

## Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat

C. Puel<sup>1</sup>, A. Quintin<sup>1</sup>, A. Agalias<sup>3</sup>, J. Mathey<sup>1</sup>, C. Obled<sup>2</sup>, A. Mazur<sup>1</sup>, M. J. Davicco<sup>1</sup>, P. Lebecque<sup>1</sup>, A. L. Skaltsounis<sup>3</sup> and V. Coxam<sup>1\*</sup>

<sup>1</sup>Unité des Maladies Métaboliques et Micronutriments, INRA Theix, 63122 Saint Genès-Champanelle, France

<sup>2</sup>Unité Nutrition et Métabolisme Protéique, INRA Theix, 63122 Saint Genès-Champanelle, France

<sup>3</sup>Division of Pharmacognosy, University of Athens, Panepistimioupolis, Zografou, 15 771 Athens, Greece

(Received 20 October 2003 – Revised 18 March 2004 – Accepted 29 March 2003)

The present study was designed to evaluate the effect of olive oil and its main polyphenol (oleuropein) in ovariectomised rats with or without inflammation. Rats (6 months old) were ovariectomised or sham-operated as control. Ovariectomised rats were separated into three groups receiving different diets for 3 months: a control diet with 25 g peanut oil and 25 g rapeseed oil/kg (OVX), the control diet with 50 g olive oil/kg or the control diet with 0.15 g oleuropein/kg. The sham-operated group was given the same control diet as OVX. Inflammation was induced 3 weeks before the end of the experiment by subcutaneous injections of talc (magnesium silicate) in one-half of each group. The success of ovariectomy was verified at necropsy by the atrophy of uterine horns. Inflammation, oleuropein or olive oil intakes did not have any uterotrophic activity, as they had had no effect on uterus weight. The plasma concentration of  $\alpha$ -1-acid glycoprotein (an indicator of inflammation) was increased in OVX rats with inflammation. With regard to bone variables, osteopenia in OVX was exacerbated by inflammation, as shown by a decrease in metaphyseal and total femoral mineral density. Both oleuropein and olive oil prevented this bone loss in OVX rats with inflammation. At necropsy, oleuropein and olive oil consumption had had no effect on plasma osteocalcin concentrations (marker of bone formation) or on urinary deoxypyridinoline excretion (marker of bone resorption). In conclusion, oleuropein and olive-oil feeding can prevent inflammation-induced osteopenia in OVX rats.

### Olive oil: Oleuropein: Bone loss: Ovariectomised rat

With the continuing demographic shift in population toward an older society, all industrialised countries face a growing prevalence of chronic age-related conditions. Development of degenerative diseases such as osteoporosis, characterised by a low bone-mass and microarchitectural deterioration (Consensus Development Statement, 1997) and a steep age-related incidence, has a major impact on the health of elderly populations in the western world. Hip fractures are associated with considerable morbidity and even lead to an overall mortality of 15–30% (Browner *et al.* 1996). Vertebral fractures also lead to reduced survival, probably due to clustering of co-morbidity (Cooper *et al.* 1993).

It has been suggested that the decline in sex hormones at the time of menopause heightens the risk of osteoporosis (Ershler *et al.* 1997). In fact, ageing and oestrogen deficiency induce inflammatory and oxidant conditions that are involved in the development of this chronic disease (Das, 2002). Indeed, plasma concentrations of proinflammatory cytokines (IL-1, IL-6 and TNF- $\alpha$  of macrophagic or monocytic origin) increase in postmenopausal women

(Zeng *et al.* 1997). Furthermore, the *in vitro* production of cytokines by peripheral blood monocytes is greater in postmenopausal women (Cohen-Solal *et al.* 1993). In addition, Pacifi *et al.* (1991) found that in women oophorectomy-induced menopause caused an increase in cytokine production involved in bone resorption. This effect was prevented by oestrogen-replacement therapy. A high cytokine secretion may give rise to osteoclastic differentiation through the prostaglandin pathway (Horowitz & Raisz, 1999). Moreover, those cytokines are also involved in the release of free radicals (Das, 1991), which, in turn, may have a role in the development of osteoporosis by inhibiting osteoblastic recruitment and the activity of mature cells (Mody *et al.* 2001), and by increasing osteoclastic resorption (Watkins *et al.* 2001).

Research in nutrition in the past 30 years has lent support to the hypothesis that by modulating specific target functions in the body, diet can help to achieve optimal health by reducing the risk of disease (Diplock *et al.* 1999). Specifically, it has been recognised that the human diet contains, in addition to essential macronutrients, a complex

**Abbreviations:** BMD, bone mineral density, DPD deoxypyridinoline; HO, olive oil-fed group; OL, oleuropein-fed group; OVX, ovariectomised control group; SH, sham-operated control group.

\* **Corresponding author:** Mrs Véronique Coxam, fax + 33 473 62 4638, email coxam@clermont.inra.fr

array of naturally occurring bioactive molecules, including the phytochemicals endowed with antioxidant and anti-inflammatory properties. Antioxidant nutrients may enhance bone formation and reduce the production of free radicals that contribute to bone resorption (Keys *et al.* 1986). Thus, there is an increasing rationale for focus on the Mediterranean diet, known for its antioxidant nutrients (within Europe the lowest incidence of osteoporosis has been reported in the Mediterranean area (Kanis, 1993; European Commission, 1998; Table 1)). This diet is mainly characterised by its high content of olive oil, which contains a series of phenolic minor compounds such as hydroxytyrosol and oleuropein, known to scavenge superoxide radicals and inhibit neutrophils respiratory burst (Visioli *et al.* 1998).

Consequently, we have examined the consumption of olive oil and its main polyphenol (oleuropein) as a possible way of preventing bone loss in the ovariectomised rat with or without chronic inflammation.

