disease, occurring in 0.7 to 1% of the population worldwide. It is estimated that approximately 75% of RA patients are women (Hresko *et al.*, 2018; Prado *et al.*, 2018). The etiology of RA is not completely understood although several risk factors have been identified. Genetic factors are predicted to contribute to approximately 50% of RA with an increased risk in people with a family history of RA. Several single-nucleotide polymorphisms and genes of the inherited tissue type major histocompatibility complex antigen HLA-DR4 are also associated with increased disease risk (Smolen *et al.*, 2016). Modifiable risk factors associated with RA include smoking, obesity, low fish intake, and periodontal disease, which are predicted to contribute to approximately 40% of its etiology (Nielen *et al.*, 2004; Rantapaa-Dahlqvist *et al.,* 2003).

Modification of the cytokine network, which are mediators of chronic inflammation is often targeted in the clinical management of inflammatory diseases like RA. This is demonstrated by a movement away from traditional treatments comprising nonsteroidal anti-inflammatory drugs (NSAIDs) toward disease-modifying anti-rheumatic and biological drugs (Hresko *et al.*, 2018). However, while these medications have demonstrated clinical efficacy, they are associated with several adverse immune-related

effects including an increased risk of serious infections and tuberculosis (Rondaan *et al.,* 2014). Consequently, there is increasing scientific interest in naturally-sourced ingredients as an additional anti-inflammatory and pain-relieving treatment option for RA (Suroowan and Mahomoodally, 2018).

Promising findings via *in vitro, in vivo*, and clinical studies have demonstrated RA anti-inflammatory and pain-relieving effects from olive tree polyphenols and Mediterranean diets, of with olives comprise a major component. For example, it was shown that adherence to a Mediterranean diet decreased inflammatory activity, increased physical function, and improved vitality in RA patients (Skoldstam *et al.*, 2003). In another study, adherence to a Mediterranean diet resulted in significant reductions in pain, early morning stiffness, and improvements in general health up to 6 months later (McKellar *et al.,* 2007). Further direct evidence of the potential benefits of olive tree polyphenols in RA is obtained from a study demonstrating faster reductions in cell damage, restoration of oxidative balance, and improvements in interleukin(IL)-6 suppression from the combined administration of a dry olive leaf extract and methotrexate during high disease activity in early-phase RA (Cabarkapa *et al.,* 2016). Moreover, several in vitro studies have confirmed the anti-inflammatory effects of hydroxytyrosol, tyrosol and oleuropein which are polyphenols derived from olive leaves, baby leaves, olive fruits, and olive oil. These polyphenols act on prostaglandin E2 (PGE2), leukotriene B4 (LTB4), tumor necrosis factor-α (TNF- α), IL-6, IL-1, and high-sensitivity C-reactive protein (hs-CRP) (Camargo *et al.*, 2014; Richard *et al.,* 2011; Zhang *et al.,* 2009b). Moreover, similar anti-inflammatory actions of oleocanthal, a component found in olive oil,

and the NSAID ibuprofen was identified (Beauchamp *et al.*, 2005).

Although anti-inflammatory effects from olive tree polyphenols have been identified, these results are primarily derived from in vitro and preclinical studies. Therefore efficacy from robust clinical trials are needed to substantiate the potential therapeutic efficacy of olive tree polyphenols in patients with RA. Consequently, the aim of this study was to examine whether supplementation with an olive tree powder (OTP) rich in polyphenols (TruOliv®) could improve clinical and laboratory parameters of disease activity in patients with RA.

