



## Oral administration of *d*-Limonene controls inflammation in rat colitis and displays anti-inflammatory properties as diet supplementation in humans

Patrizia A. d'Alessio<sup>a,\*</sup>, Rita Ostan<sup>b</sup>, Jean-François Bisson<sup>c</sup>, Joerg D. Schulzke<sup>d</sup>,  
Matilde V. Ursini<sup>e</sup>, Marie C. Béné<sup>f</sup>

<sup>a</sup> Biopark Cancer Campus, University Paris Sud-11, 94807 Villejuif, France

<sup>b</sup> Department of Experimental, Diagnostic and Specialty Medicine - University of Bologna, 40126 Bologna, Italy

<sup>c</sup> ETAP Research Centre, 54500 Vandœuvre-lès-Nancy, France

<sup>d</sup> Department of General Medicine and Gastroenterology, Charité, Campus Benjamin Franklin, Berlin, Germany

<sup>e</sup> Institute of Genetics and Biophysics, A. Buzzati-Traverso, CNR, Naples, Italy

<sup>f</sup> Hematology Laboratory, CHU de Nantes, Nantes, France

### ARTICLE INFO

#### Article history:

Received 16 October 2012

Accepted 26 April 2013

#### Keywords:

TNF- $\alpha$

NF- $\kappa$ B

Orange peel extract (OPE)

### ABSTRACT

**Aims:** To further explore the anti-inflammatory properties of *d*-Limonene.

**Main methods:** A rat model was used to compare evolution of TNBS (2,5,6-trinitrobenzene sulfonic acid)-induced colitis after oral feeding with *d*-Limonene compared to ibuprofen. Peripheral levels of TNF- $\alpha$  (Tumor Necrosis Factor alpha) were assessed in all animals. Cell cultures of fibroblasts and enterocytes were used to test the effect of *d*-Limonene respectively on TNF $\alpha$ -induced NF- $\kappa$ B (nuclear factor- $\kappa$ B) translocation and epithelial resistance. Finally, plasmatic inflammatory markers were examined in an observational study of diet supplementation with *d*-Limonene-containing orange peel extract (OPE) in humans.

**Key findings:** Administered per os at a dose of 10 mg/kg p.o., *d*-Limonene induced a significant reduction of intestinal inflammatory scores, comparable to that induced by ibuprofen. Moreover, *d*-Limonene-fed rats had significantly lowered serum concentrations of TNF- $\alpha$  compared to untreated TNBS-colitis rats. The anti-inflammatory effect of *d*-Limonene also involved inhibition of TNF $\alpha$ -induced NF- $\kappa$ B translocation in fibroblast cultures. The application of *d*-Limonene on colonic HT-29/B6 cell monolayers increased epithelial resistance. Finally, inflammatory markers, especially peripheral IL-6, markedly decreased upon OPE supplementation of elderly healthy subjects submitted or not to 56 days of dietary supplementation with OPE.

**Significance:** In conclusion, *d*-Limonene indeed demonstrates significant anti-inflammatory effects both in vivo and in vitro. Protective effects on the epithelial barrier and decreased cytokines are involved, suggesting a beneficial role of *d*-Limonene as diet supplement in reducing inflammation.

© 2013 Elsevier Inc. All rights reserved.

### Introduction

In the course of inflammatory responses, an intricate sequence of events allows activated leukocytes to reach endangered areas. To this avail, among other mechanisms, endothelial adhesion molecules are activated by pro-inflammatory cytokines through complex signaling pathways. ICAM-1 up-regulation elicited by TNF- $\alpha$  has indeed been well documented for hemorrhagic recto-colitis (Vanier, 2005) notably via the NF- $\kappa$ B pathway (Li et al., 2005; Andresen et al., 2005). The adhesion properties maintained by such molecules also involve the cytoskeleton and in particular actin to sustain the integrity of the epithelial barrier of the intestine (Farhadi et al., 2003). Moreover, among the mechanisms contributing to inflammatory lesions in Inflammatory Bowel Disease

(IBD) (Tebelind et al., 2006), oxidative stress (McKenzie et al., 1996; Lih-Brody et al., 1996; Pavlick et al., 2002; McCafferty, 2000), together with the production of other pro-inflammatory cytokines such as IL-6 (Panaccione et al., 2005), plays an important role.

These patho-physiologic pathways therefore provide several targets and markers to test the activity of potentially anti-inflammatory drugs. The latter include a large variety of pharmacological compounds, from industry-designed chemicals to more recently developed biological compounds (Danese, 2011). Another class of therapeutic agents involves active principles derived from plants, which have long been empirically used and more recently introduced in cancer research. For example, together with other cyclic monoterpenes, *d*-Limonene ((4R)-1-methyl-4-isopropenylcyclohex-1-ene) indeed inhibits tumor growth (Crowell, 1999; Crowell et al., 1992). Moreover, it was recently shown to also have potentially beneficial effects in colon cancer (Chidambara Murthy et al., 2012). Orange Peel Extract (OPE) contains large amounts of *d*-Limonene, moreover identified for its specific anti-inflammatory activities by in vitro screening (d'Alessio, 2002, 2004, 2005).

\* Corresponding author at: Biopark Cancer Campus 1, Mail Pr G. Mathé and CHU Paul Brousse 94807 Villejuif, France. Tel.: +33 6 87 47 80 32 (cell).

E-mail address: [endocell@wanadoo.fr](mailto:endocell@wanadoo.fr) (P.A. d'Alessio).

URL: <http://www.aisa-tx.com> (P.A. d'Alessio).

