ISSN 0025-7680

**ORIGINAL ARTICLE**

MEDICINA (Buenos Aires) 2000; 60: 482-486

# EFFECTS OF DEPOT MEDROXYPROGESTERONE ACETATE ON THE CALCIUM METABOLISM OF ADULT OVARIECTOMIZED RATS

**ERIBERTO A. ROVERI, GUSTAVO CHAPO, IRENE GRAPPIOLO, RODOLFO C. PUCHE**

*Laboratorio de Biología Osea, Facultad de Ciencias Médicas, Universidad Nacional de Rosario*

**Abstract** This paper describes experiments designed to test the effect of depot medroxyprogesterone acetate (DMPA) on calcium metabolism of adult ovariectomized rats. The 24 animals were randomly assigned to control or treated groups. Treated rats received 15 mg of DMPA i.m. per week, during four or twelve weeks. Controls received solvent alone. The variables characterizing the metabolism of Ca (daily rates of intestinal absorption and excretion, bone accretion and resorption and the sizes of the exchangeable pools and their rate constants) were measured with the aid of $^{45}Ca$ according to Aubert and Milhaud. No effects were observed at four weeks of treatment. After twelve weeks, treatment produced serum levels of 46.5 ± 5.6 nmoles of medroxyprogesterone/L, reduction of bone turnover (Ca accretion and resorption rates) and of the size of the slow exchanging Ca compartment. The increase in true Ca intestinal absorption was compensated by the increased endogenous fecal Ca excretion. The mass of body Ca was not affected by treatment.

**Resumen** ***Efecto del acetato de medroxiprogesterona depot sobre el metabolismo cálcico de ratas adultas ovariectomizadas.*** Este trabajo describe una investigación sobre los efectos del acetato de medroxiprogesterona (depot) (DMPA) sobre el metabolismo del calcio de ratas adultas castradas. Los animales fueron distribuidos al azar en dos grupos: 12 controles y 12 tratados. Los animales tratados recibieron 15 mg de DMPA i.m. por semana, durante cuatro o doce semanas. Los controles recibieron solvente. Las variables que caracterizan el metabolismo del calcio: tasas de absorción y excreción intestinal de Ca (en mg/día), deposición y reabsorción ósea (en mg/día) y el tamaño y las tasas de intercambio de los compartimientos de intercambio rápido y lento (en mg y mg/día, respectivamente) se midieron in vivo mediante la técnica de Aubert y Milhaud utilizando $^{45}Ca$. El tratamiento no produjo efectos comprobables a las cuatro semanas. Después de doce semanas de tratamiento, la concentración plasmática de medroxiprogesterona fue de 46.5 ± 5.6 nmoles/L, se redujo el turnover óseo (tasas de deposición y reabsorción óseas) y el tamaño del compartimiento de intercambio lento de calcio. El aumento en la absorción intestinal de calcio fue compensado por la excreción aumentada de calcio fecal endógeno. La masa de calcio corporal no fue afectada por el tratamiento.

**Key words:** medroxyprogesterone acetate, bone, calcium metabolism, true Ca absorption, endogenous fecal Ca excretion

Medroxyprogesterone acetate* is a synthetic steroid used as a contraceptive by young women. It is also administered with estrogens to postmenopausal women with intact uteri to prevent the development of endometrial hyperplasia and carcinoma. Clinical trials and experimental works have been done with different doses and pharmaceutical forms of the drug, administration routes and continuous or cyclical dosages. Due to the divergency of results, consensus on the effect of medroxyprogesterone on bone tissue has not been reached. We decided to investigate the effects of the drug on the metabolism of calcium in adult ovariectomized rats. The latter are assumed to be an endocrine model of the ovarian failure in women. To the best of our knowledge antecedents are restricted to two papers. In ovariectomized young adult beagles[1] continuous administration of DMPA (100 mg, i.m., every two months, during nine months) is reported to produce decreased resorption at the corticoendosteal surfaces and increased formation in periosteal surfaces. On the other hand, ovariectomized rats treated with MPA (0.3 mg/Kg/day during 30 days) showed no effects on the metabolism of bone (assessed with static and dynamic morphometric studies[2]).