METHODS

Study population

79 male and female patients aged between 20 and 80 years were recruited during July 2018 from patients referred to a rheumatology clinic (ESSEHA) located in Casablanca, Morocco. To be eligible for enrolment, patients required a diagnosis of rheumatoid arthritis for greater than one year based on the criteria established by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) (Aletaha *et al.,* 2010). The details of this study were explained to all volunteers and a written consent was obtained from all voluntary participants prior to study enrollment. Patients were ineligible for study participation if they were smokers, pregnant, lactating, taking the contraceptive pill, diagnosed with metabolic syndrome as defined by the Adult Treatment Panel III, suffered from an inflammatory disorder, were taking NSAIDs and/or cytokine inhibitors, or had a history of an allergy or intolerance to olive products. Participants were also excluded if that had a white blood cell count ≤3.5×109/L, hemoglobin level

≤8.5g/dl, platelet count ≤100×109/L, creatinine level ≥2.0 mg/dl or an aspartate aminotransferase level ≥2.5 times the upper normal limit. Participants also agreed to avoid the consumption of olive tree products (e.g., olive oil and olive fruits), other antioxidant supplements, nutrients high in omega-3 polyunsaturated fatty acids (e.g., fish), and herbs and nutraceutical supplements known to affect inflammation and immune function for at least 3 weeks prior to study commencement and then throughout the study duration. Participants were also required to maintain their normal diet throughout the study duration.

Study design and intervention

This was a 9-week, randomized, double-blind, placebo-controlled study. The study design is summarized in figure 1 and the Eligible participants were randomly assigned to take capsules containing OTP or a placebo using a computer-generated random-number sequence. Investigators and participants were blinded to treatment allocation.

Following an overnight fast of at least 12 hours, participants attended the clinic to undergo a screening visit which included a tender and swollen joints examination, completion of self-report questionnaires (the Mediterranean Diet Questionnaire and International Physical Activity Questionnaire), blood draw, urine collection, and an anthropometric and blood pressure measurement. Participants were also asked to record their dietary intake for 3 days prior to their screening visit. Study personnel provided necessary explanations about food intake estimations. All assessments were repeated at the end of the study (9 weeks later). Participants and all study personnel (including investigators) had free and continuous access to clinic services for advice and consultation during this study.

Participants received vegetable capsules (hydroxpropyl methylcullose) containing 500mg of OTP (TruOliv®) or a matching placebo (maltodextrin excipient). All participants received instructions about capsule intake and storage and were asked to take one capsule, twice daily (1gram a day) prior to their meal. Supplement and placebo intake was weekly controlled. The OTP (TruOlive®) was manufactured by Atlas Olive Oils and is and extract derived from olive leaves, olive baby leaves, olive fruit, and olive oil using a physical extraction process without the use of solvents, purification processes or any chemicals. The extraction utilizes a freeze-drying process to optimize polyphenol levels. It is worth noting that this OTP is derived from olive trees planted in a specific rocky desert and drought-dominant environment in Morocco where temperatures reach 127°F in the summer. Moreover, this highly rocky environment restricts the roots of the olive tree from sourcing nutrients in the soil. These olive trees are under stress and produce abnormally high quantity of antioxidants (mainly hydroxytyrosol) to defend themselves and survive.

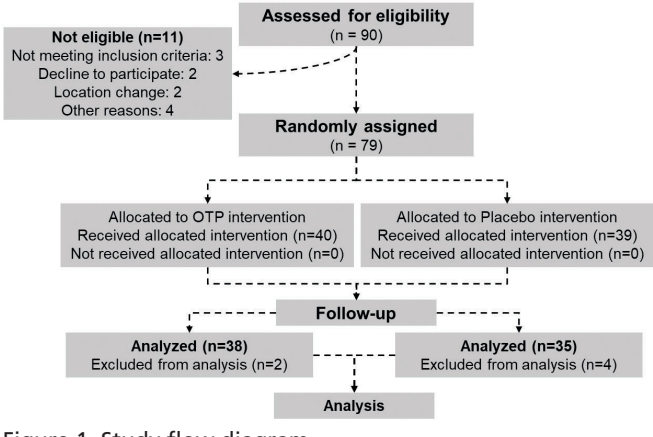

Figure 1. Study flow diagram.

OUTCOME MEASURES

An 8ml fasting blood sample was collected at baseline and week 9 from each participant's antecubital vein. Blood samples were collected in EDTA and SST tubes. All erythrocytes, plasma, serum and urine samples were stored as 1 mL aliquots at 80°C until analysis.