## Materials and methods

### Animals and treatments

The present study was conducted in accordance with current legislation on animal experiments in France. Female Wistar rats (*n* 80, 6 months old) were purchased from a laboratory colony (National Institute for Agricultural Research (INRA), Clermont-Ferrand/Theix, France) and housed individually at 21°C with a 12 h light–dark cycle. They were sham-operated (*n* 20) or surgically ovariectomised (*n* 60) under anaesthesia using chloral hydrate (Fluka Chemie AG, Buchs, Switzerland; 80 g/l saline solution (9 g NaCl/l); 4 ml/kg body weight, intraperitoneally). After an adaptation period of 7 d with a semipurified standard diet (Table 2; INRA, Jouy en Josas, France) mixed with 25 g peanut oil (Lesieur Alimentaire, Asnieres sur Seine, France) and 25 g rapeseed oil (Auchan Alimentaire, Lyon, France)/kg, the rats were randomly assigned to four groups: (1) untreated (SH: sham-operated controls receiving the control diet); (2) untreated (OVX: ovariectomised controls also given the control diet); (3) ovariectomised rats given the control diet supplemented with 0.15 g oleuropein/kg (OL); (4) ovariectomised rats animals given the semipurified diet mixed with 50 g extra virgin olive oil (Puget, Rueil Malmaison, France)/kg (HO). Oils were stored sheltered from the light. Diets were prepared every week and stored at 4°C. During the 80 d experimental

period, the quantity of diet given to each rat per d was adjusted to the mean level consumed by SH animals the previous day, in order to prevent ovariectomy-induced hyperphagia. Three weeks before the end of the experiment (day 59), inflammation was induced in ten animals of each group by four separate subcutaneous injections of talc (magnesium silicate; 3.2 g per animal) in sterile saline (9 g NaCl/l). On days 41 and 80, blood samples were taken and the 24 h urine samples collected (started at 09.00 hours) to measure urinary excretion of deoxypyridinoline (DPD), a marker of bone resorption, and osteocalcin, a marker of bone formation. At necropsy (day 80) blood samples were collected to assess osteocalcin,  $\alpha$ -1-acid glycoprotein and the ferric-reducing ability of plasma. The spleen, heart and uterine horns were removed and weighed. The hearts were washed in ice-cold saline (9 g/l NaCl), placed in liquid N<sub>2</sub> and stored at –80°C until the susceptibility to peroxidation was carried out. Left and right femurs were cleaned from adjacent tissues and collected for mechanical testing and bone mineral density (BMD) measurements respectively.

### Oleuropein preparation

Leaves of *Olea europea* var. *koroneiki* were collected in Crete, dried at room temperature and pulverised. The powdered leaves (7 kg) were then completely extracted with dichloromethane, methanol and water. The methanol extract was evaporated to dryness and a 130 g sample was subjected to chromatographic fractionation with a CH<sub>2</sub>Cl<sub>2</sub>–MeOH solvent system of increasing polarity, to give twenty fractions. Fractions 8–9 were eluted with CH<sub>2</sub>Cl<sub>2</sub>–MeOH (90:10, v/v) and yielded 50 g oleuropein. The purity of oleuropein was determined by HPLC and NMR spectral analysis as described by Tsarbopoulos *et al.* (2003).

### Bone mineral density

BMD was assessed by dual-energy X-ray absorptiometry with a Hologic QDR-4500 A X-ray bone densitometer (Hologic, Massy, France). The total right femur BMD, as well as the BMD of two sub-regions, one corresponding to the distal femur metaphyseal zone, rich in cancellous bone, and the other to the diaphyseal area, rich in cortical bone, were determined. The intra- and inter-assay CV for femoral assays were 0.22 and 0.24 % respectively.

**Table 1.** Age-specific prevalence values for vertebral fractures in European Union member states\* (Mean values per 10 000 women)

	Age group (years)							
	50–54	55–59	60–64	65–69	70–74	75–79	80–84	≥85
Denmark	1220	1630	2140	2740	3450	4280	5230	7560
Finland	1220	1630	2140	2740	3450	4280	5230	7560
Italy	743	996	1300	1670	2110	2610	3190	4610
Portugal	846	1130	1490	1900	2400	2970	3630	5250
Spain	846	1130	1490	1900	2400	2970	3630	5250

\* From the European Commission (1998).

**Table 2.** Composition of the soyabean-protein-free and fibre-free powdered semipurified diet consumed by female Wistar rats\*

Ingredient	g/kg
Casein	200
Maize starch	660
Cellulose	50
Vitamin mixture (vitamin E-deficient)†	10
Mineral mixture (Ca and P deficient)‡	25
DL-Methionine	3
Choline bitartrate	2

\* Supplied by INRA (Jouy en Josas, France); casein (Union des caséinerie, Surgères, France), Maize starch (Cerestar, Saint-Maur, France), cellulose (Durieux, Mame la Vallée, France), vitamin mixture (Roche, Neuilly sur Seine, France), mineral mixture (Prolabo, Fontenay sous bois, France), DL-methionine and choline bitartrate (Jerafrance, Jeufosse, France).

† With DL- $\alpha$ -tocopheryl acetate 1.1 mg/kg.

‡ With Ca 4.2 g/kg; P 4.01 g/kg; Mg 1.25 g/kg.

### Femoral mechanical testing

After collection of the left femurs in NaCl (9 g/l), the length and the mean diameter of the diaphysis were measured with a precision caliper (Mitutoyo, Telford, Shropshire, UK). Femoral failure load was then determined, using a three-point bending test (Turner & Burr, 1993), with a Universal Testing Machine (Instron 4501, Instron, Canton, MA, USA). Each bone was secured on the two lower supports (diameter 4 mm, length 20 mm) on the anvil of the testing machine. The upper roller diameter was 6 mm and the cross-head speed was 0.5 mm/min. The span of the specimen that was loaded was 20 mm to ensure that 85–90% of the bone flexure was caused by bending.

### Marker of bone formation

Osteocalcin in plasma was measured by RIA using rat  $^{125}$ I-labelled osteocalcin, goat anti-rat osteocalcin antibody and donkey anti-goat second antibody (Biochemical Technologies, Stoughton, MA, USA). The sensitivity was 0.01 nmol/l. The intra- and inter-assay CV were 6.8 and 8.9% respectively.