* DMPA refers to a microcrystalline suspension of medroxyprogesterone acetate, administered by intramuscular injection. As a contraceptive the dose is 150 mg of the drug every three months. Other doses of this pharmaceutical form are stated in each case. MPA refers to either tablets administered orally or to intramuscular injection of the drug in solution, at the doses indicated in each case.

Received: 3-XII-1999 Accepted: 3-V-2000

**Postal address:** Dr. Rodolfo C. Puche, Laboratorio de Biología Osea, Facultad de Ciencias Médicas, Santa Fe 3100, 2000 Rosario, Argentina
Fax: (54-0341) 480-4552 e-mail: rpuche@unrctu.edu.ar

## Material and Methods

*Animals.* Twenty four-IIM/Fm adult inbred rats, subline "m"[3], weighing 220-250 g, were ovariectomized under ether anesthesia. They were left to recover for one week before starting the experiments. The success of ovariectomy was assessed through vaginal cytology for five days after surgery. All experiments were done according to the principles stated in the NIH guide[4]. The rats were randomly assigned to control (n = 12) or treated (n = 12) groups. They were housed in individuals metabolic cages with food and water ad libitum at constant room temperature (23-24 °C), with natural light cycle. All animals received a semisynthetic diet in powder form: total wheat flour 85%, casein 12%, sodium chloride 2.5%, calcium carbonate 0.7% and salts mixture[5] 0.05%. Final Ca and P contents of the diet were 5.0 mg/g and 6.2 mg/g, respectively. Calcium metabolism studies (see below) were carried out in control and treated (see below) animals after four and twelve weeks of treatment.

*The dose of DMPA.* Treated rats received an intramuscular injection of medroxyprogesterone acetate (DMPA, 0.1 ml/week of Depo-Provera 150®, containing 15 mg of the drug). Control animals were injected with solvent (0.1 ml of an isotonic solution of polyethyleneglycol, polysorbate and sodium chloride). The dose of DMPA employed in this experiment is at the highest level reported not to affect adversely body weight increase, mortality, behavior, and length of the tibia (Bertazzoli C., unpublished data, quoted in[6]). Intramuscular administration of DMPA was elected to attain permanently raised plasma levels of this steroid.

*Calcium metabolism studies.* Body weight and intake of food and water were recorded daily. Urine and feces were collected for analysis the last week of the experiments. Calcium of urine and feces was measured by atomic absorption spectropho-tometry[7].

The rates of intestinal Ca absorption and excretion, bone Ca accretion and resorption and the sizes of the exchangeable pools and their rate constants were measured with the aid of $^{45}Ca$ according to Aubert and Milhaud[8]. The rate of bone Ca accretion ($V_{o+}$) was estimated according to the relationship:

$$\text{Radioactivity of the skeleton} = V_{o+} \int R_s \, dt$$

The second term of the righ side of the equation shows the integral of the change of specific activity ($R_s$) of plasma calcium as a function of time. The rate of Ca resorption ($V_{o-}$) was estimated as the difference between Ca balance minus $V_{o+}$. The rates of Ca accretion, Ca resorption and the size of the exchangeable Ca pools were expressed per gram of skeletal Ca.

Seventy-two hours after $^{45}Ca$ injection the rats were sacrificed by exsanguination under ether anesthesia. Blood plasma was stored at –20 °C for determinations of calcium[6], medroxyprogesterone[9] and alkaline phosphatase (total activity[10] and bone isoenzyme[11]). Urine was collected during this interval to measure total hydroxyproline[12]. The rats were then eviscerated, their carcasses cleansed as much as possible of muscle and incinerated. The ashes were dissolved in 20 ml of hot HCl 1 M and diluted to 100 ml. Aliquots of this solution were used to measure Ca[6] and $^{45}Ca$[13].

*Statistical analysis.* Unpaired t tests were used for the assessment of the differences[14]. We chose to study six rats per group in order to have 70-80% power of detecting a 25-30% change in most informative variables (e.g., the rates of bone Ca accretion rate or true Ca absorption) with a significance level of 0.05 (two tailed).

## Results

The dose employed of DMPA in these experiments produced serum levels of 46.5 ± 5.6 nmoles/L, at the time $^{45}Ca$ was injected (twelfth week).

As displayed in Table 1, four weeks of treatment did not affect the variables defining the metabolism of Ca were not affected after with DMPA.