Primary Outcome Measures 1: Inflammatory Measures

Serum quantification of PGE2, LTB4, TNF-α, and cytokines IL-1 and IL-6 was performed using high-sensitivity enzyme-linked immunosorbent assay kits (DIA, Belgium), while serum hs-CRP level was determined by a turbidometric assay using a commercial kit at a wavelength of 500 nm.

Primary Outcome Measure 2: Measures of Disease/ Pain Activity:

The visual analog scale (VAS), a clinically validated subjective measure of pain, was used to evaluate pain intensity by participants, according to the protocol devised by (DeLoach *et al.,* 1998). Participants were instructed to draw a line to a maximum of 100 millimeters (mm) depicting their pain intensity (0mm equals no pain and 100 mm equals very severe pain).

A verbal rating scale (VRS) was also used to assess the extent of pain relief using a 5-point VRS where 0 equals no relief, 1 equals a little (perceptible) relief, 2 equals some (meaningful) relief, 3 equals a lot of relief, and 4 equals complete relief. The VRS is validated and commonly-used subjective measure of pain intensity (Jensen *et al.,* 1986).

A Disease Activity Score (DAS28) was calculated by the European League Against Rheumatism (EULAR) (Wells *et al.,* 2009). This validated outcome measure for RA is based on the number of tender and swollen joint, serum hs-

CRP concentration, and the global health (GH) results as calculated by the patient's 100mm VAS score (converted to cm). The calculation of DAS28 is shown below:

DAS28 (CRP) = [0.56 √TJC] + [0.28 √SJC] + [0.36 Ln (CRP + 1)] + [0,014 (GH)]

Secondary Outcome Measures:

Anthropometric measures: A calibrated scale and wall-mounted stadiometer with a precision of 0.1cm, and a semi-automatic oscillometer (Boso Medicus smart Semi-automatic Blood Pressure Monitor, Germany) were used to measure anthropometric parameters and blood pressure.

Measure of compliance to capsule intake:

Urinary hydroxytyrosol content, considered an objective estimate of OTP intake, was measured by High Performance Liquid Chromatography (HPLC). First, hydroxytyrosol was extracted from acidified urine (hydrochloric acid, 0.6 N of final concentration) and then quantified by a Shimadzu chromatograph device equipped with a reverse phase C18 column (250mm L. × 4.6 mm I.D., 5 µm) according to the protocol described by Visioli *et al.* (2000).

Measure of adverse effects: Possible adverse effects of OTP administration throughout the study period (e.g., oral symptoms, digestive disorders, fullness, allergic skin response, and other intervention-related symptoms) were assessed by medical practitioners and study personnel. In addition, a 5-point scale (0 = poor, 1 = fair, 2 = good, 3 = very good, and 4 = excellent) was used to assess the global satisfaction assessment in response to treatment (GAST).

Measures of Dietary Intake and Physical Activity: Participants were asked to complete a 3-day food diary at baseline, week 4, and week 9. andbased on this dietary recordusing Participants were also asked to complete the

Mediterranean Diet questionnaire (Estruch *et al.,* 2006) at baseline and week 9 to examine level of adherence to the Mediterranean diet. This measure provides a validated estimate of adherence to a Mediterranean diet (ref). Moreover, physical activity was assessed using the psychometrically-validated International Physical Activity Questionnaire (physical exercise was categorized as high, moderate, or low) (Craig *et al.*, 2003).

STATISTICAL ANALYSIS

Data were statistically analyzed using GraphPad Prism version 6.00 (GraphPad Prism Inc, San Diego, California). For the baseline characteristics, continuous variables are expressed as mean values ± standard deviation (SD), and categorical variables are expressed as frequencies (percent). For inflammatory biomarkers, pain intensity, and pain relief mean values are expressed with 95% confidence intervals (CIs). Normal distribution of data was checked using the Kolmogorov-Smirnov test. The difference between baseline groups characteristic was performed using the independent-samples T-test for normally continuous data, the Mann-Whitney U test for non-normally continuous data, and the $\chi 2$ test for categorical data. The independent-samples T-test was also used to compare mean changes from baseline to the end of the study (9 weeks) between OTP and placebo groups. Results with two-sided p-values of <0.05 were considered statistically significant.