In order to further assess these properties *in vivo*, we used an experimental rat model of TNBS-induced intestinal inflammation treated with *d*-Limonene (Hoffman et al., 2010). Concomitantly, *in vitro* experiments were conducted to better explore the mechanisms of action of *d*-Limonene. We also examined the anti-inflammatory properties of a *d*-Limonene-containing OPE-supplemented diet in healthy elderly humans aged 65–85 years old, enrolled in the RISTOMED study ([www.ristomed.eu](http://www.ristomed.eu)).

## Materials and methods

### Drugs

*d*-Limonene (C<sub>10</sub>H<sub>16</sub>, CAS Number 5989-54-8) of 97% purity was purchased from ETAP (Sigma-Aldrich, Saint-Quentin Fallavier, France) and prepared each day as a fresh solution by dissolving it in sunflower oil (Olvea, Saint Léonard, France) as vehicle. Ibuprofen was stored and reconstituted according to the manufacturer's recommendations. TNBS (2,5,6-trinitrobenzene sulfonic acid) was purchased from Fluka (Buchs, Switzerland) as a 40° alcoholic solution at a concentration of 50 mg/mL and reconstituted according to the manufacturer's recommendations. OPE, containing 95% *d*-Limonene, was purchased from Golgemma (Esperaza, France) and soft gel capsules containing sunflower oil (Olvea) and OPE were prepared for oral administration to humans.

### Animal model

#### Induction of colonic inflammation

Thirty Wistar HsdBrlHan female rats (175–200 g) were obtained from EOPS (Harlan Breeding Centre, Gannat, France).

Colonic inflammation was induced by a single rectal administration of TNBS dissolved in a 40° alcoholic solution at a concentration of 50 mg/mL. Rectal administration of 0.4 mL of the TNBS solution was carried out on anesthetized rats (2 mg/kg Calmivet, Vetoquinol, Lure, France and 50 mg/kg Kétamine, Vibrac, Carros, France) restrained in dorsal *decubitus* position. The rats were maintained in this position for at least 30 min to avoid premature outflow of the TNBS solution.

#### Study of anti-inflammatory effects of *d*-Limonene in rat colon

Thirty animals were allocated to five experimental groups (N = 6 in each group): i) a "control" group without colon inflammation and daily oral treatment with sunflower oil; ii) a "TNBS" group with colon inflammation and treatment with sunflower oil; iii) a "TNBS + *d*-Lim 10" group with colon inflammation and treatment with 10 mg/kg of *d*-Limonene; iv) a "TNBS + *d*-Lim 100" group with colon inflammation and treatment with 100 mg/kg of *d*-Limonene; v) a "TNBS + ibuprofen" group with colon inflammation and treatment with 50 mg/kg of ibuprofen. The latter, although liable to induce digestive damage was used here as an efficient anti-inflammatory drug in this short-term model, and as a positive control of colitis management. For groups iii to v, two log doses (10 and 100 mg/kg) of freshly prepared *d*-Limonene or ibuprofen were administered orally on a daily basis, three days before (pre-treatment) and then five days after the induction of colon inflammation according to classical pharmacological trials.

Blood samples were taken from each rat 24 h after TNBS administration. TNF- $\alpha$  assays were carried out by ELISA (Miltenyi Biotec, Paris, France) on all collected serum samples.

### Animal follow-up

Daily supervision allowed to determine the day of death and to perform immediate autopsies. This supervision also allowed the segregation of weak or moribund animals following principles and guidelines of ASAB (ASAB, 2006), Canadian Council on Animal Care (2003) and UK legislation of *in vivo* aspects in inflammation research (Brain, 2006). The protocol used for this study as all the SOAPS in use

at ETAP was approved by the Director's Office of Veterinary Services and the local Committee on the Ethics of Animal Experiments in Nancy, and authorized by the French government (Governmental authorization no. A 54-547-1).

### Colon macroscopic scoring

The animals were sacrificed under anesthesia 24 h after the last oral treatment with vehicle (sunflower oil), *d*-Limonene or ibuprofen. Colon specimens were sampled, macroscopically scored and stored in preservative (Roti®-Histofix 4%, Carl Roth, Karlsruhe, Germany) to carry out histo-pathological analysis with classical pathological methods after paraffin embedding. A macroscopic score between 0 and 15 was attributed to all samples. Score 0 meant no colon inflammation. Colon inflammation was scored between 1 and 4 when minor, 5 and 9 when important, 10 and 14 when severe and 15 and 19 when necrotic. The lesions observed were characteristic of ulcero-necrotic colitis, up to supuration and necrosis.

### Colon microscopic scoring

Histo-pathological analysis was carried out on 9 sections from each colon specimen (by P. Roignot, director of the Pathology Centre of Dijon, France). A score of 0 corresponded to a normal colonic wall, without any edema, necrosis, epithelial atrophy, inflammatory infiltrate or dysplasia. A score of 1 indicated moderate uni- or bifocal lesions and a score of 2 moderate multifocal lesions. A score of 3 was assigned to acute necrotizing inflammatory lesions with sub-acute inflammatory lesions background and a score of 4 described seriously affected colon specimens with acute necrotic multifocal inflammatory lesions. A score of 5 characterized total necrosis of the colon.

### *In vitro* studies

#### NF- $\kappa$ B activity assessment

Mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Saint Aubin, France) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 1% glutamine at 37° C in 5% CO<sub>2</sub>.

MEF transfection was carried out using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). All transfections included Igkappa-Luc reporter plasmid (1  $\mu$ g), TK-*Renilla* as internal control (20 ng) and a supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.

