A formulation of olive oils (oHo®) shows potent antimicrobial activities in vitro and in patients with atopic dermatitis (AD) colonized by *S. aureus*.

Other clinical results in AD and atopy

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Stratum corneum of the skin epidermal barrier (EB) and some olive oils (OOs) contain common fatty acids that exhibit similar antimicrobial actions. Atopic dermatitis (AD) is an autoimmune/inflammatory disease that affects mainly the lipid composition of the EB. A standardized formulation (blend) of organic extra virgin OOs (oHo®, Bioaveda, Spain) shows potent *in vitro* activities against *S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger* when tested in the agar diffusion test. These activities are stronger than those observed with each particular OO contained in oHo. The oHo intake, together with the application of three oHo-based dermal preparations (a gel, an emulsion and an innovative formulation of cold gelified oils), eliminate *S. aureus* colonization in patients with recalcitrant AD, including *S. aureus* infection in one case of severe iatrogenic immunosuppression. Furthermore, this oHo-combined treatment provokes the disappearance of eczema and itching in AD patients. Other positive results on asthma related to AD (atopic march), although exciting, must be taken with caution and deserve of further investigation in larger trials. Quality of life was positively changed in all patients.

**Keywords:** S. aureus; atopic dermatitis; oHo; olive oils; skin; epidermal barrier; asthma; immunity; Tregs

1. Introduction and rationale

Atopic dermatitis (AD) is a well known T helper-2 (Th2)-immune/inflammatory skin disease, being lipid alterations of the epidermal barrier (EB) the key factor that better defines the illness [1-3]. In this sense, the severe alterations of some ceramides and free fatty acids (FA) contribute highly to the dysregulated EB function [1-5]. Polarization of immune responses during AD appears to obey to disturbances in regulatory immune mechanisms mediated by interleukin-10 (IL-10) and interferon-gamma (IFN-γ), among others [3,6]. More alterations in AD concern peroxisome proliferator-activated receptors (PPARs) that, as it is well known, are regulated by monounsaturated (MUFA) and polyunsaturated (PUFA) FA substrates [3,7]. Additional physiologic mechanisms related to fat metabolism appear to be also altered in AD, then contributing to explain the extreme lipid dependence of the illness. Thus, low levels of high-density lipoprotein cholesterol (HDL-c) are frequently seen in AD [3]. These immune and lipid disturbances are the responsible for the high sensitivity of AD patients to skin colonization by *Staphylococcus aureus* (*S. aureus*) [3,6]. In fact, more than 85% of AD cases are colonized by this pathogen [6,8], being many of these germs antibiotic resistant [8].

Stratum corneum (SC) of the EB, as well as some OOs, contain common FA which exhibit antimicrobial actions [3,9]. In that concerns OOs, it is now clear some polyphenols [10,11] or FA [12] show *in vitro* antimicrobial activities, including those against *S. aureus* [13]. Unfortunately, nor the experiences in animals [14,15], neither the OO-based products for parenteral nutrition in humans [16] have shown *in vivo* antimicrobial activities.

In order to restore the EB, the topic application of different lipid compositions is now emerging for AD treatment [2,3,17]. More recently we have shown the ability of an orally given formulation (blend) of different Spanish OOs (oHo®™, Bioaveda, Spain) to induce the endogenous production of IL-10 and IFN-γ in patients with chronic kidney disease (CKD), that is thought to be provoked through an intriguing endogenous play among PPAR-α, dendritic cells (CDs) and T regulator cells (Tregs), among other immune/lipid-related mechanisms [3,18]. Moreover, oHo intake rise HDL-c plasma levels [19], and ameliorates skin xerosis and itching in CKD patients. These local and systemic beneficial immune and lipid effects have never been described by using any conventional OO in CKD patients [20-22].