RESULTS

Study compliance and adverse effects

Ninety patients were screened for study inclusion, with 11 not meeting eligibility criteria. Reasons for ineligibility are detailed in Figure 1. Seventy-nine participants were enrolled in the study and data from 73 participants was

available for statistical analysis. Data from six participants was not included in analyses (2 in OTP-group and 4 in placebo-group) as they did not follow the study protocol. No adverse effects related to the intervention were observed and an average 95% compliance to capsule intake was reported. Results shown in figure 2 illustrate the changes in urinary hydroxytyrosol concentrations (a biomarker of compliance) for both placebo and OTP groups. The content of hydroxytyrosol found in urine of OTP participant's group was significantly higher (P<.0001) compared to that of placebo group. However, it is worth noting that literature data regarding olive phenols metabolism and excretion are inconsistent (Covas *et al.*, 2006; Visioli *et al.*, 2003).

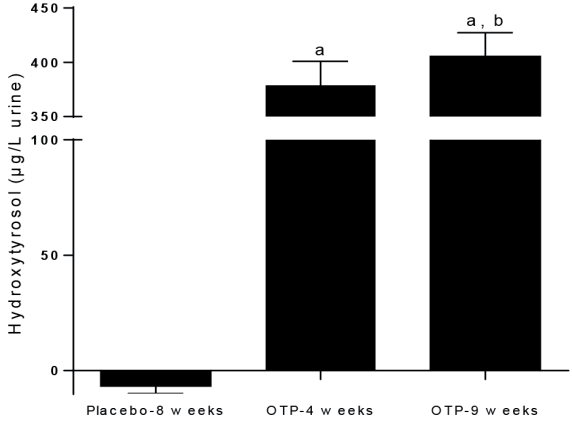

Figure 2. Change from baseline in urinary hydroxytyrosol excretion. Mean with 95% CI. [a]P<.0001, between OTP-group (at 4 or 8 weeks); [b]P= .003, between OTP-group at 4 and 8 weeks.

Baseline characteristics

The baseline characteristics of the 79 participants randomized into OTP and placebo groups are shown in

table 2. No significant differences were identified between the two study groups for all the baseline parameters. This included adherence to the Mediterranean diet (p=.296).

Table 2. Baseline characteristics of participants.

Parameter	OTP group (n=40)	Placebo group (n=39)	P value [a]
Age (years)	56.73 ± 1.61	54.31 ± 1.97	.306
Female, n (%)	32 (80)	27 (69.23)	.935
Weight (kg)	65.43 ± 3.06	63.54 ± 4.34	.957
BMI (kg/m^2)	28.17 ± 1.662	27.83 ± 1.815	.901
Disease duration (years)	7.77 ± 0.42	6.33 ± 0.56	.356
Medical history of disease, n (%)	15 (37.50)	12 (30.76)	.142
Family history of disease, n (%)	8 (20)	10 (25.64)	.708
Exercise activity habits, n (%)	16 (40)	18 (45)	.822
Alcohol drinking habits, n (%)	2 (5)	3 (7.69)	.233
15-item Mediterranean diet score	2.04 ± 0.20	1.91 ± 0.15	.307
DAS28	4.07 ± 0.67	4.40 ± 0.71	.911
Pain VAS (0–100 mm)	77.11 ± 9.84	75.65 ± 9.12	.710

Value are expressed as mean ± standard deviation or in percentage.
[a] P value (<.05) by independent t-test or Mann-Whitney test.