Twenty-four hours after transfection, cells were stimulated with TNF- $\alpha$  (10 ng/mL), with the addition of OPE in DMSO at 25  $\mu$ M or DMSO (vehicle) alone. Four hours later, the cells were lysed using a Luciferase Passive Lysis Buffer (P/N E1941, Promega, Madison WI). Cell lysates were then harvested and assayed using the Dual Glo luciferase reporter assay system (Promega). Luciferase activity, as a witness of NF- $\kappa$ B translocation, was measured using a multiplate reader (Promega), and values were normalized to *Renilla* luciferase activity. The whole protein extracts were immunoblotted with antibodies to  $\gamma$ -tubulin (Sigma-Aldrich) or  $\beta$ -actin (Santa Cruz Biotechnology Inc. Santa Cruz CA) as housekeeping proteins, as previously reported (Gautheron et al., 2010).

#### Barrier function of colonic cells

Confluent monolayers of the human colon carcinoma cell line HT-29/B6 were grown in 25 cm<sup>2</sup> culture flasks containing RPMI1640 with stable L-glutamine, 10% fetal calf serum, and 1% penicillin/streptomycin. Cells were cultured at 37° C in a humidified 5% CO<sub>2</sub> atmosphere. For electrophysiological measurements, HT-29/B6 cells were seeded on Millicell PCF filters (Millipore, Schwalbach, Germany), and experiments were performed after 7 days, yielding transepithelial resistances (R<sup>t</sup>) of 400–600  $\Omega$ ·cm<sup>2</sup>. The apical compartment was routinely filled with 500  $\mu$ L culture medium supplemented with 10% lipofundin (B. Braun, Melsungen, Germany), while the basolateral compartment bathed in

10 mL of the same medium. Solutions of OPE at grading concentrations of 75, 150, 750 or 1500  $\mu\text{M}$  were added to the medium in both compartments. All cell culture experiments were analyzed after 26 h, assessing cell numbers and density.

#### RISTOMED study

The RISTOMED study enrolled 125 healthy elderly individuals (age range 65–85 years) in three countries. They did not suffer from cancer, hypertension, or major inflammatory diseases such as colitis, arthritis, dermatitis. Participants to this European study were randomized to receive a specifically developed dietary program supplemented or not by various compounds. Here we compared the group without supplementation ( $N = 31$ ) to that ( $N = 30$ ) which also received daily soft gel capsules containing OPE. These probands were analyzed for this report by comparing, on day 1 and day 56, a number of biological parameters: ESR, CRP, WBC, fibrinogen, IL-6 and TNF- $\alpha$  as described elsewhere ([www.ristomed.eu](http://www.ristomed.eu)). A further subdivision into low, medium and high inflammatory status was performed using an inflammation score calculated with baseline serum IL-6 and TNF- $\alpha$  median levels. Low inflammation was characterized by IL-6 and TNF- $\alpha$  both below the median, and high inflammation by IL-6 and TNF- $\alpha$  both above the median. Intermediate inflammation was defined by only one of these markers being above the median.

#### Statistical analyses

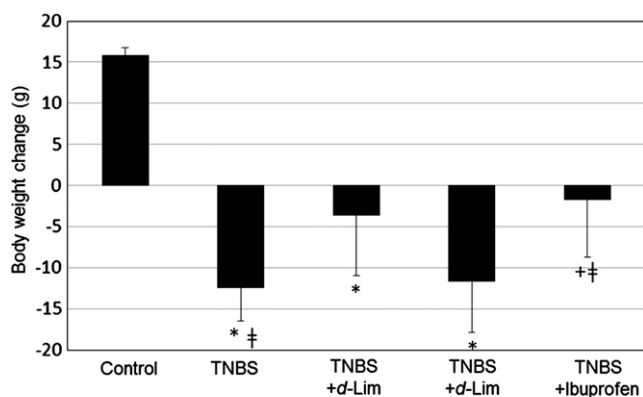
All data are presented as mean  $\pm$  SEM. Statistical analyses were carried out using the StatView 5 statistical package (SAS, Institute Inc., USA) and MedCalc Software (Mariakerke, Belgium). Non-parametric tests were applied: one-way ANOVA with Kruskal–Wallis test followed, when significant, by the Mann–Whitney U-test to compare the different study variables. For all comparisons, differences were considered to be significant at the level of  $p < 0.05$ .

## Results

#### Animal model of TNBS colitis

##### Body weight

By comparison to control rats, animals with TNBS colitis displayed a pronounced reduction in body weight on day 6 ( $p < 0.01$ ; Fig. 1). Ibuprofen resulted in less body weight reduction although the difference between groups ii and v failed to reach statistical significance ( $p = 0.06$ ). Similarly, *d*-Limonene groups iii and iv displayed a smaller decrease in



**Fig. 1.** Influence of TNBS-colitis and treatments on the body weight of rats submitted to induce colonic inflammation. Mean body weight changes obtained from the difference in body weight at day 6 and day 1. Data are given as mean  $\pm$  SEM. \* $p < 0.01$  vs control group, +  $p < 0.05$  vs control group, #  $0.05 < p < 0.10$  TNBS vs TNBS Ibuprofen and TNBS *d*-Lim 10.

body weight than observed in group ii, but again these tendencies failed to reach statistical significance ( $p = 0.09$  each).

#### Colon alterations

Colonic specimens from rats of the 5 experimental groups were obtained 6 days after induction of the TNBS colitis.