In this paper we show (a) several oHo concentrations exhibit potent *in vitro* microbicidal activities against gram positive and gram negative bacteria, and fungi (b) oral administration of oHo, together with the application of two or three oHo-based dermal products, ameliorates notably recalcitrant AD in children and adults. *S. aureus* is eliminated even in conditions of severe immunosuppression.
2. Material, patients and methods

Formulation of organic olive oils: oHo

oHo is the result of a rational combination (blend) of ≥ 3 varieties of Spanish organic OOs [23]. All OOs are mechanically extracted at very cold temperatures (18º C) from Olea europaea olives (c.v Picual, c.v. Arbequino, and c.v. Cornicabra, among others). OOs used in oHo come from Organic Agriculture applied to olive growths, and after oil extractions they are tested for the absence of herbicides, insecticides, and other endocrine disrupting chemicals (EDCs). Contrary to other conventional OOs, the resultant oHo becomes standarized year by year in spite of the annual harvest variations. Its balanced chemistry composition seems to be the responsible for the striking differences between conventional OOs and oHo when evaluated in humans [23,24]. As clear examples (a) none of conventional OOs tested in CKD patients are able to modify lipid nor protein metabolism parameters [20-22] as oHo does it [19] (b) excepting oHo [3,18], there are not literature data reporting the capacity of any conventional OO to jointly increase IL-10 and/or IFN-γ serum levels in humans (c) no conclusive beneficial effects have been reported for any conventional OOs in AD patients [25,26].

2.1 In vitro studies

oHo dermal products. Four oHo-based products for topical use [23], containing different oHo concentrations (Table 1), were evaluated in the agar diffusion test according to the rules of “Efficacy of antimicrobial preservation. European Pharmacopeia 5.3”: a) a gel (G-7%); b) an emulsion (E-10%); c) a keratolytic cream (KC-20%); and, d) an innovative formulation of cold gelified oils (CGO-87%). All products used in the different topic formulations came from organic agriculture, and none of them contain preservants or stabilishers, nor parabenes, neither corticosteroids, antibiotics or antifungals. Four pathoghens from the American Type Culture Collection (ATCC) were used in this study (Tables 1 and 2): Pseudomonas aeruginosa (ATCC 9027/CECT 111), Staphylococcus aureus (ATCC 6538/CECT 239), Candida albicans (ATCC 10231/CECT 1394), and Aspergillus niger (ATCC 16404/CECT 2574). In a separate experiment, a pathogenic S. aureus coming from an infected patient was also tested in vitro and incubated with or without oHo. Microbe survivals were evaluated on days 2, 7, 14 and 28 after their in vitro inoculation with or without oHo, and results were expressed in c.f.u x 10⁶ microorganisms/mL. oHo microbicidal effects were also compared (Table 2) with the effects obtained with (a) each particular OO contained in oHo (b) one commercial OO, and (c) the results reported in the literature at PubMed by other authors who used different OOs.

2.2 In vivo studies: patients with atopic dermatitis

Patients. This is a pilot study in adults and children who were diagnosed of recalcitrant AD by history, pattern, evolution, and skin lesions. Twenty four patients (15 females) with AD (19 children; 14 with moderate to severe seasonal asthma that needed the use of inhaled glucocorticosteroids) were initially recruited for the entry to the study. Ages ranged from 4 months to 55 years old. Two adult patients with AD also suffered from concomitant painful hand eczema colonized by S. aureus (see Fig. 1, up). Comorbid factors were documented S. aureus colonization in 17 patients (71%), and graft vs host skin disease (GvHSD) together with S. aureus colonization in one case (see Fig. 2, up). Allergic rhinitis affected 14 patients (12 children). As absolute inclusion criteria (a) all patients should be previously diagnosed by at least one dermatologist and one paediatrician or pneumologist in the case of children; (b) all patients should be habitually consumers of extra virgin OOs (conventional or organic) (c) all patients should be suffering of chronic relapsing AD in spite of treatments applied before.