Primary Outcome Measure 1: Inflammatory markers

Changes in measured inflammatory markers from baseline to week 9 are illustrated in figure 3. As measured by the independent-samples T-test, there were significant decreases in hs-CRP of participants allocated to OTP

treatment compared to those in the placebo group (p = .014 and <.0001 after 4 and 9 weeks, respectively). Mean changes from baseline in the hs-CRP levels were -0.56 (CI, -0,91 to -0.18) and -1.25 mg/L (CI, -1,75 to -0,74mg/L) after 4 and 9 weeks, respectively in the OTP group. There were greater reductions in IL-6 (p=.014 at week 4 and p<.0001 at week 9), TNF-α (p=.025 at week 4 and p<.0001 at week 9), PGE2 (p=.002 at week 4 and p<.0001 at week 9), and LTB4 (p=.0004 at week 4 and p<.0001 at week 9) in the OTP group compared to the placebo group, as measured by the independent-samples T-test. The adjusted within-group changes for the OTP group in IL-6 and TNF-α were -2.08 pg/mL (CI, -2.63 to -1.53) and -0.81 pg/mL (CI, -1.14 to -0.49). As assessed by the independent-samples T-test, there were no significant between-group differences in changes in IL-1 (p-value of 0.413 and 0.084 at 4 and 9 weeks).

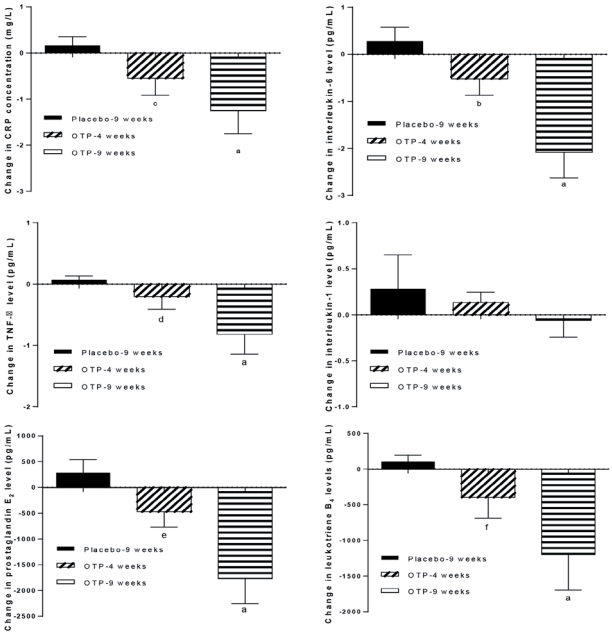

Figure 3. Change in circulating inflammatory concentrations over time in the two study groups. Error bars are 95% CIs. [a](P<.0001), [b](P=.014), [c](P=.009), and [d](P=.0247), e(P=.0017), f(P= .0004).

Primary Outcome Measure 2: Pain intensity and disease activity

Table 4 contains a summary of changes from baseline in pain intensity, pain relief, and DAS28 in both OTP and placebo groups. VAS (100-mm pain scale) values for the OPT group (50.41 ± 8.86 mm) were significantly lower than those of the placebo group (76.03 ± 10.46 mm) at 9 weeks (*P*<.0001). Similarly, pain relief score was significantly higher in the treated group compared to the placebo (*P*<.0001), even after 4 weeks of intervention

(Table 4). At week 9, more than 30% of the OTP-group members reported a high pain relief score (≥3), while the remaining participants reported a meaningful pain relief (score ≥2).

Table 4. Change from baseline in pain intensity, pain relief, DAS28. Data are expressed as mean and (95% CIs).

Parameter	OTP group (n=38)	Placebo group (n=35)	P value
Pain intensity	-26.70 (-30.39 to -20.90)	0.38 (-2.26 to 3.02)	<0.0001
Pain relief	2.14 (2.13 to 2.71)	-0.11 (-0,21 to -0,11)	<0.0001
DAS28	-1.84 (-2.43 to -2.03)	-0,13 (-0,21 to 0,09)	<0.0001
GART*	3.21 (2.93 to 3,48)	0.47 (0.28 to 0.65)	<0.0001

* Global satisfaction assessment in response to treatment. Only the average value measured at the end of the study.