TNBS colitis (group ii) induced a significant increase in colon length as a result of the inflammatory process since the animals kept feeding with an obstructed colon over the experimental procedure ( $p < 0.01$  compared to group i control rats). Both ibuprofen or *d*-Limonene at 10 and 100 mg/kg significantly impaired this modification in intestinal length ( $p < 0.01$  each compared to TNBS group ii) since transit was maintained in these animals (data not shown).

Macroscopic analysis of colon specimens (Fig. 2A), using alteration scores, disclosed significant differences between the treatment groups ( $p < 0.001$ ). Upon more precise analysis, it was observed that both ibuprofen (group v, mean score  $4.7 \pm 2.7$ ) and *d*-Limonene 10 (group iii, mean score  $6.5 \pm 1.3$ ) yielded significantly ( $p < 0.05$ ) lower scores than in group ii (TNBS, mean score  $10.7 \pm 2$ ), while this was not observed with *d*-Limonene 100 (group iv, mean score  $10.7 \pm 2.7$ ).

Histological observations of colon samples are exemplified in Fig. 2B. In control group i, normal architecture was observed with an inflammation score at 0. In TNBS-colitis rats (group ii), necrosis with acute unifocal and/or multifocal lesions characterized inflammation scores of 3 and/or 4, with occasional extended necrotic colitis (score 5, mean  $4 \pm 0.3$ ). In ibuprofen or *d*-Limonene 10 groups v and iii, only mild ulcerative colitis with moderate bifocal and/or multifocal lesions (inflammation scores 1 and/or 2, means respectively  $2.5 \pm 1.5$  and  $2.3 \pm 0.6$ ) were registered, significantly different from group ii ( $p < 0.01$ ). Conversely, no improvement was observed in group iv rats (*d*-Limonene 100, mean  $3.7 \pm 0.6$ ).

#### TNF- $\alpha$ serum levels

When compared to control rats, TNF- $\alpha$  serum levels were increased 24 h after TNBS colitis induction (Fig. 3). This effect was significantly controlled by ibuprofen ( $p < 0.05$  mean  $8.4 \pm 0.5$  pg/mL) as well as by *d*-Limonene 10 ( $p < 0.05$  mean  $17 \pm 15$  pg/mL). With the higher dose of *d*-Limonene 100 (group iv mean  $118 \pm 48$  pg/mL) however, TNF $\alpha$  levels remained similar to those in group ii (TNBS alone mean  $96.7 \pm 45$  pg/mL).

#### In vitro studies

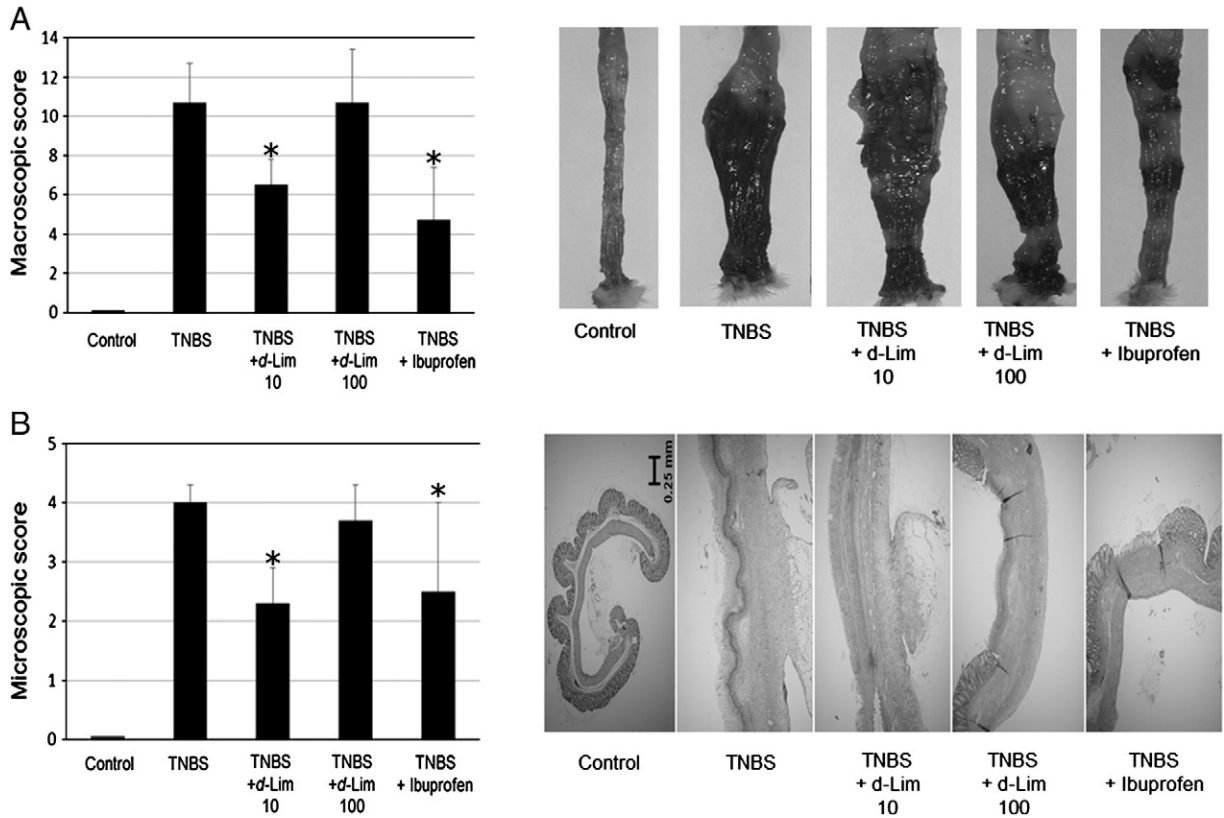
##### *d*-Limonene-induced inhibition of TNF- $\alpha$ by NF- $\kappa$ B