### Marker of bone resorption

Free DPD in urine was determined by competitive RIA, using rat monoclonal anti-DPD antibody coated to the inner surface of a polystyrene tube and  $^{125}$ I-labelled DPD (Pyrilinks-D RIA kit; Metra Biosystems, Mountain View, CA, USA). The sensitivity was 2 nmol/l. The intra- and inter-assay CV were 4 and 6% respectively. Results were expressed as nmol DPD/mmol creatinine (Robbins, 1994). The creatinine assay (Kit Bio MERIEUX SA, Marcy-l'Etoile, France) is based on a modified Jaffe's method, in which picric acid forms a coloured solution in the presence of creatinine (Cook, 1975).

### Marker of inflammation

Plasma  $\alpha$ -1-acid glycoprotein, an acute-phase protein, was determined by single radial immunodiffusion using rabbit

anti-rat  $\alpha$ -1-acid glycoprotein antibodies (Breuillé *et al.* 1998). The minimum detection level was 4  $\mu$ g/ml and the precision 1.5  $\mu$ g/ml.

### Markers of oxidative stress

The susceptibility of the heart to peroxidation was determined in tissue homogenates by measuring the thiobarbituric acid-reactive substances after a lipid peroxidation was induced with 2  $\mu$ M-FeSO<sub>4</sub>–50  $\mu$ M-ascorbate (Sigma, St Quentin Fallavier, France) for 30 min in a water-bath at 37°C, using a standard of 1,1,3,3-tetraethoxypropane as previously described (Ohkawa *et al.* 1979). The CV were 4.32 and 2.10% for the basic and stimulated methods respectively.

Ferric-reducing potential was determined using the method of Benzie & Strain (1996); this method evaluates the reduction of ferric iron to the ferrous form in the presence of antioxidant components. The colorimetric measurement was performed at 593 nm and the reaction was monitored for up to 8 min on 25  $\mu$ l samples. Results were calculated from a standard scale of FeSO<sub>4</sub>. Within- and between-run CV were <1.0 and <3.0% respectively at 100–1000  $\mu$ mol/l.

### Statistical methods

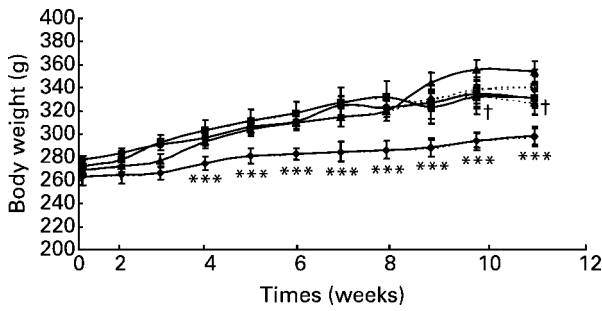
Results are expressed as mean values with their standard errors. A parametric one-way ANOVA was performed to test for any difference among groups. If the result was found to be significant ( $P < 0.05$ ), the Student–Newman–Keuls multiple comparison test was then used to determine specific differences between mean values. If a parametric ANOVA was not feasible (when there were significant differences between the SD groups by Kolmogorov–Smirnov test), a Kruskal–Wallis test followed by the Mann–Whitney Wilcoxon U-test was carried out to compare differences between groups.

## Results

### Body and uterine weight

In each group the body weight increased between day 0 and 80. However, although consuming similar amounts of food, because of pair-feeding, body weight was greater in OVX than in SH by the fourth week of the experiment and this trend was still evident on day 80 (OVX 294 (SEM 6), SH 275 (SEM 6) g;  $P < 0.001$ ). Oleuropein or olive-oil consumption did not prevent this weight gain after ovariectomy. Inflammation induced a decrease in body weight in OVX (OVX with inflammation 333 (SEM 9), OVX without inflammation 355 (SEM 9) g;  $P < 0.05$ ) (Fig. 1).

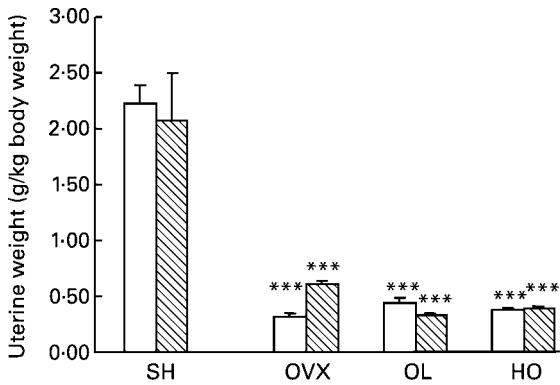
The success of ovariectomy was confirmed by uterine atrophy in OVX rats (OVX 0.32 (SEM 0.03), SH 2.21 (SEM 0.17) g uterus/kg body weight;  $P < 0.0001$ ). Neither inflammation (SH with inflammation 2.06 (SEM 0.43), OVX with inflammation 0.60 (SEM 0.03) g uterus/kg body weight), nor oleuropein nor olive oil intake had any further significant effect on this variable (OL 0.44 (SEM 0.04), HO 0.37 (SEM 0.02) g uterus/kg body weight; Fig. 2).



**Fig. 1.** Body weight in sham-operated (SH,  $\blacklozenge$ ), ovariectomised (OVX,  $\blacktriangle$ ), ovariectomised + oleuropein ( $\blacksquare$ ) and ovariectomised + olive oil ( $\bullet$ ) rats, with (—) or without inflammation (---). For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean values were significantly different from those of the SH at the same time point: \*\*\* $P$ <0.001. Mean values were significantly different from those of OVX: † $P$ <0.05.