Data for DAS28 show a significant decrease in disease activity reported by the treated participants compared to those allocated to the placebo ($P<.0001$). Patients in OTP group with baseline active RA (DAS28 score ≥ 3.2) showed a good therapeutic response by the end of the study (DAS28 score of 2.23 ± 0.54), which indicates an RA remission (DAS28 score ≤ 2.6). Table 4 shows also the global satisfaction assessment in response to treatment, included the assessment of patient's anxiety. Very good satisfaction regarding the intervention (score of 3.21 of the 5-point categorical scale) was reported by participants received OTP, compared ($P<0.0001$) to those of placebo group. High degree of participant's satisfaction was correlated to a significant decrease in circulating inflammatory biomarkers level, pain intensity, and disease

activity score, indicating the efficacy of the treatment with OTP.

FOOD, ENERGY, AND NUTRIENT INTAKE

Results shown in table 3 demonstrate that there was no significant change in dietary/nutrient intake during the study period for both the OTP and placebo groups. In general, all participants have met the daily recommended diet for this study, and the consumption of olive products and any other products known to have anti-inflammatory effects was avoided.

Table 3. Mean energy/nutrient intake and weight change over the 9-week study period.

Parameter	OTP group (n=45)	Placebo group (n=45)
Energy (cal)		
Baseline	1355.00 ± 209,54	1809.00 ± 130.3
9 weeks	1402.00 ± 205,11	1695.00 ± 318.4
P value [a]	0.603	0.445
Fat (g)		
Baseline	63.90 ± 11.40	78.32 ± 13.47
9 weeks	64.17 ± 11.95	75.06 ± 12.95
P value [a]	0.747	0.833
PUFAs (g)		
Baseline	11.33 ± 2.01	11.90 ± 1.22
9 weeks	12.77 ± 2.91	10.67 ± 1.43
P value [a]	0.711	0.802
MUFAs (g)		
Baseline	22.15 ± 1.87	23.22 ± 1.34
9 weeks	23.52 ± 2.23	22.54 ± 1.57
P value [a]	0.193	0.114
SFAs (g)		
Baseline	13.11 ± 1.52	12.93 ± 1.97
9 weeks	15.86 ± 1.62	15.57 ± 2.11
P value [a]	0.515	0.749

Parameter	OTP group (n=45)	Placebo group (n=45)
Weight (kg)		
Baseline	65.43 ± 3.06	63.54 ± 4.34
9 weeks	66.01 ± 4.77	63.97 ± 4.66
P value [a]	0.794	0.906

Data are expressed as mean ± standard deviation. PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids. a Paired Student t test ($p<0.05$).

Physical activity and Mediterranean diet??

DISCUSSION

In the present study, patients with rheumatoid arthritis (according to the ACR/ELUAR criteria) were treated with a 1g daily dose of a phenolic OTP (TruOliv®) for 9 weeks. The OTP was well tolerated with no reported adverse reactions by study participants. We found that supplementation with this natural OTP displayed strong therapeutic actions against inflammation (via reductions in PGE2, LTB4, TNF-α, IL-6, but not IL-6), disease activity, and joint pain in patients with RA.

RA is characterized by a continuous and excessive influx of inflammatory cells into the synovial membrane. Inflammation of synovial membranes are believed to be the main cause of RA with high concentrations of circulating inflammatory markers, such as cytokines (IL-6 and TNF-α) and hs-CRP correlated with a propensity to pain, warmth, and redness associated with joint destruction (Liu *et al.*, 2018; Wei *et al.,* 2015). Chronic inflammation leads to cartilage damage and bone destruction (mediated by osteoclasts), which eventually causes a loss of joint function. However, inflammatory reactions and osteoclast differentiation are both mediated by a complex network of cytokines, mainly TNF-α, IL-6, IL-1, as well as other simple molecules such as eicosanoids (PGE2 and LTB4) (Boissier

et al., 2012; Smolen and Redlich, 2014). RA treatment most commonly comprises biologic treatments targeting specific immunologic pathways. In particular, it has been reported that the inhibition of TNF-α and IL-6 has a greater effect on inflammation compared to IL-1 (Smolen and Redlich, 2014). The findings from this study demonstrate that supplementation with an OTP (containing 14% total polyphenols and 10% hydroxytyrosol) reduced circulating concentrations of TNF-α, IL-6, hs-CRP, PGE2, and LTB4 in patients with RA.