Exposure of cultured fibroblasts to TNF- $\alpha$  was used to test their sensitivity, as measured by NF- $\kappa$ B induction. TNF- $\alpha$ , with or without OPE, did not alter cell viability as shown by the consistent levels of  $\gamma$ -tubulin and  $\beta$ -actin, exemplified in Fig. 4, showing no reduction in the number of cells pelleted prior to Western blotting. DMSO alone (vehicle) did not induce any translocation of NF- $\kappa$ B while this was clearly demonstrated with positive controls such as cannabis-based drugs or isopentenyladenosine (data not shown). Similarly, TNF- $\alpha$  induced a significant translocation of NF- $\kappa$ B, that was decreased over four-fold in the presence of OPE.

##### Epithelial barrier function improvement by *d*-Limonene

In order to explore whether or not *d*-Limonene could influence the degree of intestinal inflammation in response to TNBS by affecting the epithelial barrier function, colonic epithelial cells (HT-29/B6) were used as a model as described in the Materials and methods section.

Trans epithelial electrical resistance was measured as  $\Omega \cdot \text{cm}^2$ . As shown in Table 1, *d*-Limonene induced a dose-dependent increase of these values, statistically significant from 750  $\mu\text{M}$  on ( $p < 0.001$ ), indicative of a positive effect on the epithelial barrier function.

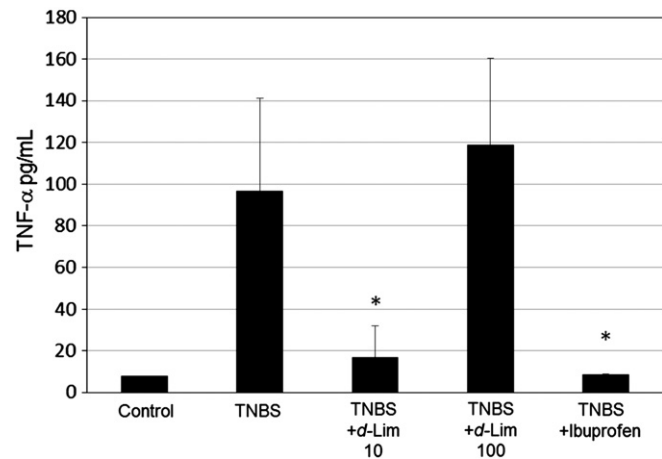


**Fig. 2.** *d*-Limonene effect on colon inflammation scores. Macroscopic (A) and microscopic (B) inflammation scores of colon samples, with corresponding macroscopic and microscopic representative snapshots. In control animals, scores are at 0, while rats with TNBS-induced colitis or fed 100 mg/kg *d*-Limonene have significantly elevated scores indicating severe inflammation. Both ibuprofen- and 10 mg/kg *d*-Limonene-fed animals display significantly less inflammation. \**p* < 0.01 vs TNBS.

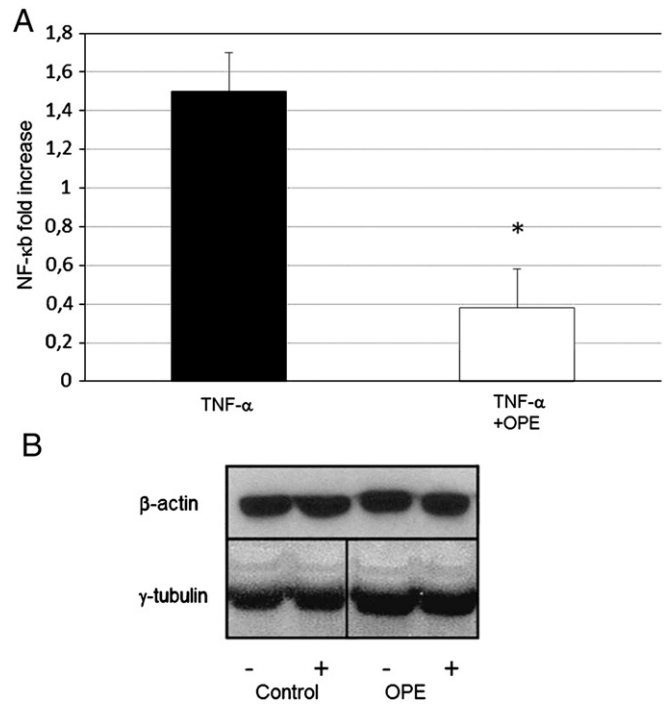
*Human studies*

*Comparison between controls and supplemented probands*

A number of biological variables were compared between day 1 and day 56 in two groups of subjects of the RISTOMED trial. As shown in Table 2, both groups were comparable at baseline, except for higher levels of TNF- $\alpha$  in the group receiving diet alone compared to the group supplemented with OPE. This finding was only available at the end of the trial when biological analyses were performed, since diet allotment was not based on inflammatory parameters. At the end of



**Fig. 3.** *d*-Limonene effects on TNF- $\alpha$  levels. The elevated mean serum TNF- $\alpha$  levels from rats with TNBS induced colitis compared to controls are significantly decreased when animals are treated with ibuprofen or 10 mg/kg *d*-Limonene but not 100 mg/kg *d*-Limonene. \**p* < 0.01 vs TNBS.