As expected, older animals showed lower bone Ca accretion and resorption rates[15]. After twelve weeks of treatment with DMPA several variables were affected. The rate of bone Ca accretion was decreased in both groups, being significantly lower in DMPA treated rats. The resorption rate was affected in the same direction without statistical significancy between groups, probably because it is the result of bone Ca accretion minus calcium balance, and its calculation drags the errors of previous measurements. The larger reduction in accretion and resorption rates probably explains the smaller increase in the size of the slowly exchanging Ca compartment in DMPA treated rats. Conversely, the rapidly exchanging Ca compartment it is no affected by treatment.

The increases in the rates of true Ca absorption and endogenous fecal Ca excretion compensated each other. Ca balance was not affected.

The indicators of bone metabolism: alkaline phosphatase (bone isoenzyme) or hidroxyproline excretion were not affected. Total bone mass did not differ between groups.

## Discussion

According to Verhaar et al.[15] MPA ($10^{-14}$ M) stimulates the mitogenesis and differentiation of osteoblast-like cells harvested from female bone explants. The obvious inference that MPA may have an anabolic effect on the metabolism of bone does not correlate with the observations and results of experimental (and clinical) research. Consequently we decided to investigate the effects of the drug on the metabolism of Ca in the ovariectomized rat model.

Treatment with DMPA for 12 weeks to spayed rats increased true Ca absorption (a finding that deserves further studies). The fact that serum calcium levels were not affected probably indicates functioning parathyroid glands with a lower than normal secretion rate. The latter may have caused the significant reduction in bone turnover (decreased bone Ca accretion and resorption rates). The effect on bone turnover, however, was not correlated with hydroxyproline excretion or alkaline phosphatase levels. The decrease in bone turnover could explain the increase in endogenous fecal Ca

TABLE 1.– *Calcium metabolism in control and DMPA-treated spayed rats*

| | Four weeks treatment | | Twelve weeks treatment | | |
|---|---|---|---|---|---|
| | Controls | Treated | Controls | Treated | p |
| Variable | | | | | |
| Body weight at sacrifice | 296 ± 9 | 313 ± 13 | 331 ± 27 | 384 ± 14 | |
| Body weight increase (% of initial) | 11 | 16 | 35 | 31 | |
| Food intake g/d | 14.9 ± 0.9 | 16.8 ± 1.3 | 11.2 ± 0.7 | 13.2 ± 0.8 | |
| Skeletal calcium, grams | 2.26 ± 0.07 | 2.31 ± 0.12 | 2.41 ± 0.09 | 2.45 ± 0.1 | |
| Skeletal calcium, g/100 g bw | 0.76 ± 0.02 | 0.77 ± 0.01 | 0.71 ± 0.03 | 0.70 ± 0.03 | |
| Fast exchangeable Ca compartment | | | | | |
| Size (mg Ca/g sk. Ca) | 3.6 ± 0.1 | 3.1 ± 0.5 | 7.9 ± 0.4 | 7.3 ± 0.2 | |
| Rate of exchange, h-1 | 6.1 ± 0.4 | 5.9 ± 0.4 | 6.7 ± 0.4 | 6.5 ± 0.3 | |
| Slowly exchangeable Ca compartment | | | | | |
| Size (mg Ca/g sk. Ca) | 13.5 ± 2.0 | 13.0 ± 0.8 | 22.7 ± 1.7 | 16.0 ± 0.6 | 0.004 |
| Rate of exchange, h-1 | 0.71 ± 0.01 | 0.77 ± 0.02 | 0.63 ± 0.13 | 0.54 ± 0.07 | |
| Skeletal Ca accretion rate, mg Ca/d | 38.4 ± 3.8 | 33.5 ± 3.6 | 12.2 ± 0.6 | 8.7 ± 0.3 | 0.0004 |
| Skeletal Ca resorption rate, mg Ca/d | 13.1 ± 0.6 | 13.7 ± 4.3 | 8.1 ± 2.3 | 6.8 ± 2.0 | |
| True Ca absorption, mg/d | 43.8 ± 4.7 | 48.1 ± 5.6 | 36.2 ± 4.7 | 52.7 ± 5.0 | 0.051 |
| Endogenous fecal Ca excretion, mg/d | 10.2 ± 1.6 | 17.5 ± 2.8 | 13.5 ± 3.2 | 0.2 ± 0.04 | 0.038 |
| Urinary Ca, mg/d | 0.30 ± 0.08 | 0.40 ± 0.08 | 0.35 ± 0.04 | 0.32 ± 0.05 | |
| Plasma Ca, mg/dL | 10.1 ± 0.3 | 9.5 ± 0.3 | 10.3 ± 0.3 | 10.2 ± 0.4 | |
| Urinary hydroxyproline, mg/d/100 g bw | 45.1 ± 7.0 | 46.7 ± 4.0 | 41.1 ± 7.5 | 38.0 ± 2.4 | |
| Plasma alkaline phosphatase, KA Units | n.m. | n.m. | 27.8 ± 1.2 | 29.8 ± 3.1 | |
| Idem Bone isoenzyme, KA Units | n.m. | n.m. | 5.6 ± 0.2 | 5.2 ± 0.5 | |
| Plasma MPA, nmoles/L | n.m. | n.m. | 0.1 ± 0.1 | 46.5 ± 5.6 | 0.00001 |