*Bone mineral density*

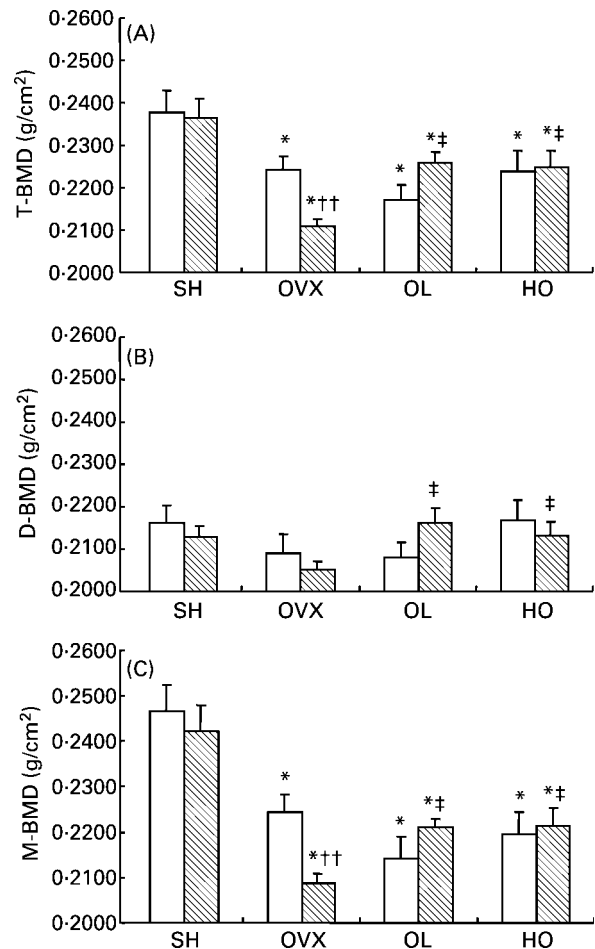
No significant differences were reported in bone area between groups. BMD reflects bone mineral content:bone area ratio (Table 3). Ovariectomy induced a significant decrease in total BMD (OVX 0.2243 (SEM 0.0031), SH 0.2378 (SEM 0.0050)  $g/cm^2$ ;  $P$ <0.001). Inflammation exacerbated this osteopenia, but was devoid of any effect in SH rats (OVX with inflammation 0.2109 (SEM 0.0018)  $g/cm^2$ ,  $P$ <0.01; SH with inflammation 0.236 (SEM 0.0043)  $g/cm^2$ , NS). Oleuropein and olive oil reduced this bone loss in OVX rats with inflammation (HO with inflammation 0.2249 (SEM 0.0038), OL with inflammation 0.2258 (SEM 0.0028)  $g/cm^2$ ). However, they had no effect in those without inflammation (HO 0.2240 (SEM 0.0048), OL 0.2170 (SEM 0.0036)  $g/cm^2$ , NS compared with the values in OVX control rats). The same pattern was observed for metaphyseal BMD, whereas for diaphyseal BMD only olive oil and oleuropein consumption were able to elicit significant changes compared with the values measured in OVX rats with inflammation (Fig. 3) (Table 3).



**Fig. 2.** Uterine weight in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats, with (□) or without (▨) inflammation. For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean values were significantly different from those of the corresponding SH group: \*\*\* $P$ <0.001.

*Bone turnover*

An age-related decrease in bone formation was demonstrated by a decrease in plasma osteocalcin levels throughout the experimental period. At day 41, ovariectomy elicited an increased bone turnover: plasma osteocalcin concentrations osteocalcinaemia, (ng/ml) and urine DPD excretion were greater than in control rats (OVX 33.1 (SEM 1.7), SH 21.1 (SEM 2.2) ng/ml; OVX 165.0 (SEM 9.5), SH 86.1 (SEM 7.4) nmol DPD/mmol creatinine respectively). Bone resorption was still high at the end of the experiment (day 80: OVX 175.4 (SEM 23.0), SH 91.5 (SEM 10.2) mmol DPD/mmol creatinine), whilst osteoblastic activity was not different anymore with intact (SH) animals (OVX 16.7 (SEM 2.3), SH 15.8 (SEM 0.8) ng/ml). As far as inflammation is concerned, a greater degree of osteocalcinaemia was observed in OVX with inflammation than



**Fig. 3.** Bone mineral density (BMD) in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats, with (□) or without (▨) inflammation. (A), total femur (T-BMD); (B), femur diaphysis (D-BMD); (C), femur metaphysis (M-BMD). For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean values were significantly different from those of the corresponding SH group: \* $P$ <0.05. Mean values were significantly different from those of OVX without inflammation: †† $P$ <0.001. Mean values were significantly different from those of OVX with inflammation: ‡ $P$ <0.05.

OVX. Finally, only the oleuropein-containing diet was able to partially improve bone resorption in OVX rats (Fig. 4).

#### Femoral mechanical testing

Femoral failure load was similar in OVX with inflammation (100 (SEM 3) N) and SH with inflammation (101 (SEM 4) N). However, it was significantly greater in OL with inflammation and HO with inflammation ( $P < 0.05$ ) than in OVX with inflammation (Fig. 5).

#### Marker of inflammation

Plasma  $\alpha$ -1-acid glycoprotein concentration was similar in all groups. This marker was increased after inflammation in oestrogen-deficient animals, but was unchanged in intact rats (OVX 22.6 (SEM 4.3), OVX with inflammation 35.9 (SEM 7.5)  $\mu\text{g/ml}$ ,  $P < 0.05$ ; SH 17.78 (SEM 2.35), SH with inflammation 18 (SEM 3.03)  $\mu\text{g/ml}$ ). This pattern was prevented by olive oil (HO with inflammation 22.8 (SEM 1.7)  $\mu\text{g/ml}$ ;  $P < 0.005$ ) (Fig. 6).