**Fig. 4.** *d*-Limonene protection against TNF- $\alpha$  involves NF- $\kappa$ B. A. Ratio of Luciferase activity, witness of NF- $\kappa$ B translocation normalized to *Renilla* luciferase in lysates from I $\kappa$ B transfected cells activated by TNF- $\alpha$  alone (black bar) or with the addition of *d*-Limonene-containing OPE (white bar) showing approximately four fold less activation in *d*-Limonene treated cells *p* < 0.01. B. Protein extracts from I $\kappa$ B transfected fibroblasts immunoblotted for  $\gamma$ -tubulin and  $\beta$ -actin, confirming cell integrity in both conditions.



**Table 1**  
OPE containing 95% of *d*-Limonene: effect on epithelial barrier function.

	Control	OPE			
		75 $\mu$ M	150 $\mu$ M	750 $\mu$ M	1500 $\mu$ M
R <sup>t</sup>	107 $\pm$ 3%	106 $\pm$ 4%	112 $\pm$ 3%	132 $\pm$ 3%	153 $\pm$ 4%
n	9	6	6	6	6
p		n.s.	n.s.	<0.001	<0.001

OPE containing 95% of *d*-Limonene prepared in sunflower oil was applied to colonic HT-29/B6 epithelial cell monolayers in 4 different concentrations, namely 75, 150, 750 and 1500  $\mu$ M, respectively. Electrical resistance was measured (in  $\Omega \cdot \text{cm}^2$ ) and is presented in percent of initial resistance R<sup>t</sup>. Mean  $\pm$  SEM is given for each group,  $p < 0.05$  was considered significant.

the trial, all values were statistically similar to baseline, except for the erythrocyte sedimentation rate (ESR), significantly lowered in both groups. It may also be noted that, although not significant, there was a clear decrease in IL-6 levels in the group supplemented with OPE while this parameter increased in the group receiving diet only (Table 2). The higher baseline levels of TNF- $\alpha$  in the group with diet alone remained high, and the low levels of the supplemented group also were unchanged.

#### Influence of the baseline inflammation score

Based on the observations mentioned above, suggesting an effect of diet and/or OPE on inflammation, we further subdivided the probands according to their baseline inflammatory status as described in Materials and methods. As shown in Fig. 5 no significant variation of IL-6 levels was seen in the group receiving diet only, whether they were classified as normal/intermediate or high inflammatory status. The same was noted for normal/intermediate subjects in the group supplemented with OPE, while supplemented individuals with an initially high inflammatory status had a significant decrease of IL-6 levels at day 56 ( $p = 0.02$ ).

## Discussion

Steroids, mesalazine and more recently anti-TNF $\alpha$  antibodies have been developed to treat inflammatory diseases including IBD (van der Woude and Hommes, 2007). In this way, clinical episodes can usually be managed, despite numerous side effects.

Consideration has also been given to oxidative stress targeting enterocytes as a promoter of such affections, especially with regard to inflammatory diseases. In this line, compounds derived from natural substances, mostly plants, have acquired a new status of interesting pharmacological candidates for the development of novel drugs preventing, maintaining and/or curing many body disabilities (Fiorino et al., 2010; Ardizzone and Bianchi Porro, 2005; Bosani et al., 2009). Intestinal inflammatory diseases are strategically interesting to investigate the efficacy of new anti-inflammatory molecules (Danese, 2011).

Along these lines, the studies reported here clearly demonstrate beneficial anti-inflammatory effects of orally administered *d*-Limonene in a rat model of TNBS colitis, as well as in a human trial, at the same low doses. In the animal model, these effects were remarkably similar to those of the classically used ibuprofen in similar settings, especially

lower inflammation scores, as well as decreased serum TNF- $\alpha$  levels. In the human trial, similarly, subjects with high inflammatory scores benefited from OPE supplementation through a significant decrease of peripheral IL-6 levels. These data nonetheless suggest that *d*-Limonene acts by suppressing the pro-inflammatory activity of cytokines. Indeed, in a model of cultured fibroblasts treated with OPE containing *d*-Limonene, a lessened responsiveness to TNF $\alpha$ -induced NF- $\kappa$ B translocation was evidenced. This supports the anti-inflammatory effect of this compound as an active process implying well-defined cell-signaling pathways. It is indeed furthermore possible that a decreased activation of NF- $\kappa$ B could be responsible for the lowered production of TNF- $\alpha$  observed in vivo.

In the model of cultured enterocytes, application of *d*-Limonene contained in OPE significantly induced increased resistivity, in a dose-dependent fashion. This is actually in keeping with the lesser efficacy of *d*-Limonene fed in vivo at higher doses in rats. Indeed, because the compound is given orally, it may first increase the strength of the epithelial barrier, perhaps through cytoskeleton modifications. As a “side-effect”, it is likely that this could have then impaired an efficient penetration of the compound in the inflamed colon. This would therefore result in a lessened systemic anti-inflammatory effect. Conversely, a better balance between strengthening of the intestinal epithelial barrier and bioavailability of the fed compound would be obtained at lower doses (Miller et al., 2011).