*p values refer to control versus treated comparison at 12 weeks*
*n.m.: not measured*

excretion that counterbalanced the increase in true Ca absorption. These series of self neutralizing effects may explain the absence of effect on the skeletal calcium mass.

Discussion of the present results compared with other experimental studies in spayed animals is not straightforward due to differences in end points and the absence of measurements of plasma levels of medroxyprogesterone. The lack of MPA effects on bone tissue of rats reported by Isserow et al.[2] may be attributed to a low dose and short time of administration. Korombolova et al.[1] carried out a nine month study in dogs, with static and dynamic histomorphometric analyses. They reported a net bone modelling effect in the ribs: increased resorption at corticoendosteal surfaces and increased formation at periosteal surfaces.

The reader sould also note the difficulties in discussing the present data compared with the effects of DMPA in human beings. First, serum levels of the drug were not measured in clinical reports investigating the effect of DMPA or MPA on bone mass, with one exception[17]. Second, experiments with spayed rats may not be the adequate experimental model. The majority of women receiving DMPA have functioning ovaries and a temporary menopausal state induced by the drug.

The dose of DMPA employed in these experiments produced, at the 12th week of treatment, a serum concentration of 46.5 ± 5.6 nmoles/L. These levels are similar to those reported for human beings receiving 1 g of DMPA by the i.m. route every 2 weeks or 100 mg MPA per os per day[18]. When used as a contraceptive in young women, DMPA is reported to produce serum levels of 4.1 ± 1.1 nmoles of medroxyprogesterone/L[17].

Clinical investigations on the effects of medroxyprogesterone on bone tissue deserves further comments. When MPA+estrogen are administered to postmenopausal women, the prevailing effect on bone is that of the latter hormone. Grey et al. reported that the MPA (5 mg/d, per os) enhanced the spinal bone density response to oestrogen[19]. Others, however, have reported that continuous estrogenic therapy plus MPA (10 or 20 mg/day, per os, during the second half each month, for one year) did not improve BMD of the lumbar spine[20]. Very recently Nand et al.[21] reported that the daily administration of 1.25 mg of estrone sulfate together with 2.5, 5.0 or 10.0 mg MPA prevented the bone loss in postmenopausal women. After adjustment for several covariates, the 10.0 mg group had smaller increases than the others.

Adverse reports on the effect of DMPA on bone tissue have been published for treated premenopausal women.

Cundy et al.[22] reported that DMPA, by reducing circulating estrogen levels, could produce a temporary menopausal state, thus inducing some bone loss in some users. This study was criticized because it did not have pretreatment data, there was inadequate matching of control groups and it did not investigate the influence of other factors such as duration and amount of smoking, alcohol, parity and other contraceptive methods used[23, 25]. A second study by Cundy et al.[26] with improved design and that of Scholes et al.[27], reported similar results. In a third study, Cundy et al.[28] suggested that if estrogen levels returned to normal following cessation of depot DMPA, spinal bone density increased almost to pretreatment levels. Other investigators, however, have reported no effect of DMPA on bone mass[29, 31]. In a fourth study, Cundy et al.[32] concluded that a higher dose of MPA (50 mg/day per os), induced hypogonadism and produced significant early loss of trabecular bone. Grecu et al.[33] suggest that in human beings, DMPA is an effective therapy against the adverse effects of glucocorticoids on bone. His report supports the hypothesis of a competitive antagonism at the bone level between progesterone and glucocorticoids.