Methods. Classic emollients, moisturizers, and/or so-called hydration agents (including conventional topic OOs), oral and/or topic glucocorticosteroids (OGC and/or TGC), and topic inhibitors of calcineurin (TIC) were withdrawn respectively at the beginning, two weeks, and one month before the entry to this study. oHo was orally given to all patients at daily doses dependent on body mass index. G-oHo was applied twice daily in all body surface during a short shower, and 15 minutes later the E-oHo was applied in all eczematous lesions. In case of severe skin lesions, the use of CGO was recommended to 15 minutes after the emulsion. Patients were independently evaluated by Drs Villarrubia and Vidal Asensi, and photos were taken in all patients before the entry and every 15 days throughout the study. Responses were defined as complete (CR) when skin signs (eczema, scratching lesions) and symptoms (itching, sleep disturbances) disappeared completely. All patients, or their parents in the case of children, were asked for the acceptance of products, as well as for the changes experienced in their quality of life.
3. Results and discussion

3.1 In vitro studies

As shown in Table 1, oHo provoked the complete disappearance of all germs tested, whichever the concentration of oHo used, early after 2 days of in vitro incubation. Evaluation at days 7, 14 and 28 continued showing the absence of living germs (data not shown).

Table 1  Effects of different oHo concentrations on microbe survival after 2 days of in vitro incubation.

<table>
<thead>
<tr>
<th>Germs</th>
<th>Positive control</th>
<th>oHo products and concentrations (%)</th>
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<tr>
<td></td>
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<td>G (7), E (10), KC (20), CGO (87)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>C. albicans</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>A. niger</td>
<td>2.0</td>
<td>0</td>
</tr>
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Results in c.f.u x 10^6 microorganisms/mL represent the mean of four experiments. G: gel; E: emulsion; KC: keratolytic cream; CGO: cold gelified oils (see the text).

Interestingly, the in vitro inoculation of the S. aureus coming from the infected patient described before provoked the colony formation at 24 hours of plaques incubation. The presence of oHo abrogated the growth of germs (data not shown).

Thus, herein we show by the first time that an OO, elaborated through the blend of different OOs, exhibits potent microbicidal in vitro and ex vivo activities. These effects are detected on gram+ (S. aureus) and gram- (P. aeruginosa) bacteria, and on fungi (C. albicans and A. niger). Our results do not show the contradictory microbicidal effects reported by other authors that use different OOs [10-13]. The effects of oHo on gram- bacteria are surprising, because it is generally thought oleic and palmitoleic MUFA, both contained in oHo as well as in the SC of the skin, do not show microbicidal actions against P. aeruginosa [9]. Other authors confirm microbicidal effects of OOs on gram+ bacteria are superior to those seen on gram- bacteria [10]. In this sense, when each OO contained in oHo was tested individually, results were so contradictory as showed by other authors using conventional OOs [10-13] (Table 2). On the contrary, only oHo shows significant potent effects against the gram- P. aeruginosa (Table 2).

Table 2  oHo microbicidal effects. Comparison with the microbicidal effects obtained with each individual olive oil (OO) contained in the final oHo composition, and with the obtained with a conventional bivarietal OO (CBOO) and with the results reported in the literature for other conventional OOs.

<table>
<thead>
<tr>
<th>Olive oils</th>
<th>Microbicidal activity against:</th>
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<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td><strong>oHo:</strong></td>
<td></td>
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<tr>
<td>• OO1</td>
<td>-</td>
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<tr>
<td>• OO2</td>
<td>+</td>
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<tr>
<td>• OO3</td>
<td>+</td>
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<tr>
<td>CBOO</td>
<td>-</td>
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OO1, 2, 3: monovarietal organic olive oils contained in oHo; CBOO: conventional bivarietal extra virgin OO bought in the market; COO: data reported in the literature at PubMed for conventional olive oils; COO: conventional OOs. +++: complete elimination of germs; +: mild microbicidal effects; -: negative results; n.d: no data. *It is generally assumed monounsaturated fatty acids kill better gram+ than gram- bacteria.

These results demonstrate the extreme variability (diversity) among OOs, overall in that concern their biological behaviour in humans. Although firstly stated by us [23,24], other authors have recently shown mixtures of polyphenols from different olive growths exhibit more potent antioxidant and antiinfectious in vitro activities than each individual isolated polyphenols [30,31]. These differences are also manifested at the genomic level [32,33], thus definitively proving the existence of differences between the commonly, and erroneously, designated under the generic name of olive oil.