Hung et al. (2008) used a similar in vitro model as ours to show that lycopene is able to inhibit TNF $\alpha$ -induced ICAM-1 mRNA and protein expression. This phenomenon could also be involved here and explain the overall beneficial effects of *d*-Limonene, either pure or contained in OPE. The use of this model by several authors (Hung et al., 2008; Bisson et al., 2008) provides evidence that the in vitro assay that we used was of relevant significance. We have previously used *d*-Limonene in an in vitro model of HUVEC challenged with TNF- $\alpha$  and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as well as mechanical induced lesions demonstrating similar protective properties (d'Alessio, 2005, 2012). Moreover, the effect of another monoterpene, geraniol, inhibiting TNF $\alpha$ -induced leukocyte adhesion has long been known (Yamawaki, 1962). The protective influence of *d*-Limonene on the epithelial barrier could also indeed be due to protein changes in tight junctions (Barnes, 2004). Similar effects have been observed from another food component, quercetin, taken up by enterocytes and affecting claudin-4 expression (Amasheh et al., 2008). Moreover, NF- $\kappa$ B dependent signaling, involved both in colorectal cancer (Sakamoto and Maeda, 2010) and in chronic colitis (Hassan et al., 2010), was here clearly addressed by *d*-Limonene at the low dose of 25  $\mu$ M.

## Conclusion

The pronounced anti-inflammatory effects of *d*-Limonene in an animal model of colitis and as OPE dietary supplement in humans suggest that this compound could be worthwhile in multimodal anti-inflammatory therapy concepts (d'Alessio et al., 2012). In vitro experiments on cell cultures provided further insight in the subcellular mechanisms involved.

**Table 2**  
Comparison of biological parameters on days 1 and 56 of dietary supplementation alone or with the supplementation of soft gel capsules containing OPE.

	Diet			Diet $\pm$ OPE		
	T1	T56	p	T1	T56	p
ESR (mm/1 h)	24.9 (3.4)	18.9 (3.1)	$p = 0.03$	21.4 (2.5)	15.1 (2.7)	$p = 0.05$
WBC (G/L)	6.07 (0.24)	5.89 (0.25)	ns	5.92 (0.21)	5.68 (0.23)	ns
CRP (g/L)	3.6 (0.6)	3.8 (4.5)	ns	3.6 (0.9)	2.9 (0.8)	ns
Fibrinogen (g/dL)	37.7 (1.7)	38.2 (1.3)	ns	37.9 (1.9)	34.9 (1.8)	ns
IL-6 (pg/mL)	29.9 (9.1)	34.7 (10.9)	ns	30.1 (12.3)	19.0 (5.4)	ns
TNF- $\alpha$ (pg/mL)	60.2 (27.7)	64.6 (28.0)	ns	8.1 (5.1)	8.4 (3.7)	ns

ESR: erythrocyte sedimentation rate; WBC: white blood cells; CRP: C-reactive protein.

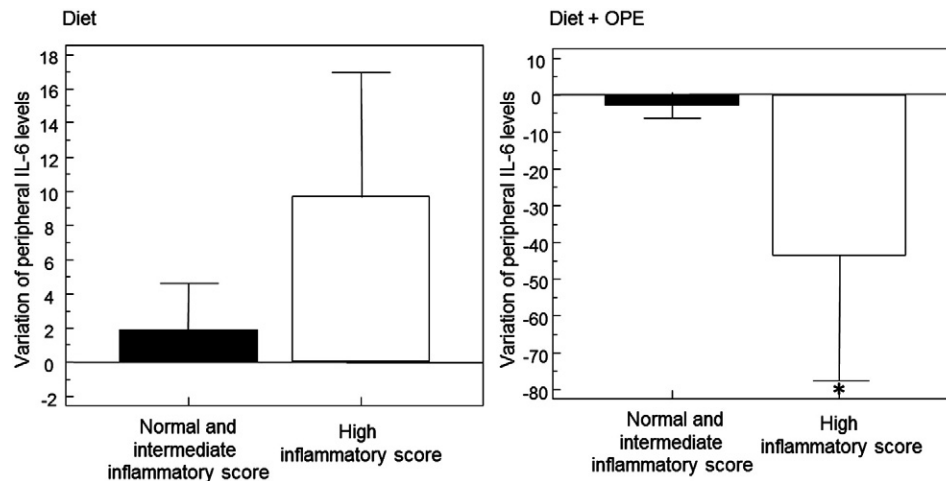


Fig. 5. OPE supplementation. Serum IL-6 levels significantly decrease in subjects with a highly inflammatory profile at baseline following OPE supplementation. \* $p < 0.02$ .

#### Conflict of interest statement

No competing interests to declare.

#### Acknowledgments

This study was funded by a grant from the French Ministry of Research to Pr. Patrizia d'Alessio: "Award for Innovative Research" (2005) and by the European grant Capacities no 222230 "Ristomed" (2009).

We would like to thank Dr. Patrick Roignot from the Pathology Centre in Dijon (France) for performing the histopathological analysis of the colon specimens.

We would like to thank M. William Sibran, student of the Sup'Biotech Paris School, for performing the manuscript's layout.