It is concluded that further studies are needed to understand the effects of DMPA on bone tissue. Clinical research have, to date, failed to produce a clear definition of whether DMPA has an anabolic or catabolic effect on bone tissue. Experimental models should reproduce more closely the endocrine situation of the sexually mature women undergoing chronic treatment with DMPA.

**Acknowledgements:** The work reported in this paper was partially supported by a grant (BID 802/OC-AR, PICT N° 05-00000-01876). RCP is Principal Investigator, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. Depot medroxyprogesterone acetate was kindly supplied by Upjohn Argentina S.A.

## References

1. Karambolova KK, Snow GR, Anderson C. Surface activity on the periosteal and corticoendosteal envelopes following continuous progestogen supplementation in spayed beagles. *Calcif Tissue Int* 1986; 38: 239-43.
2. Isserow JA, Rucinski B, Romero DF, Mann GN, Liu CC, Epstein S. The effect of medroxyprogesterone acetate on bone metabolism in the ovariectomized, tamoxifen treated rat. *Endocrinology* 1995; 136: 713-9.
3. Calderari S, Font MT, Garrocq O, Martínez SM, Puche RC, Tarres MC. The inbred IIM/Fm stock. *Rat Newsletter* 1991; 25: 28-9.
4. Anonymous. US Department of Health and Human Services. NIH Publication N° 86-23. Revised 1985.
5. Bernhart FW, Tomarelli RM. A salt mixture supplying the National Research Council estimates of the mineral requirements of the rat. *J Nutr* 1966; 89: 495-500.
6. Gruppo Montedison Farmitalia. Medrossiprogesterone acetato - MPA. Studi Farmacologici e Clinici. Brochure 311276. Direzione Medica e Documentazione, Gruppo Montedison Farmitalia, Milano, Italia, 1976.
7. Willis JB. Determination of calcium and magnesium in urine by atomic absorption spectrophotometry. *Analyt Chem* 1961; 33: 556-9.
8. Aubert P-P, Milhaud D: Méthode de mesure des principales voies du métabolisme calcique chez l'homme. *Biochem Biophys Acta* 1960; 39: 122-39.
9. Kaiser DG, Carlson RG, Kirton KT. GLC determination of medroxyprogesterone acetate in plasma. *J Pharm Sci* 1974; 63: 420-3.
10. King EJ, Armstrong AR. A convenient method for determining serum and bile phosphatase activity. *Canad Med Ass J* 1934; 31: 376-81.
11. Puche RC, Caferra DA, Rosillo I. Bone isoenzyme of serum alkaline phosphatase measured with wheat germ agglutinin. *Clin Chem* 1988; 34: 1372-4.
12. Nobbs BT, Walker AW, Davis TJ. A simplified method for the estimation of urinary total hydroxyproline *Clin Chim Acta* 1975; 64: 219-25.
13. Lutwak L. Estimation of $^{45}Ca$ by liquid scintillation counting. *Analyt Chem* 1959; 31: 340-3.
14. Snedecor GW, Cochran WG, Statistical Methods, Ames IO. Iowa State University Press 1966.
15. Puche RC, Alloatti R, Ledesma S. Growth and development of the bone mass of two strains of inbred rats. *Bone Miner* 1988; 4: 341-53.
16. Verhaar HJJ, Damen CA, Duursma SA, Scheven BAA. A comparison of the action of progestins and estrogen on the growth and differentiation of normal adult human osteoblast-like cells in vitro. *Bone* 1994; 15: 307-11.
17. Taneepapanichskul S, Intaraprasert S, Theppisai U, Chaturachinda K. Bone mineral density during long term treatment with Norplant implants and depot medroxy-progesterone acetate A cross sectional study of Thai women. *Contraception* 1997; 56: 153-5.
18. Hesselius I, Johansson EDB Medroxyprogesterone acetate (MPA) plasma levels after oral and intramuscular administration in a long term study. *Acta Obstet Gynecol Scand* 1981; (Suppl) 101: 65-70.
19. Grey A, Cundy T, Ecans M, Reid I. Medroxyprogesterone acetate enhances the spinal bone mineral density response to oestrogen in late post-menopausal women. *Clin Endocrinol* 1996; 44: 293-6.
20. Adachi JD, Sargeant EJ, Sagle MA, et al. A double-blind randomised controlled trial of the effects of medroxy-progesterone acetate on bone density of women taking oestrogen replacement therapy. *Brit J Obstet Gynaecol* 1997; 104: 164-70.
21. Nand SL, Wren BG, Gross BA, Heller GZ. Bone density effects of continuous estrone sulfate and varying doses of medroxyprogesterone acetate. *Obstet Gynecol* 1999; 93: 1009-13.
22. Cundy T, Evans E, Roberts H, et al. Bone density in women receiving depot medroxyprogesterone acetate for contraception. *BMJ* 1991; 303: 13-6.
23. Hinchley H. DMPA and bone density. *BMJ* 1991; 303: 467.
24. Kubba A. DMPA and bone density. BMJ 1991; 303: 467.
25. Tobias JH, Chow J, Chambers TJ. DMPA and bone density. *BMJ* 1991; 303: 468.
26. Cundy T, Cornich J, Roberts H, Elder H, Reid IR. Spinal bone density in women using depot medroxyprogesterone contraception. *Obstet Gynecol* 1998; 92: 569-73.
27. Scholes D, Lacroix AZ, Ott SM, Ichikawa LE, Barlow WE. Bone mineral density in women using depot medroxy-progesterone acetate for contraception. *Obstet Gynecol* 1999; 93: 233-8.
28. Cundy T, Evans E, Roberts H, et al. Recovery of bone density in women who stopped using medroxyprogesterone acetate. *BMJ* 1994; 308: 247-8.