Olive products contain several polyphenols including hydroxytyrosol, tyrosol oleuropein, and oleocanthal which are responsible for its anti-inflammatory effects. In particular, the polyphenols in the administered OTP (particularly hydroxytyrosol), has been shown to act directly on DNA to reduce the expression of inflammatory mediators and/or inhibit their biosynthesis pathways through a similar mechanism as glucocorticoids and/or NSAIDs. For example, several studies have confirmed an inhibitory effect of hydroxytyrosol on PGE2 levels through the suppression of inducible cyclooxygenase (COX-2, key enzyme of PGE2 biosynthesis pathway from arachidonic acid) in isolated human monocytes (Lu and Wahl, 2005; Rosignoli *et al.*, 2013; Zhang *et al.,* 2009a) and murine macrophages (Richard *et al.*, 2011). Moreover, hydroxytyrosol and oleuropein displayed a strong in vivo inhibitory effect against COX-2 and PGE2 in mice with dextran sulfate sodium-induced colitis (Giner *et al.,* 2011; Sanchez-Fidalgo *et al.,* 2012). Additionally, results from other cell culture models show that hydroxytyrosol in its pure form or carried in a natural matrix (e.g. *via* an OTP or olive oil) can inhibit the synthesis of LTB4, TNF-α, IL-6, IL-1 and hs-CRP (Bitler *et al.,* 2005; Camargo *et al.,* 2014; Maiuri *et al.,* 2005; Richard *et al.,* 2011; Zhang *et al.*, 2009b). Findings from clinical studies have demonstrated

such inhibitory effects against inflammatory markers in patients with stable coronary disease (Estruch *et al.*, 2006; Fito *et al.*, 2007). The results of the current study were consistent with previous in vitro and in vivo investigations demonstrating the positive effects of olive oil and a Mediterranean diet rich in olive tree polyphenols in patients with RA. (Cabarkapa *et al.*, 2016; McKellar *et al.*, 2007; Skoldstam *et al.*, 2003). However, in our study we demonstrated the therapeutic and anti-inflammatory effects of an OTP as a stand-alone treatment for RA. We note that the observed changes were not caused by dietary changes over the course of treatment as there were no nutritional/dietary changes observed, as measured by our diet questionnaire and 3-day food record. In particular, there were no changes in PUFAs which have been shown to have a therapeutic effect in RA (Park *et al.*, 2013).

To further understand the potential mechanisms of OTP polyphenols, it is important to note that NSAIDs work by inhibiting the activity of cyclooxygenase enzymes (COX-1 and/or COX-2). However, COX inhibition with NSAIDs (celecoxib, rofecoxib, diclofenac) has been shown to cause an increase in TNF-α levels in rheumatoid synovial membrane cultures as well as in blood (Page et al., 2010; Rosignoli et al., 2013). In contrast, we found that the OTP resulted in a significant decrease in TNF-α. This suggests that hydroxytyrosol and/or other OTP's polyphenols target differing immunological pathway(s) leading to the decreased levels of IL-6 and TNF-α. Killeen *et al.* (2014) highlighted the potential of hydroxytyrosol to drive new therapeutic opportunities by reducing nuclear factor kappa β (NF-$\kappa\beta$) activation and its nuclear translocation. NF-$\kappa\beta$ triggers the expression of more than 150 genes including those encoding cytokines, TNF-α, IL-1, and IL-6 (Makarov, 2001). Richard *et al* (2011) found that the decrease of cytokines levels in murine macrophages

following treatment with hydroxytyrosol was correlated with a low expression of NF-κβ p65. Hydroxytyrosol has also been shown to influence NF-κβ activity in endothelial (Scoditti et al., 2012) and neural cells (St-Laurent-Thibault *et al.,* 2011). This suggests that the anti-inflammatory effects of OTP's polyphenols may be by inhibiting COX-2 enzyme activity and/or reducing the expression of NF-κβ.