The spleen weight increased after inflammation in OVX rats, while SH animals were protected (OVX 1.86 (SEM 0.08), OVX with inflammation 2.54 (SEM 0.09) g spleen/

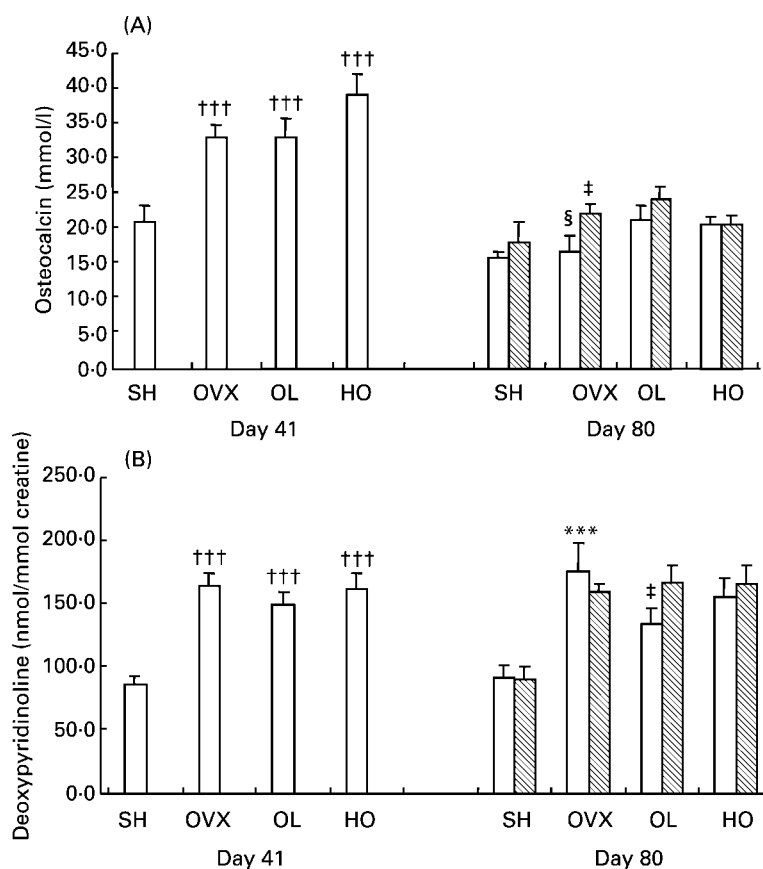
kg body weight,  $P < 0.001$ ; SH 2.18 (SEM 0.18), SH with inflammation 2.31 (SEM 0.16) g spleen/kg body weight). Both diets prevented this effect (HO with inflammation 2.15 (SEM 0.09), OL with inflammation 2.01 (SEM 0.11) g spleen/kg body weight;  $P < 0.05$ ) (Table 4).

#### Markers of oxidative stress

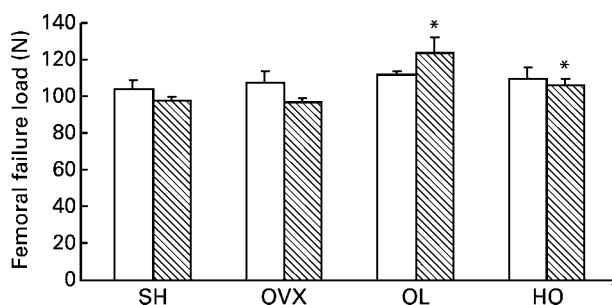
The ferric-reducing potential value, which reflects the antioxidant capacity of plasma, was not different between groups. Thiobarbituric acid-reactive substances in the heart were also not modified by any of the diets or treatments (results not shown).

#### Discussion

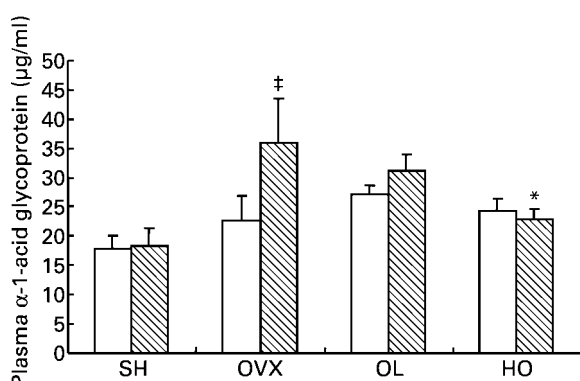
Broad-based preventive strategies designed to lower the risks of osteoporosis need to be established and implemented. This is why the concept of a healthy diet providing adequate amounts of various potent micronutrients deserves attention. A recent interest in phenolic compounds in foods has increased greatly because of the compounds' anti-inflammatory and free radical-scavenging abilities (Bors *et al.* 1990; Horcajada-Molteni & Coxam,



**Fig. 4.** Plasma osteocalcin concentrations (A) and urinary deoxyypyridinoline (B) in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats, with (□) or without (▨) inflammation before (day 41) and after (day 80) inflammation injection. For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean values were significantly different from those of SH at day 80: \*\*\* $P < 0.005$ . Mean values were significantly different from those of SH at day 41: ††† $P < 0.001$ . Mean values were significantly different from those of OVX at day 80: ‡ $P < 0.04$ . Mean values were significantly different from those of OVX at day 41: § $P < 0.05$ .



**Fig. 5.** Femoral failure load in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats, with (□) or without (▨) inflammation. For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean values were significantly different from those of OVX with inflammation: \* $P < 0.05$ .



**Fig. 6.** Plasma α-1-acid glycoprotein in sham-operated (SH), ovariectomized (OVX), ovariectomized + oleuropein (OL) and ovariectomized + olive oil (HO) rats, with (□) or without (▨) inflammation. For details of diets and procedures, see Table 2 and pp. 120–121. Values are means with their standard errors shown by vertical bars (ten rats per group). Mean value was significantly different from that of OVX without inflammation: ‡ $P < 0.05$ . Mean value was significantly different from that of OVX with inflammation: \* $P < 0.05$ .

2001). We thus assessed the effect of olive oil and its major polyphenol, oleuropein, on bone loss in ovariectomised rats with chronic inflammation: gonadal failure at the time of menopause causes osteopenia in women, and with

ageing, inflammatory and oxidant conditions drastically worsen as the body becomes unable to eliminate free radicals and prevent inflammation (Das, 2002).

In the present experiment, the animals were given a control diet in which fat was replaced by olive oil (50 g/kg diet). This amount is equivalent to 61 ml/d for a 70 kg man. According to Quaranta & Rotundo (2000), the average daily consumption of olive oil is about 55 ml in Mediterranean countries; consequently, the dose given was in the order of magnitude of the current nutritional doses in human subjects. The amount of oleuropein in the oleuropein-supplemented diet was about twice the level in the diet containing 50 g olive oil/kg.