#### References

- Amasheh M, Schlichter S, Amasheh S, Mankertz J, Zeitz M, Fromm M, et al. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. *J Nutr* 2008;138:1067–73.
- Andresen L, Jorgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J. Activation of nuclear factor  $\kappa$ B in colonic mucosa from patients with collagenous and ulcerative colitis. *Gut* 2005;54:503–9.
- Ardizzone S, Bianchi Porro G. Biologic therapy for inflammatory bowel disease. *Drugs* 2005;65:2253–86.
- ASAB Ethical Committee. Guidelines for the treatment of animals in behavioral research and teaching. *Anim Behav* 2006;71:245–53.
- Barnes PJ. COPD: is the light at the end of the tunnel? *Curr Opin Pharmacol* 2004;4:263–72.
- Bisson JF, Menut C, d'Alessio P. Anti-inflammatory senescence activates 5203-L molecule to promote healthy aging and prolongation of lifespan. *Rejuvenation Res* 2008;11:399–407.
- Bosani M, Ardizzone S, Bianchi Porro G. Biologic targeting in the treatment of inflammatory bowel diseases. *Biologics* 2009;3:77–97.
- Brain S. UK legislation of in vivo aspects in inflammation research. In: Stevenson CS, Marshall LA, Morgan DWCA, editors. *In vivo models of inflammation*; 2006.
- Canadian Council on Animal Care. *Guide to the care and use of experimental animals*. In: Olfert DVM ED, Cross DVM BM, McWilliam, editors; 2003.
- Chidambara Murthy KN, Jayaprakasha GK, Patil BS. D-Limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cells. *Life Sci* 2012;91:429–39.
- Crowell PL. Prevention and therapy of cancer by dietary monoterpenes. *J Nutr* 1999;129:775–85.
- Crowell PL, Lin S, Vedejs E, Gould MN. Identification of metabolites of the anti-tumor agent *d*-Limonene capable of inhibiting protein isoprenylation and cell growth. *Cancer Chemother Pharmacol* 1992;31:205–12.
- d'Alessio P, Bennaceur A, Ostan R, Franceschi C. New targets for the identification of an anti-inflammatory anti-senescence activity. In: Nagata Tetsuji, editor. 978-953-51-0144-4; 2012.

- d'Alessio P. Endothelium as pharmacological target. *Curr Opin Investig Drugs* 2002;2:1720–4.
- d'Alessio P. Aging and the endothelium. *J Exp Gerontol* 2004;39:165–71.
- d'Alessio P. Composition for prevention or treatment of cell degeneration using a molecule able to maintain the reversibility of expression of adhesion molecules and the polymerization of actin fibers; 2005 (PCT; WO 2005/105074).
- d'Alessio P. Use of a monoterpene to increase tissue repair (EP Q7291145.6); 2012.
- Danese S. New therapies for inflammatory bowel disease: from the bench to the bedside. *Gut* 2011. <http://dx.doi.org/10.1136/gutjnl-2011-300904>.
- Farhadi A, Banan A, Keshavarzian A. Role of cytoskeletal structure in modulation of intestinal permeability. *Arch Iran Med* 2003;6:49–53.
- Fiorino G, Rovida S, Correale C, Danese S. Emerging biologics in the treatment of inflammatory bowel disease: what is around the corner? *Curr Drug Targets* 2010;11:249–60.
- Gautheron J, Pescatore A, Fusco F, Esposito E, Yamaoka S, Agou F, et al. Identification of a new NEMO/TRAF6 interface affected in incontinencia pigmenti pathology. *Hum Mol Genet* 2010;19:3138–49.
- Hassan A, Ibrahim A, Mbodji K, Coëffier M, Ziegler F, Bounoure F, et al. An  $\alpha$ -linolenic acid-rich formula reduced oxidative stress and inflammation by regulating NF- $\kappa$ B in rats with TNBS-induced colitis. *J Nutr* 2010;140:1714–21.
- Hoffman JC, Pawlowski NN, Kühl AA, Höhne W, Zeitz M. Animal models of inflammatory bowel disease: an overview. *Pathobiology* 2010;70:121–30.
- Hung CF, Huang TF, Chen BH, Shieh JM, Wu PH, Wu WB. Lycopene inhibits TNF $\alpha$ -induced endothelial ICAM-1 expression and monocyte-endothelial adhesion. *Eur J Pharmacol* 2008;586:275–82.
- Li JH, Yu JP, Yu HG, Xu XM, Yu LL, Liu SQ. Expression and significance of nuclear factor  $\kappa$ B p65 in colon tissues of rats with TNBS-induced colitis. *World J Gastroenterol* 2005;11:1759–63.
- Lih-Brody L, Powell S-R, Collier KP, Reddy GM, Cerchia R, Kahn E, et al. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 1996;41:2078–86.
- McCafferty DM. Peroxynitrite and inflammatory bowel disease. *Gut* 2000;46:436–9.
- McKenzie SJ, Baker MS, Buffinton GD, Doe WF. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 1996;98:136–41.
- Miller JA, Thompson PA, Hakim IA, Chow HH, Thomson CA. *d*-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment. *Oncol Rev* 2011;66:31–42.
- Panaccione R, Ferraz JG, Beck P. Advances in medical therapy of inflammatory bowel disease. *Curr Opin Pharmacol* 2005;5:566–72.
- Pavlick KP, Laroux FS, Fuseler J, Wolf RE, Gray L, Hoffman J, et al. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* 2002;33:311–22.
- Ristomed, acronym of the European Capacities study n° 222230. [www.ristomed.eu](http://www.ristomed.eu).
- Sakamoto K, Maeda S. Targeting NF- $\kappa$ B for colorectal cancer. *Expert Opin Ther Targets* 2010;14:593–601.
- Tebelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acid acetate and propionate: a study with relevance to inflammatory bowel disease. *World J Gastroenterol* 2006;13:2826–32.
- van der Woude CJ, Hommes DW. Are we ready for top-down therapy for inflammatory bowel diseases: pro. *Expert Rev Gastroenterol Hepatol* 2007;1:243–8.
- Vanier B. Intercellular adhesion molecule-1 (ICAM-1) in ulcerative colitis. *Dig Dis Sci* 2005;54:313–27.
- Yamawaki T. Pharmacological effects of geraniol. *Nippon Yakurigaku Zasshi* 1962;58:394–400.