29. Gbolade E, Ellis S, Murby V, Randall S, Kirkman R. Bone density in long term users of depot medroxyprogesterone acetate. *Brit J Obstet Gynecol* 1998; 105: 790-4.
30. Taneepapanichskul S, Intaraprasert S, Theppisai U, Chaturachinda K. Bone mineral density in long term depot medroxyprogesterone acetate acceptors. *Contraception* 1997; 56: 1-3.
31. Virutamasen P, Wangsupachart S, Reinproyoon D, Kriengsinyot R, Leepipatpaiboon S, Gua C. Trabecular bone in long term depot medroxyprogesterone acetate users. *Asia Oceania J Obstet Ginaecol* 1994; 20: 269-74.
32. Cundy T, Farquhar CM, Cornich J, Reid IR. Short term effects of high dose oral medroxyprogesterone acetate on bone density in premenopausal women. *J Clin Endocrinol Metab* 1996; 81: 1014-7.
33. Grecu EO, Weinschelbaum A, Simmons R. Effective therapy of glucocorticoid-induced osteoporosis with medroxyprogesterone acetate. *Calcif Tissue Int* 1990; 46: 244-99.

- - - -

*A famous Victorian story reports the reaction of an aristocratic lady to the primary heresy of her time: "Let us hope that what Mr. Darwin says is not true; but if it is true, let us hope that it will not become generally known". Teachers continue to relate this tale as both a hilarious putdown of class delusions (as if the upper crust could protect public morality by permanently sequestering a basic fact of nature) and an absurdist homely about the predictable fate of ignorance versus enlightenment. And yet, I think we should rehabilitate this lady as an acute social analyst and at least a prophet. For what Mr. Darwin said is, indeed, true. It has also not become generally known, at least in our nation.*

En un famoso cuento victoriano, una señora airstocrática, frente a la primera herejía de su tiempo, reacciona de esta manera: "Espero que lo que dice el Sr. Darwin no sea cierto; pero si fuera cierto, espero que no llegue a difundirse". Los maestros siguen contando este cuento tanto como un ejemplo comiquísimo de las deluciones de clase (como si la clase alta pudiera proteger la moralidad pública secuestrando un hecho básico de la naturaleza) como un dicho absurdo sobre el destino predecible de la ignorancia versus la explicación. Y sin embargo, creo que tendríamos que rehabilitar a esta señora como una aguda analista social y por lo menos una profeta. Porque lo que dijo el Sr. Darwin es, indudablemente, la verdad. Además, en general no ha sido reconocido como tal, por lo menos en nuestra nación.

Stephen Jay Gould

*Darwin's more stately mansion. Science* 1999; 284: 2087