*The case of ovariectomised rats with inflammation*

With regard to bone variables, a significant decrease in femoral BMD was elicited by ovariectomy, as previously shown by Miller *et al.* (1991). As in human subjects (Uebelhart *et al.* 1991), this bone loss probably resulted from a faster bone turnover, as indicated by higher plasma osteocalcin concentrations associated with bone-formation rate (Riggs & Melton, 1986) and increased urinary DPD excretion associated with bone resorption (Eastell *et al.* 1992) in OVX rats than in SH (Fig. 4). However, in our experimental conditions, mechanical properties (assessed by the femoral failure load), which depend on bone architecture quality, remained unchanged. This discrepancy could be explained by the fact that this variable reflects only the diaphyseal bone quality, which reacts more slowly than cancellous bone. Indeed, the lack of change in diaphyseal BMD confirms a lesser susceptibility of this tissue, as previously reported by Kalu (1991).

A further bone loss was elicited by inflammation in the OVX rats; this loss reached 6% of the total BMD. This alteration of trabecular bone mass was, however, quite small compared with a 64% decrease of BMD in 1.5-month-old rats (Minne *et al.* 1984; Vukicevic *et al.* 1994). In young growing animals a decrease in osteoblastic activity and reduced growth could explain a greater effect than in our present adult animals. In trying to explain the pathogenesis of this osteopenia, Vukicevic *et al.* (1994) have shown that modulation of TNF-α is involved. Moreover, an increased production of NO and an activation of

**Table 3.** Bone mineral content (BMC), bone area and bone mineral density (BMD) measured in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats with or without inflammation (inf)\*

(Mean values with their standard errors for ten rats per group)

Treatment group	BMC (g)		Bone area (cm <sup>2</sup> )		BMD (g/cm <sup>2</sup> )	
	Mean	SEM	Mean	SEM	Mean	SEM
SH	0.4468	0.0173	1.8764	0.0438	0.2378	0.0050
SH inf	0.4392	0.0160	1.8541	0.0401	0.2366	0.0043
OVX	0.4261	0.0101	1.8988	0.0246	0.2243	0.0031
OVX inf	0.4020	0.0094	1.9050	0.0383	0.2109	0.0018
OL	0.4111	0.0110	1.8932	0.0281	0.2170	0.0036
OL inf	0.4217	0.0118	1.8684	0.0486	0.2258	0.0028
HO	0.4342	0.0202	1.9026	0.0470	0.2240	0.0048
HO inf	0.4391	0.0142	1.9505	0.0440	0.2249	0.0038

\* For details of diets and procedures, see Table 2 and pp. 120–121.

**Table 4.** Spleen weight in sham-operated (SH), ovariectomised (OVX), ovariectomised + oleuropein (OL) and ovariectomised + olive oil (HO) rats with or without inflammation (inf)†

(Mean values with their standard errors for ten rats per group)

Group	Spleen weight (g/kg body weight)	
	Mean	SEM
SH	2.18	0.18
SH inf	2.31	0.16
OVX	1.86	0.08
OVX inf	2.54***	0.09
OL	1.91	0.07
OL inf	2.01*	0.11
HO	2.16	0.15
HO inf	2.15*	0.09

Mean values were significantly different from those of the OVX inf: \* $P < 0.05$ .Mean value was significantly different from those of the OVX: \*\*\* $P < 0.001$ .

† For details of diets and procedures, see Table 2 and pp. 120–121.

the inducible NO synthetase in the bone marrow space have also been demonstrated (Armour *et al.* 1999). In the same way, in the present study the talc-induced-inflammation also led to an increase in inflammatory variables, such as a splenomegaly and an increase in plasma  $\alpha$ -1-acid glycoprotein. Spleen hypertrophy could be considered as a sign of activation of the immune system; the spleen plays a major role in the immune system by synthesising antibody-producing lymphocytes and filtering damaged cells of blood. Conversely, in our present study, there was no modification of thiobarbituric acid-reactive substances (peroxidation marker) or in the ferric-reducing potential (antioxidative capacity of plasma), as measured at the end of the experiment. It is thus possible that a steady-state was reached, but more probably, oil in the diet might have contained enough  $\alpha$ -tocopherol to avoid any further free radical production after ovariectomy or inflammation. Indeed, the standard dietary fat mixture (peanut oil–rapeseed oil (50:50, w/w)) and olive oil supplied 1540 and 1380  $\mu$ g  $\alpha$ -tocopherol/kg body weight per d respectively. Vitamin E is usually an excellent inhibitor of lipid peroxidation (Chaudiere, 1994), possibly explaining the similarity between groups.

Oleuropein or olive oil prevented (at least partially) overweight in ovariectomised rats and the loss of weight after inflammation.  $17\beta$ -Oestradiol has been shown to restore body weight in ovariectomised rats (Coxam *et al.* 1996). Oleuropein is a polyphenol belonging to the secoiridoid class, which includes lignans, and has a stereochemistry analogous to recognised phyto-oestrogens; thus, this polyphenol could act as a phyto-oestrogen. However, the oestrogenic potency of oleuropein has not been investigated.

With regard to bone health, a protective effect of both diets was observed in inflammatory conditions. Indeed, femoral failure load and diaphyseal BMD were increased with oleuropein and olive oil in OVX with inflammation. In the same way, BMD was also improved at the trabecular level. Our present results are quite similar to those

previously reported in a model for breast cancer: rats fed on a polyphenol-supplemented virgin olive oil-containing diet with inflammation induced by carrageenan (Martin-Moreno *et al.* 1994). It is well known that oleuropein elicits anti-inflammatory effects by inhibiting the lipoxygenase activity and the production of leucotriene  $B_4$  (De la Puerta *et al.* 1999). Olive oil is rich in phenolic compounds, particularly oleuropein, hydroxytyrosol and tyrosol, which possess anti-inflammatory effects (De la Puerta *et al.* 1999). Moreover, it is well established that the anti-inflammatory effect of olive oil depends on the polyphenol content (Martinez-Dominguez *et al.* 2001) and that a synergistic effect between phenolic compounds is possible. Visioli *et al.* (1998) has shown that extra virgin olive oil gives better protection against LDL oxidation *in vitro* than oleuropein or hydroxytyrosol. Even if olive oil polyphenols are absorbed in rats (Tuck *et al.* 2001), as in human subjects (Vissers *et al.* 2002), in our experimental conditions they did not increase the antioxidant capacity of plasma, again probably because protection was already at a maximum value.