Chronic inflammation in RA induces proliferation of the synovium leading to formation of pannus and joint destruction, where neovascularization (angiogenesis) is a major contributor (Lee *et al.*, 2001; Semerano *et al.*, 2011). A high correlation between RA progression and an important pro-angiogenic factor, vascular endothelial growth factor (VEGF) has been observed in RA patients (Lee *et al.,* 2001; Sone *et al*., 2001). Pro-angiogenic factors VEGF and angiopoietins (Ang)-1 activation in the synovial membrane are a multi-targeted mechanism involving the cytokines IL-1β, TNF-α, and COX-2 (Pettit *et al.,* 2001; Scoditti *et al.*, 2012). Our unpublished investigations suggest that hydroxytyrosol derived from the studied OTP, modulates the angiogenic response of endothelial cells by repressing VEGF (isoforms A, B, and C), Ang-1 and Ang-2 gene expression (Fortesa *et al.*, 2012; Scoditti *et al.*, 2012).

In RA, pain intensity is strongly correlated with high PGE2 level (Kamei *et al.,* 2004; Prochazkova *et al.*, 2009; Scher *et al.,* 2007), explaining the effectiveness of NSAIDs as pain relief agents. Similar pain relief effect of the NSAID, ibuprofen has been reported for oleocanthal by Beauchamp *et al* (2005). The decrease in circulating inflammatory markers, particularly in PGE2 level, is likely a major contributor of pain reduction observed in our study.

Study Limitations and Directions for Future Research

Although our study demonstrated the positive effects of an OTP on inflammation and RA symptoms, there remain several unanswered questions that require investigation in larger-scale studies. Our study revealed the efficacy of an OTP over a 9-week period, however, its benefits, tolerability, and safety over a longer duration is uncertain. Symptomatic changes and inflammatory effects following treatment discontinuation also requires investigation. Moreover, the efficacy of the OTP as an adjunct to pharmacological medications would be important to examine, particularly in unresponsive RA patients or those exhibiting only moderate improvements from traditional treatments. We also only used a single dose of 1g a day, so its efficacy at either a lower or higher dose is uncertain. The dose-escalating effects in non-responders or those exhibiting only a partial treatment response will be also helpful. The effects of the OTP in different populations, particularly in those where the consumption of olive products is less common will further substantiate the therapeutic effects of the OTP, as we recruited RA patients from Morocco where olive consumption is high. Finally, the effects of the OTP in patients with early-diagnosed RA versus long-standing RA, and its therapeutic efficacy compared to standard olive oil, or other olive tree extracts will be important to examine. RA is also associated with differences in intestinal microbial composition compared to healthy controls (du Teil Espina *et al.*, 2018). A Mediterranean diet and olive consumption have been shown to alter microbial compositions, potentially presenting as another therapeutic mechanism of action (Martin-Pelaez *et al.,* 2017; Mitsou *et al.*, 2017). An examination of microbial changes along with its relationship to symptomatic improvement may help to further clarify the physiological actions of an OTP.

CONCLUSION

In summary, chronic inflammation and pain are hallmark features of RA. Our findings demonstrate that the administration of rich-polyphenols extract from the olive tree was associated with significant decreases in circulating inflammatory markers, pain intensity, and disease activity in RA patients. We suggest that the anti-inflammatory effect of olive polyphenols, as natural components of OTP, may be linked to i) NF-κβ-dependent inhibition of cytokines (IL-6 and TNF-α), ii) similar actions to NSAIDs comprising the inhibition of COX-2, and iii) VEGF and Ang-1 repression. Further research should focus on the anti-angiogenic activity of hydroxytyrosol in synovial membrane, as a future target of new anti-inflammatory drugs based on hydroxytyrosol structure.

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