In summary, our in vitro data contribute, among others [3,18,23], to establish definitive differences between oHo and other OOs, and strongly support the consistent results obtained with oral oHo, and its dermal preparations, in the treatment of AD (see below). Furthermore, these in vitro results provide a good rational for the use of oHo in other...
cutaneous infections. In this last case, the anecdotic results described above suggest oHo could be useful in the prevention and/or treatment of catheter infections in hemodialysis patients or in other catheterized patients.

3.2 In vivo studies in patients with recalcitrant atopic dermatitis

At the time of the first evaluation (day +60) after the combined treatment with oral oHo plus dermal oHo-products: (a) itching and sleep disturbances have disappeared in 95% of children and in 80% of adults (b) eczema, lesions of scratching and S. aureus colonization have disappeared in 84% of children and in 80% of adults. Itching and eczema relapses occurred always in patients who stopped oHo intake or changed it by the intake of other conventional or organic OOs, whilst S. aureus relapses appeared more frequently in those patients who withdrawal topic oHo-products or changed them by the application of other conventional OO-based creams bought in the market. Patients who were non-responders (16% of children and 20% of adults) to this initial procedure received the same oHo products associated to TGC (metilprednisolone or fluticasone, at a half of the recommended doses, in finger tips), and all patients responded clinically without skin signs of corticosteroid side-effects.

In Fig. 1 we represent the clinical evolution of an adult male patient with a long history of AD and painful hand eczema colonized by S. aureus, before (up) and after (down) 90 days treatment with oral oHo+G+E+CGO. Patient continues with treatments, and free of illness 18 months later. In Fig. 2 we show a definitive case of severe recalcitrant AD in a young male patient suffering of AD from infancy, who also developed a graft vs host skin disease (GvHSD) as a consequence of an antileukemic semiallogeneic bone-marrow transplant. In spite of strong immunosuppressive treatment (cyclosporine plus OGCs) plus antibiotics, lesions of AD and GvHSD, together with multiple foci of S. aureus infection, were generalized and persistent. The treatment with oral oHo+G+E+CGO provoked the initial clearance of all lesions and S. aureus disappearance in 45 days. This patient continues treatment just now (30 months).

Due to the absence of side-effects, and the well known relapsing nature of the illness, patients or their parents decided to continue treatments just to now. After 2 years follow-up (with oHo treatments) in those 14 children initially recruited with asthma and atopic dermatitis (atopic asthma), the clinical evaluation showed the results exposed at Fig. 3. As shown in the figure asthma prevalence appeared to decrease according as clinical AD began to disappear. Moreover, seasonal asthma attacks changed their intensity from severe-moderate to moderate-mild in all cases at second year. However, as stated in the same figure, these results must be taken with caution due to two main reasons: 1º. The low
number of patients included in the study; 2nd. The fact atopy can spontaneously disappear in more than 60% of patients over the age of 12 years [34], as it is the case of 5 of our cases included in this study. Anyway, it is tempting to speculate that amelioration of AD, through the lipid restoration of the EB provoked by oHo [3], could favourably interfere with the so-called atopic march leading to asthma. As suggested by others, it seems EB recovery could avoid thymic stromal lymphopoietin (TSLP) production, which is thought to be crucial for asthma development in atopy [34] (Fig. 4).

**Fig. 3. Amelioration of atopic dermatitis (AD) by oral plus dermal oHo treatments x 2 years appears to interfere with atopic march. Results on asthma and allergic rhinitis (AR) must be taken with caution. Larger controlled trials are clearly needed.**

From our results it appears oHo mitigates itching and eczema when orally given, and that its topic application is effective in more severe eczematous lesions, overall in those colonized by *S. aureus*. The first clinical manifestation of the oHo efficacy is the quick disappearance of itching, which occurs between 7 to 15 days after oHo intake. Ezcema and *S. aureus* clearances occur between 15 to 90 days, and do not appear to depend on severity and extension of lesions. The mechanisms responsible for these last features deserve of further research. Interestingly, *S. aureus* elimination is achieved even in conditions of extreme iatrogenic immunosuppression (see clinical case at Fig. 2) or in the group of initial non-responders who were rescued with oHo plus TGGs. This feature, together with the absence of TGC-side-effects evoked by the association of oHo, open a new way in the treatment of all forms of AD or other cutaneous allergic/infectious disorders.