#### The case of ovariectomised rats

Osteopenia due to ovariectomy was unchanged by any diet, as shown by values of BMD and markers of remodelling that changed, although on day 80 urinary DPD excretion decreased in OL rats while osteocalcinemia remained constant. This lack of effect of both olive oil and oleuropein consumption in OVX rats (an experimental model for postmenopausal osteoporosis) compared with an improvement in bone health when an inflammation was induced experimentally (a model for senile osteoporosis) could be explained by an indirect action on bone metabolism through an improvement of the inflammatory status. It is possible that inflammation elicited by OVX was not great enough (only a trend towards higher  $\alpha$ -1-acid glycoprotein levels was demonstrated). It is well known that only molecules with oestrogenic activity are effective in the prevention of bone loss associated with early menopause. It is possible that the intrinsic oestrogenic activity of those polyphenols was not enough to directly target bone cells.

#### The case of sham-operated animals

The reduced susceptibility of sham-operated animals to inflammation was probably due to the protective effect of steroid hormones. Indeed, oestrogens inhibit pro-inflammatory cytokine production (IL-1, TNF- $\alpha$ ) involved in orosomucoid synthesis (Fournier *et al.* 2000). However, this protective action does not exclude other pathways such as, for example, control of immune cells recruitment and activation: Cuzzocrea *et al.* (2000) have shown, in an ovariectomised rat model with carrageenan-induced pleurisy, an attenuated inflammatory response and a decrease in polymorphonuclear cell migration and activation after  $17\beta$ -oestradiol treatment. Moreover, inducible NO synthetase activity was decreased, as well as the subsequent formation of free radical species. Again, as in OVX rats, inflammation was not associated with a significant oxidative stress development, even though there was a trend to

higher malondialdehyde levels (after lipid peroxidation induction). However, inflammation induced a significant body-weight loss due to anorexia, because rats bearing talc granulomas eat less than intact animals (Vukicevic *et al.* 1994).

In conclusion, in the rat, inflammation exacerbated osteopenia induced by ovariectomy, making this model suitable for the study of senile osteoporosis. The present study demonstrates that oleuropein and extra virgin olive oil were able to elicit protective effects on bone loss in this model of ovariectomy associated with inflammation, probably by modulating variables of inflammation (such as  $\alpha$ -1-acid glycoprotein). However, they did not have any effect on BMD when inflammation was not performed. These present results strengthen the current hypothesis that micronutrients could be of importance in degenerative conditions involving changes in the inflammatory status.

### Acknowledgements

The authors thank F. Rambourdin, C. Lab and A. Bellanger for technical assistance.