To our sense, it seems oHo works “from inside to outside” through the modulation of the immune/inflammatory host response, while providing the FA needed to maintain the integrity of the skin EB. In support of this idea we know oHo is able to increase HDL-c [19], IL-10 and IFN-γ [3] serum levels, and that serum levels of these molecules are low in AD patients [3,6,7]. The mechanisms leading to the joint production of IL-10 and IFN-γ have been postulated [3,18] as due to activation of a particular subset of Treg cells (Tregs IL-10+/IFN-γ+) that play a crucial role in the regulation of immune mechanisms that prevent the appearance of GvHD while maintaining and adequate antitumoral reactivity [35]. After the initial description by some of us [36] of the now called Treg cells, the case shown at Fig 2 seems to be the first clinical case described in the literature about a complete remission of a recalcitrant AD and GvHSD, and disappearance of *S. aureus* colonization, in which Treg cells work in vivo (Fig. 2, down), and under the control of a special olive oil: oHo.

Although there are no data comparing the incidence and prevalence of AD in habitually OO consumers, it seems it is similar to that observed in non OO-consumers. Equally, even if tradition of hundred of years in our area (Jaén, Andalousia, Spain) refers the topic use of OOs for several skin diseases, no scientific data are available in AD. Moreover, many physicians of our region – including Dermatologists- empirically use virgin OOs to moisturize and treat skin infections, but not scientific results have been reported just to now. Only two non-Spanish publications addressed this question by showing no conclusive effects [25,26] in AD patients. There are no publications showing the therapeutic activity of orally given OO in AD.
organic origin of oHo, together with is careful management during oil extractions and conservation, avoid these dangerous possibilities [23].

In brief, oHo restores EB through a physical process of oleo-deposition, from inside to outside and from outside to inside, that avoids water loss and then favours internal water retention. The complete process is what we call oleohydration. In that concern the direct oHo-mediated antiinfectious activities, the in vitro results showed in Tables 1 and 2 clarify notably the situation observed in vivo in our AD patients. Whether these oHo-microbicidal activities are due to the direct actions of polyphenols or FA or both, need to be investigated.

In summary, as it has been recently proposed by us in other pathologic conditions [18], it appears oHo also works in AD by activating Treg IL-10+IFN+ cells through the intermediation of DCs primed by HDL-cholesterol and its main inducer, oHo (Fig. 4). In a first view, it means that only those OOs able to act on HDL-c metabolism, as it is for instance the case of oHo, would be the only ones able to modulate immune responses [18].

Once Tregs become activated, they produce IL-10 that acts as a potent antiinflammatoty endogenous molecule, then mitigating systemic TNF-α and IL-6 production (Fig. 4). These inhibitory effects of oHo on TNF-α and IL-6 have been reported elsewhere by us in harder inflammatory conditions, as they are patients with chronic kidney disease [3,18,23], thus demonstrating the anti-inflammatory potency of oHo. The inhibitory effects of oHo on IL-6 are important for the purposes of this study. In fact TSLP participates in the development of asthma through the induction of IL-6 [41].

Again, once the mitigation of the inflammatory response has been achieved by the oHo intake, manifested by the disappearance of itching, IFN-γ (through polymorphonuclear-mediated killing mechanisms) and FA, and/or polyphenols, exert their microbicidal activities (Fig. 4). Concomitantly, FA from oHo intake or those contained in oHo dermal products begin to repair stratum corneum, and in general EB (Fig. 4). Finally, we hope the complete EB recover could allow to the inhibition of TSLP production or other factors engaged in this complex disease, thus avoiding asthma development (Fig. 4). Further studies about this last mechanism are now in progress.

The absence of side effects, together with the high degree of compliance and acceptance of oHo products, ensure future clinical trials in AD and other skin or systemic diseases with similar immune/inflammatory patterns; i.e., psoriasis.

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