### References

- Armour KE, Van'T Hof RJ, Grabowski PS, Reid DM & Ralston SH (1999) Evidence for a pathogenic role of nitric oxide in inflammation-induced osteoporosis. *J Bone Miner Res* **14**, 2137–2142.
- Benzie FF & Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* **239**, 70–76.
- Bors W, Heller W, Michel C & Saran M (1990) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* **186**, 343–355.
- Breuilé D, Arnal M, Rambourdin F, Bayle G, Levieux D & Oblé C (1998) Sustained modifications of protein metabolism in various tissues in a rat model of long-lasting sepsis. *Clin Sci (Lond)* **94**, 413–423.
- Browner WS, Pressman AR, Nevitt MC & Cummings SR (1996) Mortality following fractures in older women. The study of osteoporotic fractures. *Arch Intern Med* **156**, 1521–1525.
- Chaudière J (1994) Some chemical and biochemical constraints of oxidative stress in living cells. In *Free Radical Damage and its Control*, pp. 25–66 [CA Rice-Evans and RH Burdon, editors]. New York: Elsevier-Science.
- Cohen-Solal ME, Graulet AM & Denne MA (1993) Peripheral monocyte culture supernatants of menopausal women induce bone resorption: involvement of cytokines. *J Clin Endocrinol Metab* **77**, 1648–1653.
- Consensus Development Statement (1997) European Foundation for Osteoporosis and the National Osteoporosis Foundation. Who are candidates for prevention and treatment for osteoporosis? *Osteoporosis Int* **7**, 1–6.
- Cook JGH (1975) Factors influencing the assay of creatinine. *Ann Clin Biochem* **12**, 219–232.
- Cooper C, Atkinson EJ, Jacobsen SJ, O'Fallon WM & Melton LJ 3rd (1993) Population-based study of survival after osteoporotic fractures. *Am J Epidemiol* **137**, 1001–1005.
- Coxam V, Bowman BM, Mecham M, Roth CM, Miller MA & Miller SC (1996) Effects of dihydrotestosterone alone and combined with estrogen on bone mineral density, bone growth and formation rates in ovariectomized rats. *Bone* **19**, 107–114.
- Cuzzocrea S, Santagati S, Sautebin L, Mazzon E, Calabro I, Caputi AP & Maggi A (2000) 17 beta-estradiol antiinflammatory activity in carrageenan-induced pleurisy. *Endocrinology* **141**, 1455–1463.
- Das UN (1991) Interaction(s) between essential fatty acids, eicosanoids, cytokines, growth factors and free radicals: relevance to new therapeutic strategies in rheumatoid arthritis and other collagen vascular diseases. *Prostaglandins Leucot Essent Fatty Acids* **44**, 201–210.
- Das UN (2002) Nitric oxide as the mediator of the antiosteoporotic actions of estrogen, statins, and essential fatty acids. *Exp Biol Med* **227**, 88–93.
- De la Puerta R, Ruiz Gutierrez V & Hoult JR (1999) Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem Pharmacol* **57**, 445–449.
- Diplock AT, Aggett P, Ashwell M, Bornet F, Fern EB & Roberfroid MB (1999) Scientific concepts of functional foods in Europe: consensus document. *Br J Nutr* **81**, S1–S27.
- Eastell R, Calvo MS, Burritt MF, Offord KP, Russell RG & Riggs BL (1992) Abnormalities in circadian patterns of bone resorption and renal calcium conservation in type I osteoporosis. *J Clin Endocrinol Metab* **74**, 487–494.
- Ershler WB, Harman SM & Keller ET (1997) Immunologic aspects of osteoporosis. *Dev Comp Immunol* **21**, 487–499.
- European Commission (1998) *Report on Osteoporosis in the European Community: Action for Prevention*. Luxembourg: Office for Official Publication of the European Communities.
- Fournier T, Medjoubi N & Porquet D (2000) Alpha-1-acid glycoprotein. *Biochim Biophys Acta* **1482**, 157–171.
- Horcajada-Molteni MN & Coxam V (2001) Flavonols and idoflavones prevent bone loss in the ovariectomized rat, a model for postmenopausal osteoporosis. In *Nutritional Aspects of Osteoporosis*, pp. 325–340 [F Burkhardt, B Dawson-Hughes and RP Heaney, editors]. San Diego, CA: Academic Press.
- Horowitz MC & Raisz LG (1999) Cytokines and Prostaglandins. In *Aging Skeleton*, pp. 195–207 [CJ Rosen, J Glowack and JP Bilezikian, editors]. San Diego: Academic Press.
- Kalu DN (1991) The ovariectomized rat model of postmenopausal bone loss. *Bone Miner* **15**, 175–191.
- Kanis JA (1993) The incidence of hip fracture in Europe. *Osteoporosis Int* **3**, 10–15.
- Keys A, Menotti A, Karvenen MJ, Arvanis C, Blackbuen H, Buzina R, Djordjevic BS, Donsas AS, Fidanza F & Keys MH (1986) The diet and 15-years death rate in the seven countries study. *Am J Epidemiol* **24**, 903–915.
- Martinez-Dominguez E, De La Puerta R & Ruiz-Gutiérrez V (2001) Protective effects upon experimental inflammation models of polyphenol-supplemented virgin olive oil diet. *Inflamm Res* **50**, 102–106.
- Martin-Moreno JM, Willett WC, Gorgojo L, Banegas JR, Rodriguez-Artalejo F, Fernandez-Rodriguez JC, Maisonneuve P & Boyle P (1994) Dietary fat, olive oil intake and breast cancer risk. *Int J Cancer* **58**, 774–780.
- Miller SC, Bowmann BM, Miller MA & Bagi C (1991) Calcium absorption and osseous organ, tissue and envelope specific changes following ovariectomy in rats. *Bone* **12**, 439–446.
- Minne HW, Pfeilschifter J, Scharla S, Mutschelknauss S, Schwarz A, Krempien B & Ziegler R (1984) Inflammation-mediated osteopenia in the rat: a new animal model for pathological loss of bone mass. *Endocrinology* **115**, 50–54.
- Mody N, Parhami F, Sarafian TA & Demer L (2001) Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Biol Med* **31**, 509–519.
- Ohkawa H, Ohishi N & Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**, 351–358.



- Pacifi R, Brown C, Puscheck E, Slatopolsky E, Maggio D, McCracken R & Avioli LV (1991) Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci USA* **88**, 5134–5138.
- Quaranta G & Rotundo V (2000) Economic and commercial prospects for olive oil in view of the changes in the common market organisation (CMO) (part one). *Olivae* **91**, 20–24.
- Riggs BL & Melton LJ (1986) Involutional osteoporosis. *N Engl J Med* **26**, 1676–1684.
- Robbins SP (1994) Biochemical markers for assessing skeletal growth. *Eur J Clin Nutr* **48**, S199–S209.
- Tsarbopoulos A, Gikas E, Papadopoulos N, Aligiannis N & Kafatos A (2003) Simultaneous determination of oleuropein and its metabolites in plasma by high-performance liquid chromatography. *J Chromatogr B* **785**, 157–164.
- Tuck KL, Freeman MP, Hayball PJ, Stretch GL & Stupans I (2001) The *in vivo* fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats. *J Nutr* **131**, 1993–1996.
- Turner CH & Burr CB (1993) Basic mechanical measurements of bone: a tutorial. *Bone* **14**, 595–608.
- Uebelhart D, Schlemmer A, Johansen J, Gineyts E, Christiansen C & Delmas PD (1991) Effects of menopause and hormone therapy on the urinary excretion of pyridinium crosslinks. *J Clin Endocrinol Metab* **72**, 367–373.
- Visioli F, Bellomo G & Galli C (1998) Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun* **247**, 60–64.
- Vissers MN, Zock PL, Roodenburg AJ, Leenen R & Katan MB (2002) Olive oil phenols are absorbed in humans. *J Nutr* **132**, 409–417.
- Vukicevic S, Marusic A, Stavljenic A, Cesnjaj M & Ivankovic D (1994) The role of tumor necrosis factors-alpha in the generation of acute phase response and bone loss in rats with talc granulomatosis. *Lab Invest* **70**, 386–391.
- Watkins BA, Lippman HE, Bouteiller LL, Li Y & Seifert MF (2001) Bioactive fatty acids: role in bone biology and bone cell function. *Prog Lipid Res* **40**, 125–148.
- Zeng SX, Vrindts Y, Lopez M, De Groote D, Zangerle PF, Collette J, Franchimont N, Geenen V, Albert A & Reginster JY (1997) Increase in cytokine production (IL-1 beta, IL-6, TNF-alpha but not IFN-gamma, GM-CSF or LIF) by stimulated whole blood cells in postmenopausal osteoporosis. *Maturitas* **26**, 63–71.